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# Diversité phénotypique de *Brettanomyces bruxellensis* et adaptation aux procédés fermentaires

Jules Harrouard

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Diversité phénotypique de  
*Brettanomyces bruxellensis* et adaptation aux  
procédés fermentaires

Thèse soutenue par Jules HARROUARD

Soutenance : 09 décembre 2022

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## Résumé : Diversité phénotypique de *Brettanomyces bruxellensis* et adaptation aux procédés fermentaires

*Brettanomyces bruxellensis* est une levure associée à de nombreux procédés fermentaires, parfois considérée comme un agent d'altération (par exemple pour la production de vin, de bioéthanol, de bière), parfois comme un micro-organisme nécessaire et bénéfique (bières de spécialités, kombucha). Des travaux antérieurs montraient un regroupement génétique des isolats de *B. bruxellensis* en fonction de leur procédé d'origine (vin, bière, etc.), suggérant une adaptation aux environnements anthropisés. Toutefois, la diversité phénotypique de l'espèce restait mal décrite, notamment au regard des populations génétiques connues. Les principaux objectifs de cette thèse étaient donc d'étudier la diversité phénotypique de *B. bruxellensis* et d'identifier les caractères possiblement impliqués dans son adaptation aux environnements fermentaires. Pour cela, environ 150 souches représentatives de la diversité génétique connue de *B. bruxellensis* ont été caractérisées dans 5 environnements (moût de raisin, vin, moût de brasserie, bière et kombucha). Une trentaine de caractères ont été mesurés, incluant notamment des paramètres de cinétiques fermentaire et de croissance, des traits d'histoire de vie (taille des cellules, viabilité, etc.), des métabolites d'intérêt technologique (sucres consommés, acides produits, etc.). Nos résultats montrent que la variabilité des phénotypes relevés dépend fortement du milieu considéré, mais aussi des souches et des interactions génotype-environnement. Certains paramètres sont, de plus, impactés par le facteur « population » : par exemple, la population allotriploïde associée au vin présente une capacité de croissance bien supérieure dans le vin en comparaison des autres populations et des autres milieux. La capacité d'assimilation des nitrates (qui pourrait être associée à l'adaptation à certains environnements) a également été étudiée. La majorité des souches de *B. bruxellensis* sont capables d'assimiler les nitrates comme seule source d'azote, à l'exception de deux populations qui semblent avoir perdu ce caractère et qui présentent des variations dans le nombre de copies des gènes clés impliqués dans le métabolisme des nitrates.

L'objectif finalisé de cette thèse était de mieux caractériser les conséquences de la variabilité phénotypique de *B. bruxellensis* vis-à-vis de procédés de stabilisation microbiologique. L'efficacité de deux procédés innovants en cours de développement pour l'œnologie (traitement aux ultraviolets de type C et lumière pulsée) a été évaluée sur une collection de levures œnologiques incluant de nombreuses souches de *B. bruxellensis*. Ces traitements se sont révélés particulièrement efficaces contre les espèces de levure responsables des contaminations œnologiques (*B. bruxellensis* incluse). Ce travail constitue la première étude à large échelle de la diversité phénotypique de la levure *B. bruxellensis* dans les niches écologiques qu'elle colonise et de ses conséquences sur le plan fondamental (adaptation aux milieux anthropisés) et appliqué (efficacité des procédés de stabilisation microbiologique).

**Mots clefs** (6 maximum): Levure ; Etude phénotypique à large échelle ; adaptation ; boissons fermentées ; assimilation des nitrates ; stabilisation microbiologique

## Abstract: Phenotypic diversity of *Brettanomyces bruxellensis* and adaptation to fermentation processes

*Brettanomyces bruxellensis* is a yeast widely associated to fermentation processes. It is considered either as a spoiler (winemaking, brewing and bioethanol process), or as a beneficial microorganism (production of specific beers or kombucha). Previous experiments showed a strong relationship between process isolation and genetic populations, suggesting niche adaptation. However, the phenotypic variability of the species was scarcely described, particularly in the light of populations and process adaptation. The first goals of this work were to study the phenotypic diversity of *B. bruxellensis* and to identify the possible phenotypic signature of adaptation to anthropic environments. A large-scale phenotyping approach was conducted on 150 strains, representative of genetic diversity, in five relevant environments. More than 30 traits related to growth and fermentation kinetics, life-history or metabolism were measured. Our results showed that the phenotypic variability is mainly impacted by the environment, followed by the genotype and the genotype environment interaction. Interestingly, some traits also vary depending on the population and could be involved in process adaptation (*i.e.* the allotriploid wine population being fitter in wine than other populations and compared to other environments). The ability to assimilate nitrate was also assessed, as this trait could support adaptation to low-nitrogen environments. Most *B. bruxellensis* strains are able to metabolize nitrate as sole nitrogen source, with the exception of two populations. A relationship was found between this ability and the copy number variation of the genes involved in nitrate metabolization, advancing our knowledge of the possible mechanisms involved in *B. bruxellensis* evolution and adaptation.

The applied aim of this work was to evaluate the consequences of *B. bruxellensis* phenotypic variability on microbial stabilization treatments. The efficiency of two innovative control methods (ultraviolet C and pulsed light, currently assessed for winemaking) was evaluated on a collection of oenological yeasts, with a focus on *B. bruxellensis*. Both treatments were deemed as particularly efficient on wine yeast spoilers.

These results contribute to a deeper understanding of the phenotypic diversity of *B. bruxellensis* yeast species in anthropic environments and its consequences from a fundamental viewpoint (adaptation to anthropized environments) and applied one (efficiency of stabilization processes).

**Keywords:** Yeast; large-scale phenotyping; adaptation; fermented beverages; nitrates assimilation; microbiological stabilization



## Résumé vulgarisé

*Brettanomyces bruxellensis* est une levure (un champignon unicellulaire), que l'on retrouve dans de nombreux procédés fermentaires comme le vin, la bière, le bioéthanol et le kombucha. Sa présence peut être délétère (diminution de la qualité organoleptique du produit, par exemple pour le vin), ou bénéfique (indispensable pour le procédé de kombucha par exemple). Son impact économique nécessite de mieux comprendre ses caractéristiques dans les différents milieux qu'elle colonise. Pour cela, nous avons étudié un grand nombre (>150) d'individus représentatifs de la population de *B. bruxellensis* dans 5 différents environnements. Nous avons observé des différences importantes selon les individus, parfois en lien avec leur environnement d'origine. Certains caractères (capacité de croissance, capacité à utiliser des nutriments atypiques comme les nitrates) pourraient expliquer la colonisation de certains procédés. Nous avons également évalué deux méthodes de décontamination microbiologique (ultraviolets de type C et lumière pulsée), et démontré leur efficacité sur les principales levures d'altération œnologique (dont *B. bruxellensis*). Cette étude très complète permet de mieux appréhender les caractéristiques de *B. bruxellensis* expliquant sa capacité à s'adapter et à contaminer de nombreux procédés.

*Brettanomyces bruxellensis* is a yeast, a unicellular fungus, widely found in fermentation processes such as winemaking, brewing, production of bioethanol and kombucha. Its presence affects greatly the quality of the product, either positively or negatively depending on the considered process. A better understanding of the characteristic of this yeast in the fermentation processes is necessary to control its impact (desirable or undesirable). We measured various characteristics of interest in >150 individuals in five distinct environments. We observed strong variations between individuals, sometimes correlated to the isolation niche. Some traits (growth capacity, ability to use atypical nutrients such as nitrate) could explain its competitiveness in fermentation processes. We also evaluated two microbiological control methods (ultraviolet C and pulsed light) and demonstrated the efficiency of both technologies on wine yeast spoilers (including *B. bruxellensis*). This work improves our understanding of *B. bruxellensis* characteristics that could explain its ability to colonize and spoil some fermentation processes.

## Communications scientifiques

### Articles publiés et en cours de rédaction

Etienne Pilard, **Jules Harrouard**, Cécile Miot-Sertier, Philippe Marullo, Warren Albertin, Rémy Ghidossi; "Wine yeast species show strong inter- and intra-specific variability in their sensitivity to ultraviolet radiation", publié dans *Food Microbiology*, 2021

**Jules Harrouard**, Chris Eberlein, Patricia Ballestra, Marguerite Dols-Lafargue, Isabelle Masneuf-Pomarede, Cécile Miot-Sertier, Joseph Schacherer, Warren Albertin; "*Brettanomyces bruxellensis*: Overview of the genetic and phenotypic diversity of an anthropized yeast", publié dans *Molecular Ecology*, 2022

**Jules Harrouard**, Etienne Pilard, Cécile Miot-Sertier, Lena Pouget, Philippe Marullo, Giovanna Ferrari, Gianpiero Pataro, Rémy Ghidossi, Warren Albertin; "Evaluating the influence of operational parameters of pulsed light on wine related yeasts: focus on inter- and intra-specific variability sensitivity", publié dans *Food Microbiology*, 2022

**Jules Harrouard**, Cecile Miot-Sertier, Pauline Puyot, Marguerite Dols-Lafargue, Isabelle Masneuf-Pomarede, Joseph Schacherer, Alexandre Pons, Warren Albertin; "Phenotypic landscape of the spoilage yeast *Brettanomyces bruxellensis* reveals variable adaptation patterns to fermentation processes"; en préparation

**Jules Harrouard**, Louise Michelizza, Cecile Miot-Sertier, Victor Loegler, Anne Friedrich, Joseph Schacherer, Emilien Peltier, Warren Albertin; "Loss of nitrate assimilation in the yeast *Brettanomyces bruxellensis* is shaped by population structure and copy number variation"; en préparation

### Communications orales

**Jules Harrouard**, Cecile Miot-Sertier, Marguerite Dols-Lafargue, Isabelle Masneuf-Pomarede, Joseph Schacherer, Warren Albertin; "Signatures of phenotypic adaptation: the case of *Brettanomyces bruxellensis*, a yeast associated to anthropized environments.", **Levures, modèles et outils**, Octobre 2021, Strasbourg, France.

**Jules Harrouard**, Cecile Miot-Sertier, Marguerite Dols-Lafargue, Isabelle Masneuf-Pomarede, Alexandre Pons, Joseph Schacherer, Warren Albertin; "Phenotypic plasticity of the yeast species *Brettanomyces bruxellensis* across fermentation processes: toward the identification of traits underlying adaptation to anthropic environments?", **36<sup>th</sup> International Specialised Symposium on Yeasts**, Juillet 2022, Vancouver, Canada.

### Prix

**Jules Harrouard**, Etienne Pilard, Cécile Miot-Sertier, Philippe Marullo, Warren Albertin, Rémy Ghidossi; "Procédé innovant de stabilisation microbiologique des vins, utilisation des UV-C", **Prix Jean Marc Gey** (qui récompense des projets dans le domaine du management QSE, du développement durable et de la responsabilité sociale), Bordeaux INP, Mai 2021, Bordeaux, France. Lauréat de la catégorie « thèse ».

### Poster

Etienne Pilard, **Jules Harrouard**, Cécile Miot-Sertier, Warren Albertin, Remy Ghidossi; "Which level of variability in UV-C sensitivity among wine related yeasts? Which impact on wine treatment efficiency?", **Macrowine 2021**, Juin 2021.

## Liste des abréviations

4-EP: 4-ethylphenol

4-EG: 4-ethylguaïacol

4-EC: 4-ethylcatechol

4-VP: 4-vinylphenol

4-VG: 4-vinylguaïacol

4-VC: 4-vinylcatechol

AFLP: Amplified Fragment Length Polymorphism

CNV: Copy Number Variation

E: Environnement

FISH: Fluorescence In Situ Hybridization

G: Génotype

GxE: Interaction Génotype – Environnement

GC-GC-TOFMS: two-dimensional Gas Chromatography combined with Time-Of-Flight Mass Spectrometry

GMGM: Génétique Moléculaire - Génomique – Microbiologie

GWAS: Genome-Wide Association Study

HCA: Hydroxycinnamic Acid

HGT: Horizontal Gene Transfert

HHP: Hydrostatic High Pressure

LC-MS: Liquide Chromatography with Mass Spectrometre

LOH: Loss Of Heterozygosity

OIV: Organisation Internationale de la vigne et du vin

PCR: Polymerase Chain Reaction

PEF: Pulsed Electric Field

PL: Pulsed Light

QTL: Quantitative Trait Loci

RAPD: Random Amplified Polymorphic DNA

REA-PFGE: Restriction Enzyme Analysis with Pulsed Field Gel Electrophoresis

SCOBY: Symbiotic Consortium Of Bacteria and Yeast

SNP: Single Nucleotide Polymorphism

UV-C: rayonnement UltraViolets de type C

VNC: Viable but Not Cultivable

WGD: Whole-Genome Duplication

# Introduction générale

« Nothing in biology makes sense except in the light of evolution » T. Dobzhansky

L'évolution n'est pas une simple théorie. En effet, depuis sa formulation par Charles Darwin, elle permet de mieux expliquer le vivant dans toute sa diversité et est considérée comme une base fondamentale en biologie. L'étude de l'évolution et de l'adaptation évolutive ont des applications directes, tant en agronomie qu'en médecine. Dans les environnements impactés ou contrôlés par l'être humain ou « environnements anthropisés », l'étude de ces phénomènes est particulièrement intéressante car les pressions de sélection sont fortes, récentes et différentes des pressions rencontrées dans les milieux naturels. Ceci permet d'identifier les signes d'adaptation acquis par les populations anthropisées en comparaison avec les populations naturelles. En effet, les espèces qui se développent dans ces environnements sont soumises à de nouvelles pressions de sélection, ce qui entraîne la sélection des individus les plus adaptés. La forme la plus extrême d'adaptation d'une espèce à un milieu anthropisé est la domestication. Dans ce cas, l'espèce volontairement utilisée dans un objectif technologique développe une certaine dépendance à l'être humain pour sa survie dans le milieu anthropisé considéré. Les plantes et les animaux ont été historiquement les plus étudiés pour leur adaptation et pour leur domestication par l'être humain. Cependant, les micro-organismes, du fait (i) de leur temps de génération court, (ii) de leur génome de petite taille et moins complexe que celui des eucaryotes pluricellulaires, et (iii) de leur grande diversité génétique et écologique, constituent une ressource biologique de qualité qui permet des études génomiques et phénotypiques comparatives sur un nombre important d'individus. De plus, comme chez certains animaux et végétaux, la domestication de certaines espèces microbiennes remonte à la sédentarisation des populations humaines, ce qui permet l'étude des mécanismes évolutifs sur des temps relativement longs.

*Brettanomyces bruxellensis* est un micro-organisme qui a été identifié dans un nombre important de procédés anthropisés. D'un côté, cette levure est considérée comme la levure majeure des défauts microbiologiques du vin et la lutte contre les défauts qu'elle cause est un problème très actuel. Son développement représente également un problème majeur pour la fabrication industrielle de bière et de bioéthanol. D'un autre côté, cette levure est aussi recherchée car associée positivement à la production de boissons telles que le kombucha, le kéfir ainsi que de certaines bières de spécialités. Cette espèce présente une grande diversité phénotypique. Une étude de génétique des populations à l'aide de marqueurs microsatellites sur 1488 souches issues d'origines géographiques et environnementales diverses (29 pays, 5 continents, 9 niches fermentaires) a permis de révéler une grande diversité génétique. Cette étude montre que les groupes génétiques de *B. bruxellensis* sont structurés par l'environnement d'origine ainsi que par la ploïdie estimée (Avramova et al., 2018a). Ces résultats ont conduit l'équipe 'Microbiologie' de l'UMR Œnologie, en collaboration avec l'université de Strasbourg (GMGM, spécialisé dans l'étude des génomes de levures), à construire un projet financé par l'Agence Nationale de la Recherche. Les objectifs de ce projet sont d'étudier la variabilité génétique et phénotypique, et d'identifier des signatures d'adaptation évolutive de la levure *B. bruxellensis* aux boissons fermentées. Dans ce projet scientifique, mes travaux de thèse se sont principalement attachés à caractériser les phénotypes de nombreuses souches dans différents procédés fermentaires. L'objectif était de mesurer la variabilité phénotypique entre différentes populations ainsi que la variabilité phénotypique des populations par le passage d'un milieu à un autre (variabilité phénotypique intraspécifique, inter-environnement, etc...). Le but de ce travail est *in fine* d'identifier des caractères phénotypiques qui seraient liés à l'adaptation de *B. bruxellensis* à certains procédés et niches. Dans un second temps, mes travaux de thèse ont permis d'explorer des pistes innovantes pour

contrôler cette levure dans le domaine de l'œnologie, en utilisant des méthodes physiques de stabilisation microbiologique (UV-C, PL).

Le premier chapitre de cette thèse est consacré à une synthèse bibliographique rassemblant les connaissances actuelles sur l'espèce *B. bruxellensis*. Dans une première partie, les différentes niches de colonisation de cette espèce et les conséquences de son développement sur ces milieux seront présentées. Une deuxième partie détaillera la diversité génétique et phénotypique de cette espèce. Ce travail de synthèse bibliographique a fait l'objet d'un article de revue publié dans le journal scientifique *Molecular Ecology*. La troisième partie de ce chapitre 1 abordera les phénomènes d'adaptation et d'évolution chez les micro-organismes, avec un focus sur l'espèce modèle *Saccharomyces cerevisiae* et une revue des indices relevés chez *B. bruxellensis* au travers de la littérature.

Le second chapitre de ce travail présentera les travaux de caractérisation phénotypique de *B. bruxellensis*. Une étude à large échelle a été réalisée sur de nombreux individus représentatifs de la diversité génétique de l'espèce (151 souches). Ces individus ont été caractérisés dans 5 environnements distincts, représentatifs des procédés associés à *B. bruxellensis* (brasserie, œnologie et kombucha). Une approche multi-échelle a été appliquée : les cinétiques de croissance et de fermentation ont été suivies, et différentes mesures (de métabolites d'intérêt, de cytométrie de flux, de colorimétrie) ont été menées en fin d'expérience. Une approche de métabolomique permettant d'identifier les composés volatils de manière non ciblée (volatolomique) a aussi été conduite sur un sous ensemble de souches pour mieux appréhender l'empreinte aromatique de cette espèce dans ces procédés. Grâce à ces données, la recherche de signaux phénotypiques d'adaptation divergente a été réalisée. Ces travaux feront l'objet d'une publication scientifique et l'article est en préparation.

Le troisième chapitre sera consacré sur le métabolisme des nitrates chez *B. bruxellensis*. Dans la littérature, plusieurs articles ont présenté la capacité d'assimilation des nitrates de *B. bruxellensis* comme une caractéristique importante permettant à cette levure d'altérer la fabrication de bioéthanol. Cependant, ce phénotype ne semble pas partagé par l'ensemble des souches de cette espèce, et les liens génotype/phénotype ne sont pas clairs. La capacité d'assimilation des nitrates des 151 souches précédemment utilisées a donc été mesurée dans un milieu contenant des nitrates comme seule source d'azote. La présence de plusieurs gènes pouvant être impliqués dans la capacité à métaboliser les nitrates a également été étudiée, grâce à la disponibilité des séquences de très nombreux génomes de *B. bruxellensis* en cours d'assemblage par l'équipe de Joseph Schacherer (laboratoire GMGM) à Strasbourg. Ces travaux feront l'objet d'une publication scientifique et l'article est en préparation.

Le quatrième chapitre de cette thèse présentera l'étude de deux méthodes de contrôle microbiologique utilisées dans l'agroalimentaire (le traitement aux rayons ultraviolets de type C (UV-C) et la lumière pulsée (PL)), afin d'évaluer leur intérêt pour l'œnologie. L'impact organoleptique négatif majeur de *B. bruxellensis* dans les vins rend nécessaire son contrôle par les professionnels de la filière viti-vinicole. L'utilisation historique des sulfites comme agent de contrôle est actuellement remise en cause par (i) une demande des consommateurs à la limitation/réduction des intrants, (ii) la réduction de leur efficacité en lien avec le changement climatique (élévation des pH qui induit une diminution de la fraction du SO<sub>2</sub> efficace sur les microorganismes), (iii) la présence de souches de *B. bruxellensis* résistantes aux doses maximales autorisées dans les vins. La filière est donc à la recherche de nouveaux procédés de contrôle des populations microbiennes. En collaboration avec nos

collègues spécialisés en génie des procédés appliqués à l'œnologie, nous avons étudié l'impact de méthodes physiques innovantes de stabilisation (UV-C et PL). Nous avons développé une méthode de criblage de l'efficacité de ces procédés sur boîte de Pétri (milieu solide). Cette méthode a permis le criblage de 14 espèces de levures œnologiques, avec un focus important sur l'espèce *B. bruxellensis* (>100 souches). Une approche de traitement en milieu liquide, sur un nombre réduit de souches, a permis de tester l'efficacité de ces deux méthodes sur du vin fortement contaminé grâce à des pilotes disponibles et/ou construits au sein du laboratoire. Ce travail a fait l'objet de deux publications, l'une sur les UV-C, l'autre sur la PL, publiées dans le journal scientifique *Food Microbiology*.

Ce travail de thèse se concentre donc sur l'étude de la variabilité phénotypique des populations de *B. bruxellensis*, dans des milieux anthropisés et sur la réponse de ces populations à de nouvelles méthodes de contrôle des populations microbiologiques (Figure 1). La compréhension de cette diversité phénotypique permettra, à terme, de mieux appréhender les relations génotype-phénotype dans différents procédés, afin de limiter (ou, au contraire de maximiser quand c'est souhaitable) le développement de cette espèce.

# PhD: Phenotypic diversity of *Brettanomyces bruxellensis* and adaptation to anthropized processes

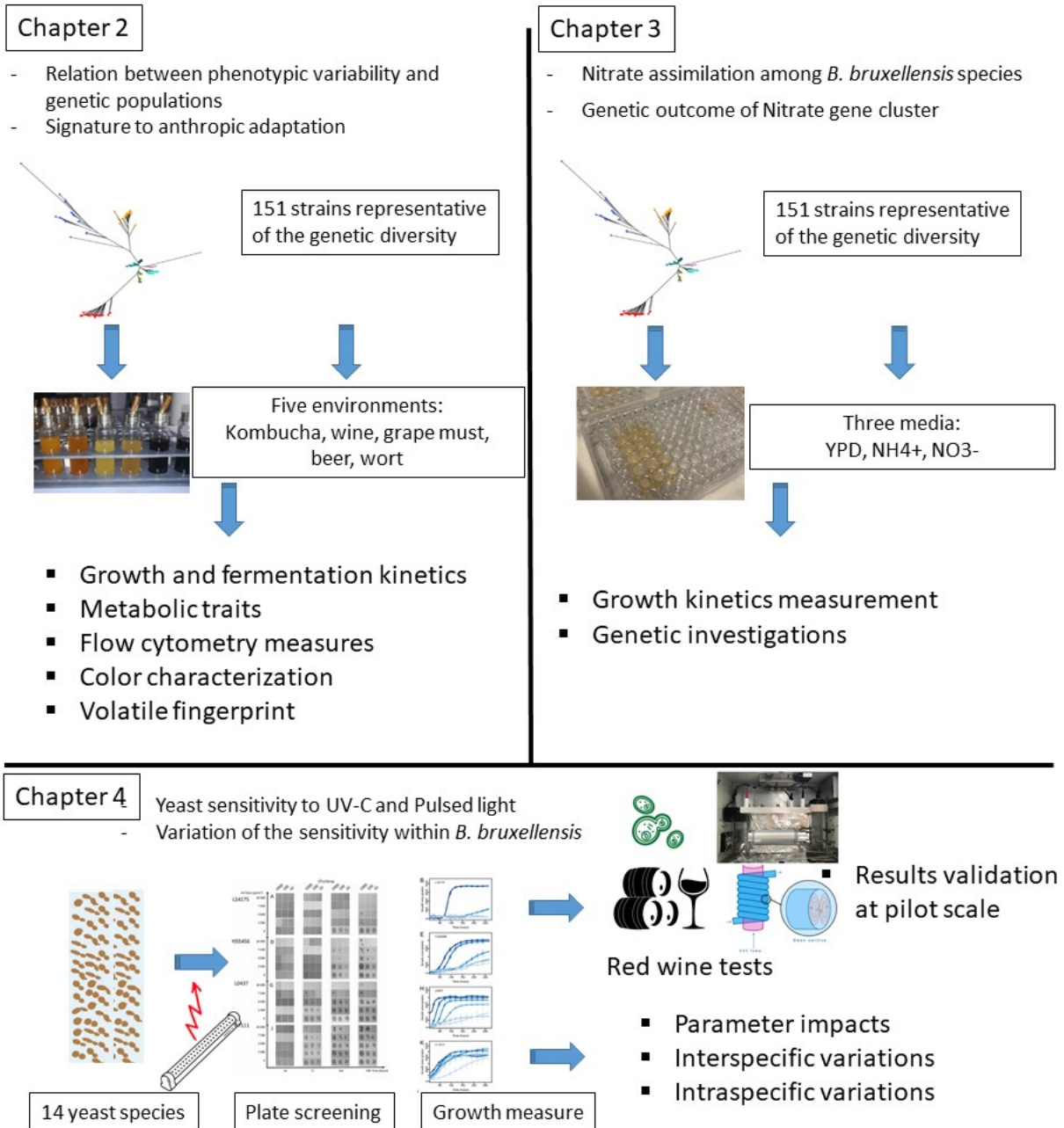


Figure 1 : Présentation schématique des principaux axes traités durant la thèse



## Chapitre 1 : Revue bibliographique

La production alimentaire étant avant tout saisonnière, le stockage et la protection des denrées alimentaires sont nécessaires. Des procédés physico-chimiques de conservation ont ainsi été développés par l'être humain au fil des âges, comme la salaison, le séchage ou encore le fumage. Cependant, ces techniques ne sont pas applicables à toutes les matières premières (et notamment les liquides). La fermentation, menée par les levures et les bactéries, est une alternative microbiologique de conservation. Elle s'accompagne d'une baisse importante du pH, liée à la production d'acides organiques et d'alcools ainsi que de composants antimicrobiens (Şanlıer et al., 2019). Les changements induits par la fermentation (principalement l'acidification) permettent la conservation des produits alimentaires et des boissons. Dans un temps où l'eau potable n'était pas toujours accessible (ce qui reste le cas dans de nombreuses régions du monde), où la gestion des déchets humains n'existait pas, la consommation de boissons fermentées, relativement « saines » du point de vue microbiologique, était un moyen pour s'hydrater sans s'intoxiquer (Selosse, 2022). C'est ainsi que la fermentation des produits alimentaires a été adoptée de manière empirique depuis la sédentarisation de l'être humain au néolithique. Les gestes nécessaires à la propagation des microbes ont été transmis de génération en génération et ont ainsi permis l'occupation des niches, sélectionnant les organismes bénéfiques. Outre la conservation des aliments, la fermentation permet d'augmenter les propriétés nutritionnelles ainsi que de détoxifier certains aliments. La fermentation du lait, du chou ou la panification par exemple, ont permis de rendre digeste des produits qui, au moment de la sédentarisation de l'être humain, ne l'étaient pas (digestion du lactose, élimination des glucosinolates dans le chou, réduction des concentrations en phytates - un facteur antinutritionnel pour l'être humain - chez les graminées) (Reale et al., 2007; Selosse, 2022). Progressivement, l'utilisation empirique des micro-organismes a joué un rôle important dans l'essor des populations agricoles sédentaires, ainsi que sur l'essor des micro-organismes eux-mêmes. En effet, les pressions de sélection retrouvées dans les milieux anthropisés sont souvent différentes des milieux naturels, fortes et régulières, ce qui peut induire des processus de divergence adaptative au sein de ces espèces microbiennes. Pour autant, les micro-organismes isolés dans les procédés fermentaires ne sont pas nécessairement adaptés à ces milieux : leur présence peut être fortuite (due au hasard), mais aussi due à une réelle adaptation à l'environnement donné, que cette adaptation soit positive pour le procédé ou négative (microorganismes sources de déviations organoleptiques, physico-chimiques, sanitaires). Seule l'étude des caractères génétiques et phénotypiques par comparaison au sein d'une espèce permet d'identifier des signes d'adaptation.

Les différentes espèces du genre *Brettanomyces* sont retrouvées dans de nombreux procédés fermentaires et/ou alimentaires comme la production de vin, de bière, de bioéthanol, des boissons à faible teneur en alcool (kombucha, kéfir), le cidre, les boissons sans alcool (soda, jus), les olives, levains, fromages, téquila (Angela et al., 2020; de Souza Liberal et al., 2007; Fadda et al., 2001; Lynch et al., 2021; Meroth et al., 2003; Morrissey et al., 2004; Neira-Vielma and Aguirre-Joya, 2020; Sonets et al., 2022; Teoh et al., 2004). Il s'agit d'une des nombreuses espèces de levures qualifiées par certains auteurs de « biotechnologiques » (Figure 2).

Dans cette partie bibliographique nous nous intéresserons aux principaux procédés fermentaires dans lesquels on retrouve *B. bruxellensis* à des niveaux de population significatifs : la production de vin, de bière, de bioéthanol et de kombucha. Nous détaillerons les effets de cette levure sur la qualité du produit final. Dans une deuxième partie, nous discuterons les particularités génétiques et phénotypiques de cette levure. Nous nous attacherons à mettre en lien la diversité phénotypique trop souvent sous-estimée et encore peu étudiée, avec la diversité génétique récemment mise en lumière.

De plus, une attention particulière sera portée sur l'écologie de cette espèce. Cette partie, qui correspond à un travail publié dans *Molecular Ecology* se termine par une ouverture sur les signes plausibles d'adaptation de *B. bruxellensis* aux différentes niches colonisées. Dans une troisième partie, les mécanismes d'adaptation seront détaillés en s'appuyant sur les études menées chez l'espèce modèle *Saccharomyces cerevisiae* et quelques données de la littérature obtenues chez *B. bruxellensis*.

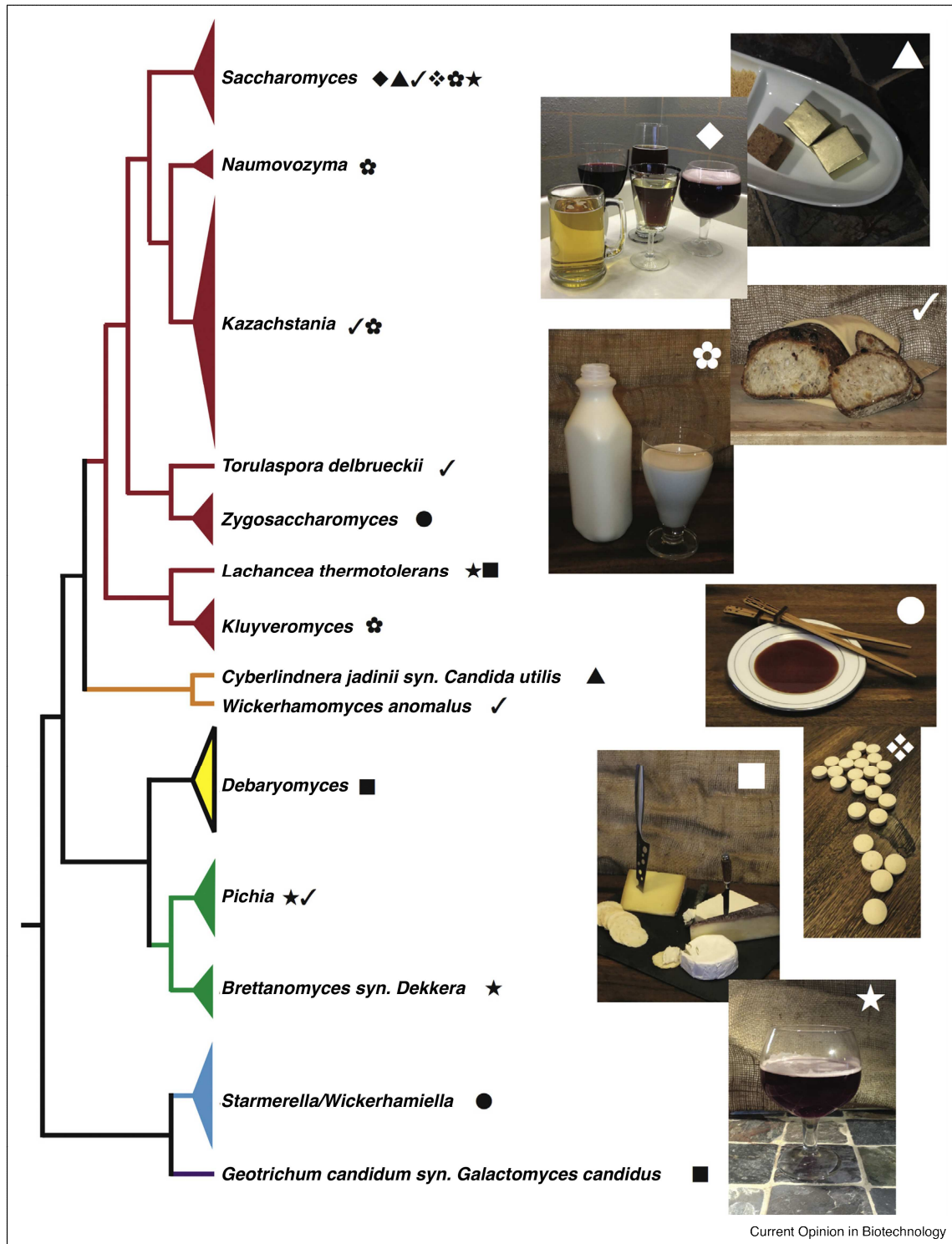


Figure 2 : Arbre phylogénétique des espèces de levures biotechnologiques. D'après Hittinger et al., (2018).

/ Les niches écologiques anthropisées colonisées par *Brettanomyces bruxellensis* :

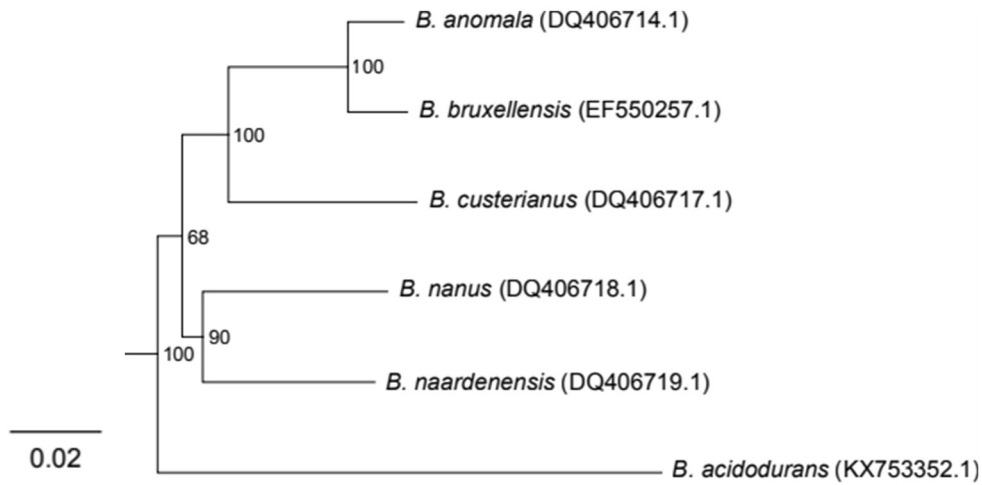


Figure 3 : Arbre de distances génétiques entre les 6 espèces du genre *Brettanomyces*. Les distances sont calculées à partir des séquences des gènes ribosomiques 26S. Adapté de Harrouard et al., (2022).

Parmi les six espèces qui composent le genre *Brettanomyces* (Figure 3), c'est l'espèce *Brettanomyces bruxellensis* qui a reçu le plus d'attention de la part des scientifiques mais aussi des industriels et du public. D'un point de vue chronologique, la littérature scientifique s'est d'abord intéressée de façon comparable à l'implication de *B. bruxellensis* dans le vin et la bière et ce, jusqu'aux années 2000 (Figure 4). Plus récemment, deux autres produits fermentaires, le bioéthanol et le kombucha ont été associés aux recherches sur *B. bruxellensis* ce qui a amené des informations nouvelles et complémentaires. Dans cette partie, nous nous intéresserons à ces quatre principaux produits dans lesquels l'espèce *B. bruxellensis* est régulièrement rencontrée. Une partie sera dédiée aux méthodes de contrôle de cette levure principalement dans le milieu œnologique, dans lequel elle est considérée comme un agent d'altération redoutable.

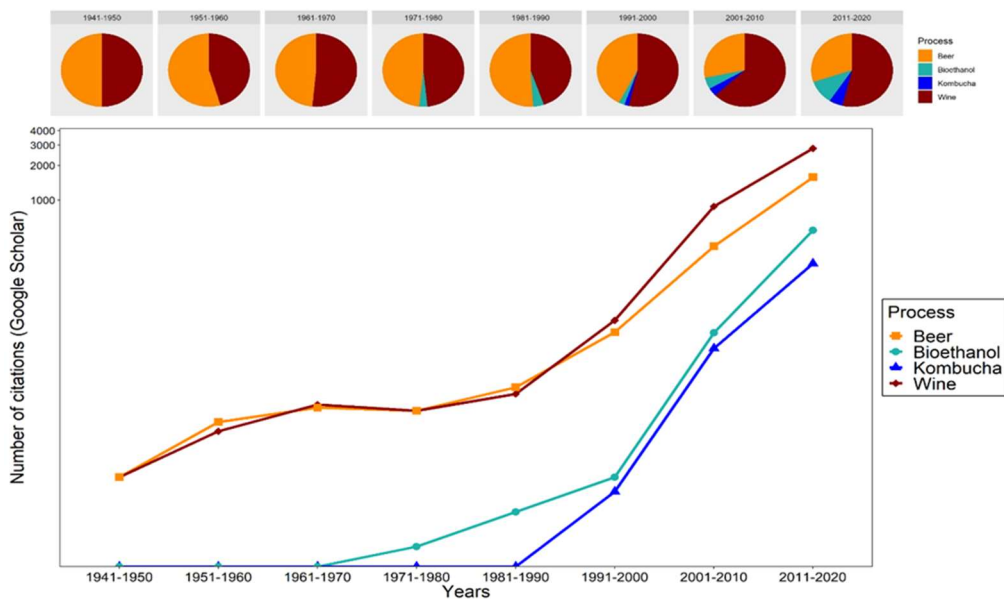


Figure 4 : Nombre de citations contenant [*Brettanomyces* or *Dekkera*] & [*bruxellensis*] & [nom du produit], par fenêtre de 10 ans, source Google Scholar (réalisé en Septembre 2020).

“Brett” et la filière œnologique

“If we let *Brettanomyces* grow in every red wine, whatever the variety and the geographic origin, all the wines will smell the same”. White, 2018 <https://daily.seventy.com/the-everything-guide-to-brettanomyces/>

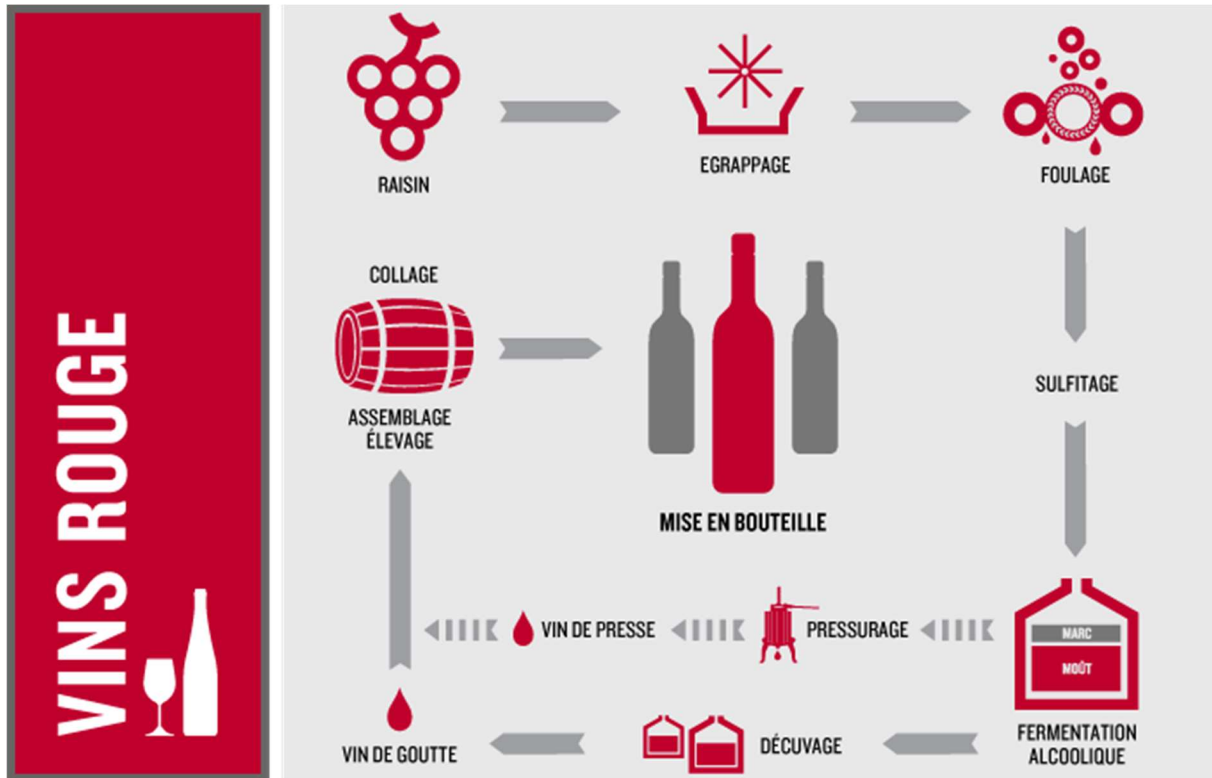


Figure 5: Schéma des principales étapes de la fabrication du vin rouge (<https://www.lapetitegoutte.fr/alcools/vins/>).

Le vin est une boisson millénaire, réalisée à partir de baies de raisin selon un procédé mettant en jeu des microorganismes et contrôlé par l'être humain (Figure 5). « Le vin est exclusivement la boisson résultant de la fermentation alcoolique complète ou partielle du raisin frais foulé ou non ou du moût de raisin » définit l'OIV (Organisation Internationale de la vigne et du vin). Le sucre présent dans les baies de raisin est transformé en éthanol par des levures. Ce processus est appelé « fermentation alcoolique » et permet la conservation du jus de raisin ainsi que l'augmentation de la richesse aromatique de ce dernier. Ces levures (principalement *S. cerevisiae*) peuvent être naturellement présentes sur les baies de raisin ou apportées par le vinificateur (pratique du levurage, réalisation de pieds-de-cuve, etc.) (Barata et al., 2012). Néanmoins, certaines espèces microbiennes peuvent altérer cette boisson, et ce, à chaque étape de la vinification (Loureiro and Malfeito-Ferreira, 2003). Surnommée « Brett » par les acteurs de la filière, *B. bruxellensis* est la levure la plus redoutée pour son impact organoleptique important et sa présence ubiquitaire en œnologie (e.g. tous les continents, toutes les étapes de la vinification). Cette espèce est très majoritairement isolée dans les vins rouges, bien que sa présence soit également notée dans des vins blancs (Dias et al., 2003a; Licker et al., 1998). Cependant, la contamination des vins blancs est peu rapportée (soit peu fréquente soit peu détectée), alors que les précurseurs des composés à l'origine de l'altération organoleptique sont aussi présents dans les vins blancs à des concentrations comparables à celles rencontrées dans les rouges. Ceci pourrait être lié à la méthode de vinification : ajout de  $\text{SO}_2$  plus important, pH et température plus

faibles après fermentation alcoolique, modalités de vieillissement et concentrations en tanins et en anthocyanes très différentes et pouvant limiter la croissance de *B. bruxellensis* (Loureiro and Malfeito-Ferreira, 2003; Malfeito-Ferreira and Silva, 2019). L'étude des capacités de croissance et d'altération des vins blancs par *B. bruxellensis* mériterait des investigations supplémentaires, et pourrait par comparaison permettre de mieux comprendre l'impact de cette levure sur le vin rouge. Néanmoins, la suite de cette partie ne concernera que le vin rouge.

Il n'existe pas de définition précise de ce qu'est un défaut du vin. En effet, la perception négative va dépendre de trois paramètres : la concentration de la(les) molécule(s) qui compose(nt) ce défaut, la matrice dans laquelle le défaut est étudié ainsi que la sensibilité du dégustateur. Dans le *Handbook* de dégustation de R. Jackson, un paragraphe y est consacré : "*Wine faults are like grammatical errors, that is, designated as such by consensus. Nevertheless, off-odors tend to have a common property. They tend to mask the wine's fragrance. Examples include the suppression induced by trichloroanisole (TCA) (Takeuchi et al., 2013) and Brett taints (Licker et al., 1999; Botha, 2010)*" (Jackson, 2017). Un défaut dans le vin sera donc lié à la présence d'un ou plusieurs composés volatiles qui auront tendance à masquer l'arôme du vin, affectant l'expression de l'origine géographique et du fruit et l'empreinte du travail du vinificateur, c'est-à-dire l'expression du terroir (Figure 6).



Figure 6: Infographie des principaux défauts volatiles des vins rouges (<https://www.wineselectors.com.au/>).

Depuis plus de 50 ans, le développement de connaissances, d'outils et de technologies liés à la fabrication du vin a permis d'améliorer considérablement la qualité globale des vins à travers le monde. Du côté des consommateurs, les attentes ont également fortement évolué ces dernières années. Les consommateurs sont passés d'une consommation journalière à une consommation moins fréquente mais plus hédoniste, avec une attente forte sur la qualité du produit. La part des consommateurs se considérant comme « néophytes » a fortement diminuée au profit des « amateurs éclairés », ces dix dernières années en France (Baromètre SOWINE/Dynata 2022). Concernant l'acte d'achat, les études menées auprès des consommateurs indiquent une vraie volonté de payer davantage pour des vins sans intrants et/ou respectant un label environnemental (Amato et al., 2017; Pelonnier-Magimel et al., 2020) (Figure 7).



Figure 7: Illustration de la volonté des consommateurs de payer des produits qui portent un label environnemental. D'après le baromètre SOWINE/DYNATA de 2022.

*B. bruxellensis* est responsable de la production de molécules associées à des arômes décrits par les professionnels du métier comme « basse-cour », « cuir », « sueur de cheval », « plastique brûlé » ou encore « clou de girofle ». Ces descripteurs sont liés à la production de phénols volatils et provoquent le rejet des consommateurs (Chatonnet et al., 1992). Le terme "phénols volatils" comprend plusieurs molécules : 4-ethylphenol (4-EP), 4-ethylguaïacol (4-EG) et 4-ethylcatechol (4-EC) ainsi que leur forme vinyle respective (4-VP, 4-VG, 4-VC) (Figure 8) (Kheir et al., 2013; Suárez et al., 2007). Les seuils de rejets (seuil à partir duquel 50% des dégustateurs considèrent l'arôme comme défaut) établis par Chatonnet et al., 1992, sont de 620µg/L et 140µg/L pour le 4-EP et le 4-EG respectivement. Les concentrations de 4-EC retrouvées dans le vin sont plus faibles (que leur seuil de rejet) et son étude est donc plus marginale. La présence de ces molécules à des niveaux de concentration supérieurs à ce seuil masque la fraîcheur, le fruité et la typicité du vin (Chatonnet et al., 1992; Tempere et al., 2016).

Les phénols volatils sont issus de la décarboxylation de précurseurs naturellement présents dans le vin provenant des baies de raisin : les acides hydroxycinnamiques (HCAs). Ces précurseurs sont retrouvés en quantité variable dans les moûts et les vins, modulant le niveau possible d'altération par *B. bruxellensis*. Les HCAs sont libérés à partir de précurseurs d'esters liés aux acides cinnamiques présents dans les cellules du raisin par réaction chimique ou enzymatique (*p*-coumaric acid, ferulic acid and cinnamic acid). Lorsqu'elle est chimique, l'hydrolyse des esters d'acide tartrique libère des HCAs au cours de la fermentation et du vieillissement. Lorsque la réaction est enzymatique, elle fait intervenir une activité cinnamyl estérase (que l'on trouve notamment chez *Oenococcus oeni*, chez les levures ou pouvant provenir de préparations enzymatiques commerciales ajoutée lors de la fermentation mais non présente chez *B. bruxellensis*) (Morata et al., 2013). Dans un second temps, les HCAs libres (acide *p*-coumarique, acide férulique, acide caféique) sont décarboxylés en hydroxystyrènes (4-vinylphénol, 4-vinylguaïacol et 4-vinylcatechol) par une cinnamate décarboxylase (Cdp) codée par le gène *PAD1* que l'on trouve dans la plupart des levures et espèces de bactéries (Godoy et al., 2014, 2008; González et al., 2017). Dans un troisième temps, les hydroxystyrènes sont ensuite réduits en dérivés d'éthyl (4-



éthylphénol, 4-éthylguaïacol, and 4-éthylcatéchol) grâce à l'enzyme vinylphénol réductase (Vprp). Très peu d'espèces possèdent cette activité enzymatique en dehors de *B. bruxellensis* (Dias et al., 2003a; Holt et al., 2018). La vinylphénol reductase (Vprp) catalyse une réaction qui consomme une molécule de NADH, et son activité, comme la vitesse globale de production de phénols, pourrait être modulée par l'éthanol et le SO<sub>2</sub> présents (Barata et al., 2008; Benito et al., 2009; Tchobanov et al., 2008). Une autre particularité de cette enzyme est l'existence d'un domaine présentant une activité enzymatique superoxide dismutase (*Sod*) (Granato et al., 2015). Cette activité pourrait diminuer le stress oxydatif provoqué par la présence de SO<sub>2</sub> (Granato et al., 2015). La production de ces molécules par *B. bruxellensis* a lieu principalement pendant la phase exponentielle de croissance ainsi qu'au début de la phase stationnaire. Visualiser la production de phénols sur des temps courts (1 mois) nécessite en effet des niveaux de populations importants, mais les liens entre production et croissance n'ont pas été clairement établis (Agnolucci et al., 2010; Cibrario et al., 2020a; Dias et al., 2003b; Schopp et al., 2013).

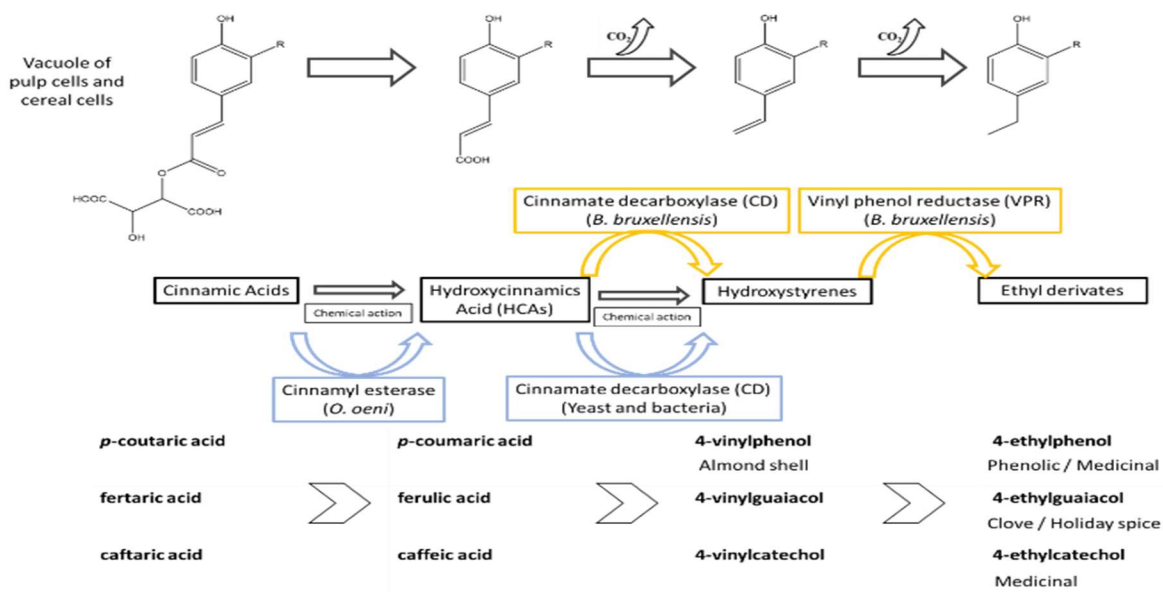


Figure 8: Conversion des acides cinnamiques en dérivés d'éthyles au travers d'hydrolyse chimiques et biologiques. Les réactions en jaune sont réalisées par *B. bruxellensis*. Les réactions en bleu peuvent être réalisées par d'autres organismes que *B. bruxellensis*.

La production de ces molécules serait variable entre les souches. Lors d'études menées en milieu modèle enrichi en précurseur et dans lequel les 33 souches étudiées parvenaient à survivre, les vitesses de production variaient en effet de 1 à 40 (Cibrario et al., 2020a). Toutes les souches étudiées présentaient donc cette activité métabolique et aucune différence claire entre groupes génétiques ou niche d'origine n'était perceptible, contrairement à ce que suggéraient d'autres auteurs (Lentz and Harris, 2015). Pour ces derniers, les isolats du vin semblaient plus efficaces que ceux de bière en termes de production de 4-EP. Néanmoins, 5 souches seulement avaient été incluses dans cette étude (Lentz and Harris, 2015).

En dehors des phénols volatils, *B. bruxellensis* produit de nombreuses molécules, notamment des esters et principalement des esters ethyliques (acétate d'éthyle, lactate d'éthyle, caprate d'éthyle...) (Figure 9) (Crauwels et al., 2015; Olaniran et al., 2017). *B. bruxellensis* modifie aussi l'acidité volatile du vin *via* la production de grandes quantités d'acide acétique (>1g/L) (Vigentini et al., 2008). Cette production d'acide acétique varierait selon la disponibilité de l'oxygène, la croissance de *B. bruxellensis*

ainsi que la souche elle-même (forte hétérogénéité au niveau des individus) (Aguilar Uscanga et al., 2003; Freer, 2002; Joseph et al., 2013; Leite et al., 2012). Un autre défaut du vin peut être aussi imputé (au moins en partie) à *B. bruxellensis* : « le goût de souris » (Grbin and Henschke, 2000). Il s'agit d'une déviation organoleptique qui serait due à 3 molécules : la 2-ethyltetrahydropyridine (ETHP), 2-acetyltetrahydropyridine (ATHP), et 2-acetylpyrroline (APY), synthétisés à partir d'acides aminés, dont 2 (l'ETHP et l'ATHP) peuvent être synthétisée par *B. bruxellensis* (pour revue : (Snowdon et al., 2006)). D'autres micro-organismes comme des bactéries lactiques pourraient aussi produire ce défaut (Heresztyn, 1986). Cependant, les raisons de la formation de ce « goût de souris » restent mal comprises, et les facteurs biotiques et abiotiques à son origine sont en cours d'étude au sein de l'UMR Œnologie notamment (thèse Pierre Moulis).

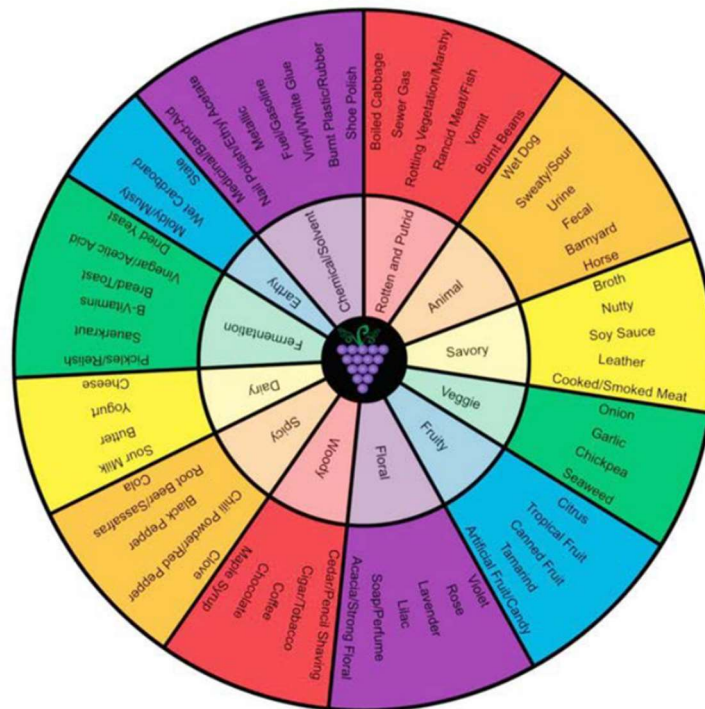


Figure 9 : Roue des arômes des descripteurs associés à *Brettanomyces* dans un contexte œnologique. D'après Joseph et al., (2017).

*B. bruxellensis* présente souvent des niveaux de population faibles, ce qui complique sa détection ainsi que son identification dans des environnements présentant par ailleurs une grande diversité microbienne (Oro et al., 2019). Sa croissance et l'altération qui s'ensuit dans le milieu œnologique, interviennent souvent après la fermentation alcoolique, avant ou après la fermentation malolactique, durant l'élevage en barrique ou après la mise en bouteille (Coulon et al., 2010; Vigentini et al., 2008). Cependant, certains praticiens rapportent des populations importantes de *B. bruxellensis* dès la fin de la fermentation alcoolique, notamment lorsque celle-ci est languissante, mais aussi en début de fermentation dans le sud de la France. Selon des observations empiriques, la fréquence de ces contaminations atypiques semble augmenter ; ceci pourrait être lié à l'évolution des pratiques (réduction importante des doses de  $\text{SO}_2$  utilisées) ou encore au changement climatique.

Il existe un débat quant à l'origine des contaminations du vin par cette levure : vignoble ou chai ? Au chai, *B. bruxellensis* est capable de pénétrer dans le bois des barriques dans les réseaux de xylèmes jusqu'à 8mm de profondeur (Fabrizio et al., 2015). Des différences peuvent être observées suivant l'espèce de bois utilisée, la chauffe du bois et l'âge de la barrique (Cartwright et al., 2018; Cartwright

and Edwards, 2020). Ainsi, des cas de contamination entre chais peuvent avoir lieu, lors de la réutilisation de tonneaux échangés entre domaines viticoles, une pratique fréquente en œnologie. Par ailleurs, la capacité de *B. bruxellensis* à survivre sur de longues périodes dans le vin, dans les barriques, sa capacité à supporter de forts taux d'alcool, ainsi que sa capacité de persistance sur les équipements (pompes, lignes de transfert) grâce à divers mécanismes physiologiques (biofilm, croissance invasive, capacité d'entrer dans un état Viable mais Non Cultivable (VNC)), sont autant d'éléments en faveur de l'hypothèse d'une présence rémanente au chai (Lebleux et al., 2020; Serpaggi et al., 2012). Des cas de contaminations par une même souche pendant plusieurs années de bouteilles de vins indiquent une capacité de survie importante dans cet environnement (Cibrario et al., 2020a). Cependant, la présence de *B. bruxellensis* a également été rapportée, bien qu'en faible quantité, sur des grappes de raisins (Oro et al., 2019; Renouf et al., 2007).

La grande fréquence de contamination des vins suggère que l'impact négatif de *B. bruxellensis* est important sur l'économie de la filière viti-vinicole. Cependant dans la littérature, il n'existe que très peu de données chiffrées au sujet de l'impact financier de cette levure. *B. bruxellensis* impacterait entre 20-25% de la production de vin rouge dans le monde (Gerbaux et al., 2000; Romano et al., 2008). Une récente étude sur trois vignobles californiens a tenté de quantifier les pertes, qui peuvent s'étaler entre 0.1-2% de la valeur marchande sur l'année 2013, valeur qui serait sous-estimée d'après l'auteur (Alston et al., 2021). Dans cette même étude, 181 professionnels répartis à travers le monde ont répondu à un questionnaire concernant la contamination par cette levure. La majorité des participants étaient dans une démarche de lutte active et d'élimination de *B. bruxellensis* quelle que soit la taille ou la géographie des productions (Alston et al., 2021). En France, 36% des vins rouges de Bourgogne contiennent une quantité de phénol volatils supérieure au seuil de perception (BIVB 2017). Une étude sur 10 000 vins a montré que 15,7% des vins à défauts présentaient une importante concentration en phénols volatils (Goode and Harrop, 2008). Le manque de données chiffrées s'explique probablement par les professionnels eux-mêmes, qui sont réticents à déclarer les problèmes de contamination par *B. bruxellensis* au sein de leur établissement. En effet, ces contaminations étaient historiquement associées exclusivement à des déficiences dans l'hygiène des chais. Les méthodes de détection et de contrôle classiquement utilisées par les praticiens pour (tenter de) maîtriser l'altération par *B. bruxellensis* seront détaillées à la fin de cette partie sur les environnements colonisés.

Implication de *B. bruxellensis* dans la filière brassicole

*B. bruxellensis* est une levure bien connue aussi dans le monde brassicole, et ce aussi bien pour son apport qualitatif que pour sa capacité d'altération. En effet, *B. bruxellensis* a été isolée pour la première fois à partir de bière, en 1904, par le laboratoire de la compagnie brassicole danoise Carlsberg (Claussen, 1904). L'objectif était de déterminer la provenance d'arômes spécifiques aux bières anglaises ; ceci lui a valu son nom de genre (« *Brettano* » et « *myces* »). Son nom d'espèce (« *bruxellensis* ») est à rapprocher de sa présence dans des bières de spécialité belges, typiques de la région bruxelloise. Grâce aux arômes spécifiques (très fruités) produits par *B. bruxellensis*, celle-ci fut le premier micro-organisme breveté (Claussen, 1904). En plus de son rôle dans l'élaboration de bières belges de spécialité (Lambic, Flanders Red, Brown ales), *B. bruxellensis* a été identifiée dans des bières de spécialités américaines (les « coolships ales ») (Bokulich et al., 2012; Claussen, 1904; De Roos and De Vuyst, 2019). Dans la plupart des cas, il s'agit de bières issues de fermentation spontanée et brassées dans des cuves ouvertes. La fermentation est menée sur plusieurs mois en trois phases successives (De Roos and De Vuyst, 2019; Spitaels et al., 2014). La première phase fait intervenir des levures et bactéries non tolérantes à l'éthanol qui produisent une grande variété de composés acides et volatils. La seconde phase consiste en (i) la conversion des sucres fermentescibles simples en éthanol par *Saccharomyces* spp., et ensuite (ii) la production d'acides par des bactéries, principalement lactiques (Tyakht et al., 2021). Enfin, la dernière phase est l'étape de maturation. Elle a lieu habituellement dans des tonneaux de bois pendant de longs mois et elle est menée principalement par *B. bruxellensis* qui va jouer deux rôles : (i) la « super-atténuation » de la bière, grâce à une forte activité  $\alpha$ -glucosidase (e.g. consommation des sucres les plus complexes venant du malt : maltotriose, maltotétraose et dextrines) et (ii) la production de composés volatils (Holt et al., 2019; Kumara et al., 1993; Serra Colomer et al., 2019). Ainsi, dans ces bières de fermentation spontanée, *B. bruxellensis* consomme les sucres résiduels (diminuant la sensation de « douceur ») et modifie les propriétés organoleptiques du produit (production d'arômes spécifiques incluant les phénols volatils, des esters ; acidification des bières par production d'acide acétique).

*B. bruxellensis* est aussi retrouvée dans d'autres types de bières (hors fermentation spontanée). Ces 30 dernières années, en réponse à la standardisation de la bière opérée par les grandes multinationales productrices, la fabrication de la bière s'est démocratisée, entraînant l'essor des microbrasseries (pour revue : Baiano, 2021; Garavaglia and Swinnen, 2018). Débuté au Royaume-Uni dans les années 1980, ce mouvement va trouver un fort écho aux Etats Unis (« Homebrewer ») avec la création d'unités de brassage « à la maison » avec une consommation pour la famille (limitation de brassage légale à 379 L/an/adulte), puis revenir en Europe pour largement s'y diffuser (Tableau 1, Figure 10) (Materna et al., 2021).

Tableau 1: Nombre de microbrasseries entre 1985 et 2015 dans les principaux pays producteurs, d'après Garavaglia and Swinnen, (2018).

Country	1985	1990	1995	2000	2005	2010	2015
Australia	3	34	26	43	93	172	358
Canada	4 <sup>a</sup>	33				277	610
Colombia			5	4	7	39	118
Germany <sup>b</sup>	894	867	1005	1024	1062	1112	1148
Germany <sup>c</sup>	632	639	759	844	894	987	1058
Hungary				110	90	50	64
Italy		1	7	60	132	311	670
Japan			17	302	256	208	222
Netherlands	7	15	30	51	78	115	380
Slovakia						14	45
Spain		1	2			46	409
USA	37	249	998	1469	1591	1756	3490

Notes<sup>a</sup>indicates that the number of microbreweries in 1984 was used Germany<sup>b</sup> and Germany<sup>c</sup> are the number of microbreweries if micros are defined, respectively, as "breweries that produce less than half of average production" and "breweries with yearly production <10.000 hl"

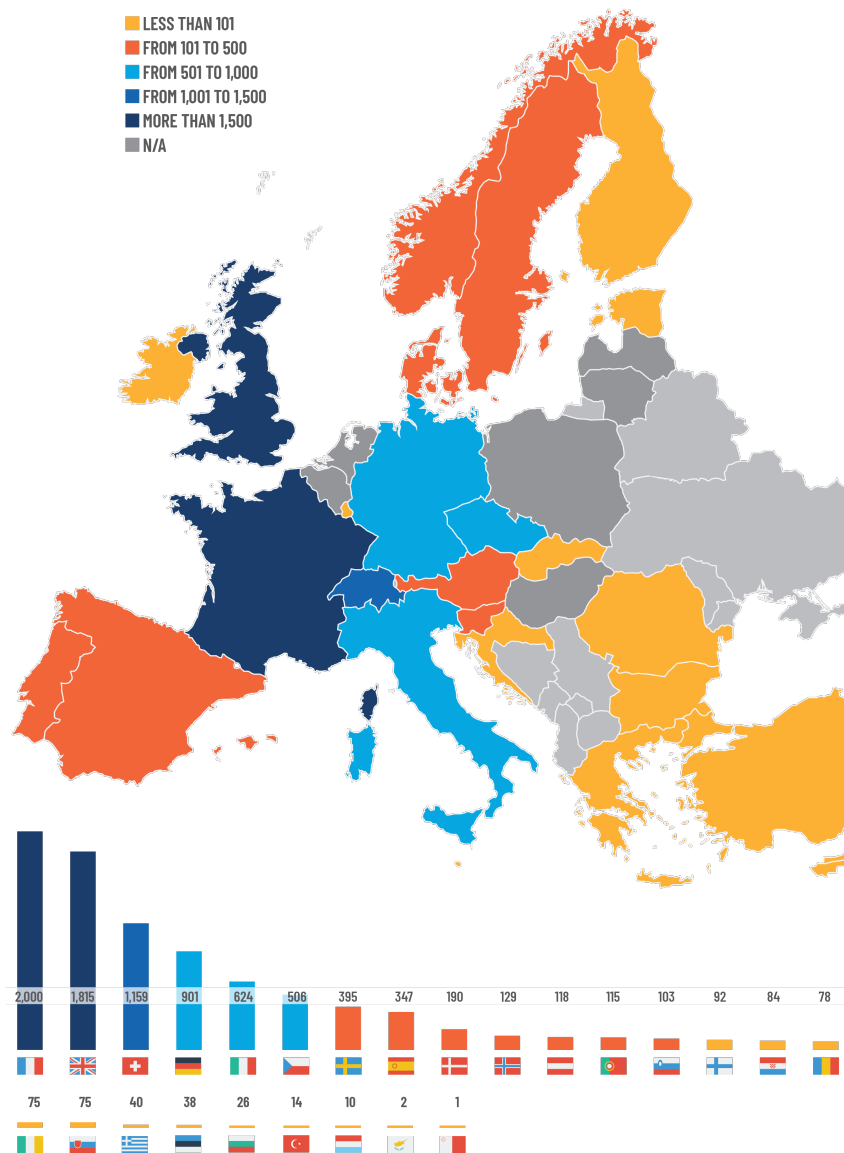


Figure 10: Répartition des microbrasseries en Europe en 2020. D'après 'The brewers of Europe' (2020). (<https://brewersofeurope.org/site/index.php>)

Avec ce développement, les consommateurs, demandeurs de produits locaux, vont découvrir des recettes originales remises au goût du jour (India Pale Ale - IPA - par exemple), permises par la flexibilité de ces petites unités de production. Dans la recherche d'originalité aromatique, les (micro) brasseurs diversifient leurs pratiques et s'émancipent de l'hégémonie du genre *Saccharomyces*. C'est ainsi que des espèces non-conventionnelles, souvent dénommées « non-*Saccharomyces* » ou « non-Sacchs », sont aujourd'hui utilisées pour produire des bières avec des arômes originaux. Le criblage d'espèces d'intérêt pour la brasserie est fréquent dans la littérature récente (pour revue : Basso et al., 2016; Canonico et al., 2019; Capece et al., 2018; Iattici et al., 2020). L'attrait persistant des consommateurs pour les bières acides ou acidulées (« funky beer », « sour beer ») a conduit les brasseurs à redécouvrir *B. bruxellensis* pour sa production d'acide acétique et de composés phénoliques (pour revue : Serra Colomer et al., 2019). *B. bruxellensis* peut être utilisée seule, pour mener l'entièreté de la fermentation alcoolique, ou bien en co-culture, en tant que second fermenteur, suivant le même principe que lors de la production des bières de fermentation spontanée, c'est-à-dire après la fermentation principale menée par *Saccharomyces* spp. (Holt et al., 2018; Serra Colomer et al., 2020b; Tyrawa et al., 2019). Des souches de *B. bruxellensis* pour les brasseurs sont commercialisées par plusieurs compagnies, souvent sous forme de crème de levure (Wyeastlab, Whitelabs par exemple).

Les esters produits par *B. bruxellensis* (esters éthyliques principalement) contribuent à l'arôme fruité, qui est le marqueur des bières Kriek belges (Crauwels et al., 2015; Olaniran et al., 2017; Tyrawa et al., 2019; Van Oevelen et al., 1977). Néanmoins, certaines souches de *B. bruxellensis* possèdent une activité estérase, ce qui peut réduire leur impact organoleptique (Steensels et al., 2015). *B. bruxellensis* présente aussi une forte activité  $\beta$ -glucosidase qui permet l'hydrolyse de glycoconjugués végétaux (Crauwels et al., 2014; Kuo et al., 2018; Vervoort et al., 2016). Les molécules d'aglycones volatiles ainsi libérées peuvent moduler l'arôme de la boisson (comme le linalol par exemple, à l'odeur florale) (Figure 11). Cette activité  $\beta$ -glucosidase permet aussi de libérer des composés volatils d'intérêt à partir du houblon ou de fruits (cerise par exemple) dans la bière (Crauwels et al., 2017; Haslbeck et al., 2017). Cependant, l'impact réel de cette activité enzymatique reste discuté car aucune corrélation n'a été observée entre l'activité  $\beta$ -glucosidase de plusieurs souches de *B. bruxellensis* et la quantité d'aglycone retrouvée dans la bière (Serra Colomer et al., 2020b). L'activité  $\beta$ -glucosidase des levures serait par ailleurs très sensible à la présence de sucres (glucose) et à l'acidification du milieu (Hernández et al., 2003). Quoi qu'il en soit, l'engouement récent pour le développement de bières « originales » permet de s'intéresser davantage à *B. bruxellensis* dans le milieu brassicole et de mieux délimiter son impact organoleptique.

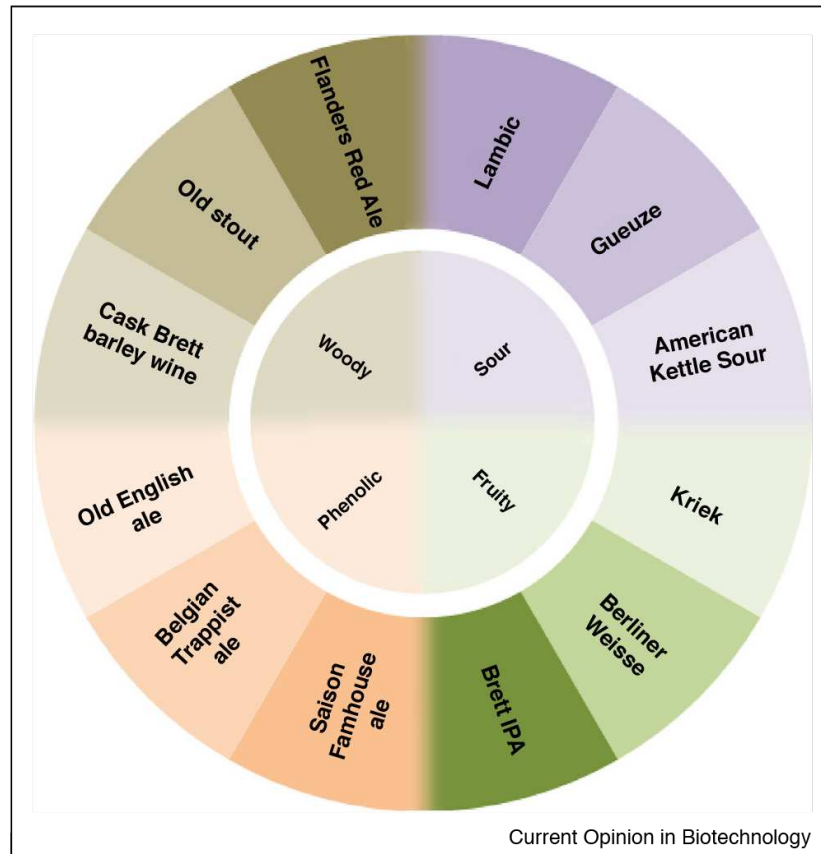


Figure 11: Les styles de bière les plus communs auxquels *B. bruxellensis* contribue. Les types de bières ont été rangées suivant leurs principales caractéristiques. D'après Serra Colomer et al., (2019).

Néanmoins, *B. bruxellensis* n'est pas toujours vue positivement par la filière brassicole : il s'agit aussi d'une levure redoutée (pour revue : Suiker and Wösten, 2022). Dans les bières « ale » et « lager » modernes, la fermentation alcoolique est conduite par *S. cerevisiae* ou *S. pastorianus* comme seule levure. Dans ces cas-là, tout autre microorganisme (comme *B. bruxellensis*) est vu comme une contamination, pouvant causer des arrêts ou ralentissement de fermentations, affectant la turbidité, la filtrabilité mais aussi les arômes de la bière (Holt et al., 2018). Ces défauts entraînent un manque d'uniformité dans la production, un rejet des consommateurs et donc conduisent bien souvent à une perte économique pour le brasseur. La capacité de *B. bruxellensis* à consommer les sucres complexes du moût de bière (*wort*), sa résistance à l'éthanol ainsi que sa persistance dans les procédés de fermentation contribuent comme dans le vin à sa problématique dans les productions brassicoles plus conventionnelles, petites ou grandes. C'est ainsi que malgré l'engouement de certains consommateurs pour les bières « Brettées », les brasseurs restent réservés quant à son utilisation. Bien que peu d'études scientifiques n'aient traité ce sujet, il est connu de manière empirique chez les brasseurs que l'utilisation de *B. bruxellensis* peut entraîner des contaminations importantes et pour une longue durée sur l'ensemble de la chaîne de production d'une brasserie. Certains microbrasseurs se refusent donc à « faire entrer le loup dans la bergerie ».

*B. bruxellensis* joue donc un rôle à part en brasserie, tantôt positif, tantôt négatif. De manière anecdotique, un festival annuel de bières « uncommon » lui est consacré à Amsterdam (*Carnivale Brettanomyces*) et dont le slogan est « Brett will eat everything » (Figure 12).

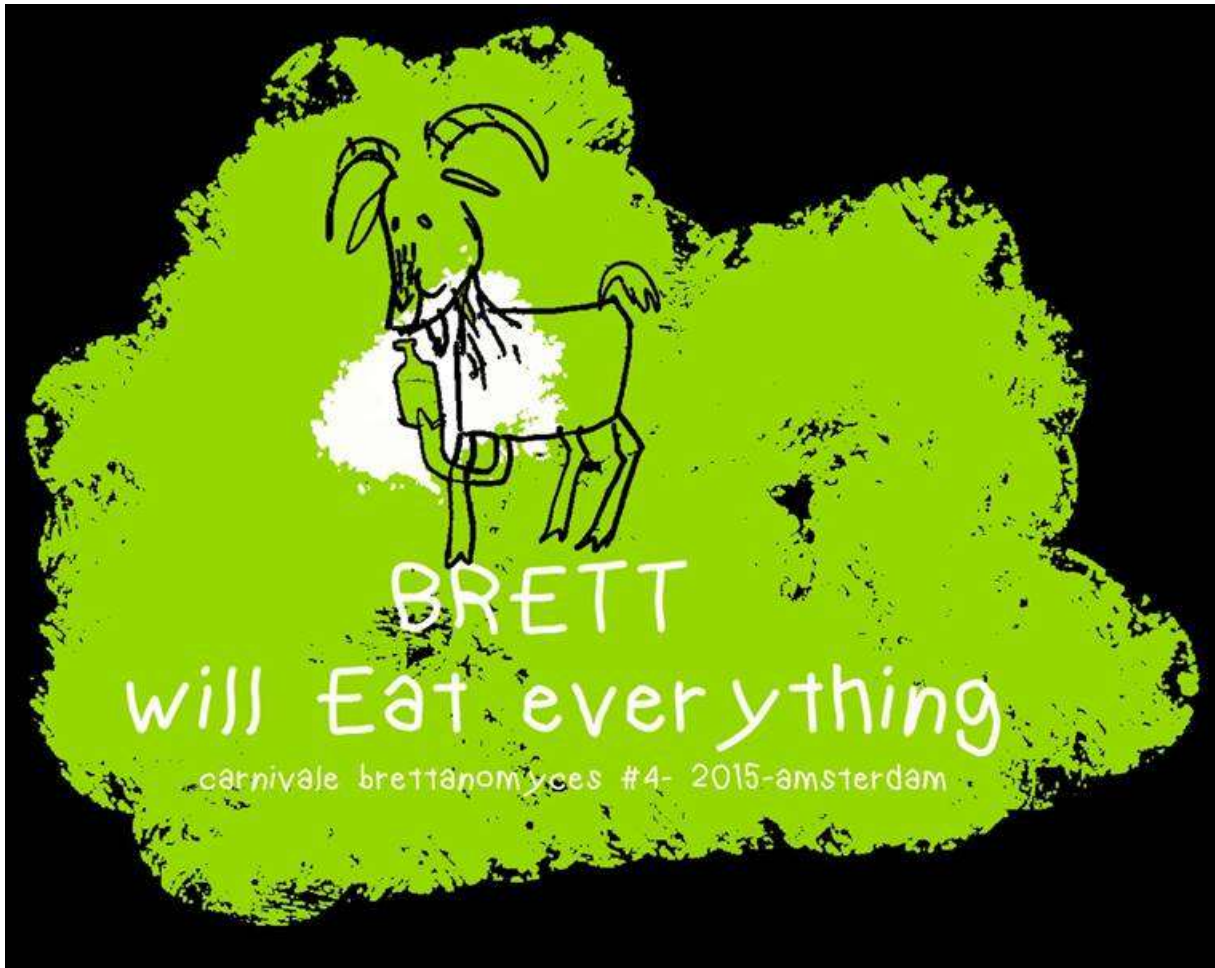


Figure 12: Poster du festival `Carnival Brettanomyces` de 2015 qui s'est tenu à Amsterdam. La chèvre représente la vision des brasseurs de *B. bruxellensis*, une levure capable de tout manger. Avec la permission de Jan Lemmens ([www.wildegist.nl](http://www.wildegist.nl)).



Filière bio-éthanol : de contamination à auxiliaire ?

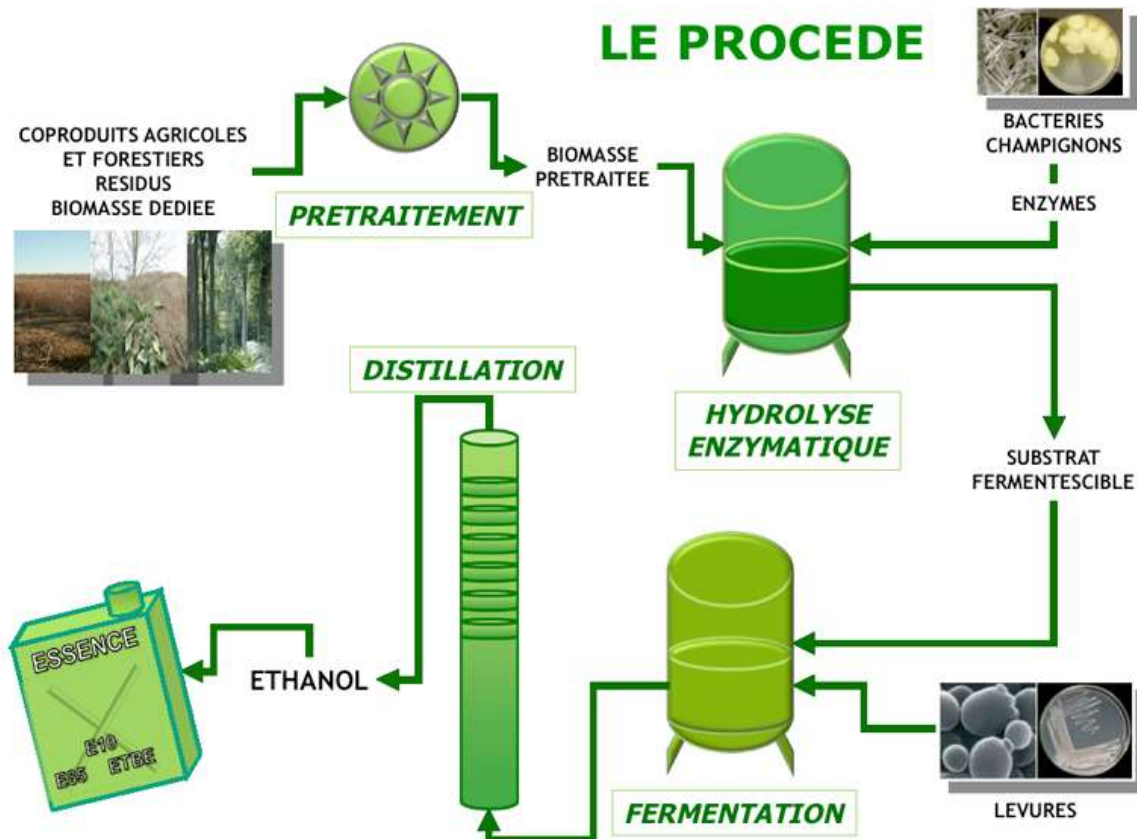


Figure 13: Chaîne de production du bioéthanol de seconde génération, d'après Challenges (<https://www.challenges.fr/automobile/dossiers/>)

Depuis environ une vingtaine d'années, *B. bruxellensis* a reçu beaucoup d'attention de la part des chercheurs pour son implication dans l'industrie du bioéthanol. Le principe de ce procédé est d'utiliser de la matière végétale pour produire de l'éthanol utilisable ensuite comme biocarburant et repose sur la réalisation d'une fermentation alcoolique à partir de végétaux. Le bioéthanol dit 'de première génération' est produit à partir de denrées alimentaires (cane à sucre, pomme de terre, maïs...). Cette utilisation, qui détourne les cultures vivrières vers la production de carburant, est en recul pour des problèmes éthiques évidents (Valentine et al., 2012) (Figure 13). Le bioéthanol de seconde génération est produit à partir de déchets lignocellulosiques ou de biomasse végétale riche en cellulose ou hémicellulose, bien qu'ils soient encore très minoritaires en terme de production (<10%) (pour revue : Bušić et al., 2018; Favaro et al., 2019; Jacobus et al., 2021). Cette biomasse végétale nécessite un prétraitement hydrolysant les sucres complexes présents en sucres fermentescibles. Ensuite, la fermentation est réalisée en grande majorité par des levures commerciales *S. cerevisiae* pour obtenir rapidement un rendement d'éthanol important. Le milieu fermentaire est régulièrement renouvelé (culture en batch, fed-batch, en continu...). Les contaminations microbiologiques sont courantes et *B. bruxellensis* est considérée comme la principale levure d'altération (Beckner et al., 2011). Ces contaminations ont pour conséquence fréquente une diminution du rendement en éthanol (de Souza Liberal et al., 2007; Seo et al., 2020). Dans certains cas, après plusieurs batch, *S. cerevisiae* est sous représentée par rapport à *B. bruxellensis* (qui présente pourtant une vitesse de croissance en théorie

plus lente que *S. cerevisiae*) (de Souza Liberal et al., 2007). Plusieurs hypothèses peuvent expliquer ces observations : *B. bruxellensis* est capable de consommer une grande variété de sucres et notamment ceux présents dans ce procédé (cellobiose, saccharose, cellulose, hémicellulose...) (Bušić et al., 2018; Codato et al., 2018; Godoy et al., 2017; Leite et al., 2012; Maria et al., 2019; Reis et al., 2014; Tiukova et al., 2014). Une deuxième hypothèse considère la production importante d'acide acétique par cette levure qui, en modifiant le pH du milieu, ralentirait la croissance de *S. cerevisiae* (Taherzadeh et al., 1997). Cependant, la pertinence de cette explication est débattue car les deux espèces partagent les mêmes tolérances pour le pH et l'acide acétique (Abbott et al., 2005; Phowchinda et al., 1995). De plus, dans les cas où *B. bruxellensis* est majoritaire, la quantité d'acide acétique et le pH ne sont pas anormaux (Pereira et al., 2012). Une autre explication repose sur la capacité de *B. bruxellensis* à utiliser les nitrates comme source d'azote contrairement à *S. cerevisiae* (Borneman et al., 2014). Les concentrations en nitrates dans les végétaux peuvent être importantes (Colla et al., 2018). L'utilisation des nitrates chez *B. bruxellensis* permet aussi l'activation du flux glycolytique, par le recyclage du NADH (Galafassi et al., 2013b; Peña-Moreno et al., 2019). Ainsi, lorsque l'azote assimilable par *S. cerevisiae* (ammonium et acides aminés) vient à manquer, *B. bruxellensis* est capable d'utiliser les nitrates pour produire plus de biomasse que *S. cerevisiae* (de Barros Pita et al., 2011).

Récemment, la capacité de *B. bruxellensis* à utiliser une grande variété de sources atypiques de carbone et d'azote (sucres habituellement peu fermentescibles, nitrate) et à réaliser la fermentation alcoolique ont conduit certains auteurs à évaluer cette espèce non plus comme agent indésirable mais comme candidate pour la production de bioéthanol (pour revue : de Barros Pita et al., 2019). De plus, alors qu'une partie de la littérature rapporte une perte en rendement d'éthanol en présence de *B. bruxellensis*, d'autres travaux montrent que *B. bruxellensis* peut conduire à des rendements similaires à *S. cerevisiae* (Blomqvist et al., 2012; Passoth et al., 2007). Plusieurs raisons peuvent expliquer ces écarts de point de vue. Les paramètres des fermentations peuvent varier : condition aérobie/anaérobie stimulant la production d'acide acétique au détriment de l'éthanol, substrat de fermentation (modulant la qualité et quantité des sucres présents ainsi que la quantité de nitrates par exemple). Des facteurs génétiques (souches différentes), physiologiques (souches acclimatées à l'environnement), ainsi que de possibles interactions microbiennes peuvent également moduler la conduite des fermentations (Bassi et al., 2018; Tiukova et al., 2014). *B. bruxellensis* est parfois associée à des bactéries dans ce procédé (*Lactobacillus fermentum* ou *Lactobacillus vini*) (Bassi et al., 2018; Passoth et al., 2007). Des co-cultures avec *S. cerevisiae* et *L. fermentum* ont montré que la croissance de *B. bruxellensis* était stimulée par la présence de *L. fermentum* (Bassi et al., 2018). Cependant, les interactions entre micro-organismes restent un pan très peu étudié chez *B. bruxellensis*. Il est toutefois probable que l'intérêt de *B. bruxellensis* pour la production de bioéthanol reste anecdotique, en effet, l'utilisation de levures génétiquement modifiées (principalement *S. cerevisiae*) est autorisée (Mohd Azhar et al., 2017). Cette réalité n'est pas favorable au développement de *B. bruxellensis* pour l'industrie du bioéthanol car ses capacités d'ingénierie génétique sont limitées comparé à *S. cerevisiae* (pour revue : Varela and Borneman, 2022). De plus, la production de bioéthanol nécessite des quantités de levains importantes, et *B. bruxellensis* est empiriquement connue au laboratoire pour être capricieuse et difficile à cultiver (observations personnelles).

## Boissons fermentées à faible teneur en alcool (Kombucha, Kéfir)

*B. bruxellensis* est régulièrement isolée dans les boissons traditionnelles peu fermentées, telles que le kombucha, le kéfir de fruit et d'eau (Patel et al., 2022; Teoh et al., 2004). Leur consommation connaît une croissance remarquable (chiffre d'affaire estimé à 2,64 milliard de dollars en 2021, avec une croissance prévue de 16% par an, d'après Kombucha Market Size, Share & Trends Analysis Report By Product (Conventional, Hard), And Segment Forecasts, 2022 – 2030), stimulée par les nombreuses allégations 'santé' et 'bien-être' associées à ces produits (présence de composés bioactifs et antioxydants, pour revue : Antolak et al., 2021). Le kombucha est une boisson à base de thé sucré originaire d'Asie de l'Est, fermentée entre 20 et 30°C durant quelques jours (entre 1 et 4 semaines généralement, Figure 14). Sa composition est donc relativement simple : de l'eau, 50 à 80g/L de saccharose (sucre de canne bien souvent), 5g/L de feuilles de thé (thé noir ou vert), et des micro-organismes (levures et bactéries) agrégés au sein d'une matrice cellulosique appelée « mère » ou « SCOBY » (acronyme pour Symbiotic Consortium Of Bacteria and Yeast) (pour revue : Jayabalan et al., 2014; Martínez Leal et al., 2018) (Figure 14). La composition microbiologique de chaque kombucha varie en fonction de la biogéographie du lieu de production et de l'origine des composants, rendant chaque kombucha unique (Bishop et al., 2022). Il s'agit d'une culture par batch, dans laquelle le SCOBY du lot précédent sert à ensemercer le lot suivant. Le premier lot est soit issu d'une fermentation spontanée dans un récipient ouvert, soit ensemencée via l'achat ou l'échange d'un SCOBY (Dantas Coelho et al., 2020). La composition microbiologique du kombucha fait l'objet d'un grand intérêt scientifique depuis quelques années (Andreson et al., 2022; Coton et al., 2017; Huang et al., 2022; Kaewkod et al., 2019; Landis et al., 2022; Rivera Flores et al., 2021; T et al., 2021; Wang et al., 2022). Bien que la composition biotique exacte varie entre chaque mère de kombucha, deux genres de bactéries sont majoritairement isolés : *Acetobacter* et *Gluconobacter*, responsables de la métabolisation de l'éthanol en acide acétique et de la production de gluconate respectivement (Macauley et al., 2001; Sengun, 2016). Les principales espèces de levures retrouvées majoritairement appartiennent aux genres *Saccharomyces*, *Zygosaccharomyces*, *Hanseniaspora*, *Pichia*, ainsi que *Brettanomyces*. Les levures participent à l'hydrolyse du saccharose en glucose et fructose, à la production d'éthanol et d'acide acétique ainsi que de métabolites secondaires d'intérêt organoleptique (esters, alcools supérieurs, aldéhydes...) (Huang et al., 2022; Tran et al., 2020). L'interaction entre levures et bactéries acétiques permet la synthèse de la matrice cellulosique du SCOBY. Cette matrice joue le rôle de protection physique, limitant l'oxygénation du milieu. La genèse de cette matrice reste encore méconnue. Une étude récente a permis d'observer par hybridation *in situ* (FISH) la dynamique de formation de biofilm dans des consortiums synthétiques (Savary et al., 2021). Les levures observées dans les biofilms sont progressivement couvertes de bactéries dans une matrice extracellulaire, renforçant l'hypothèse d'une symbiose levures bactéries. Le rôle de l'espèce *B. bruxellensis* reste cependant peu clair. Elle est présente dans la plupart des kombucha, en concentration relativement variable mais elle est bien souvent majoritaire en fin de fermentation (Andreson et al., 2022; Huang et al., 2022; Landis et al., 2022; Savary et al., 2021). Son rôle serait la production d'invertase extracellulaire pour l'hydrolyse du saccharose ainsi que la production de métabolites intermédiaires clés. Elle permettrait ainsi la croissance de bactéries productrices de biofilm ainsi que la production de composés volatils désirables spécifiques au kombucha (responsable de l'arôme de pomme par exemple) (Landis et al., 2022; Tran et al., 2022, 2020). Il est aussi possible que *B. bruxellensis* participe directement à la formation du biofilm grâce à la formation de

pseudohyphes et la production de matrice extracellulaire (Dimopoulou et al., 2019b; Joseph et al., 2007; Lebleux et al., 2020). L'impact réel de *B. bruxellensis* devrait être prochainement mieux compris, vue l'attractivité scientifique et économique grandissante de cette boisson, notamment dans les sociétés occidentales.

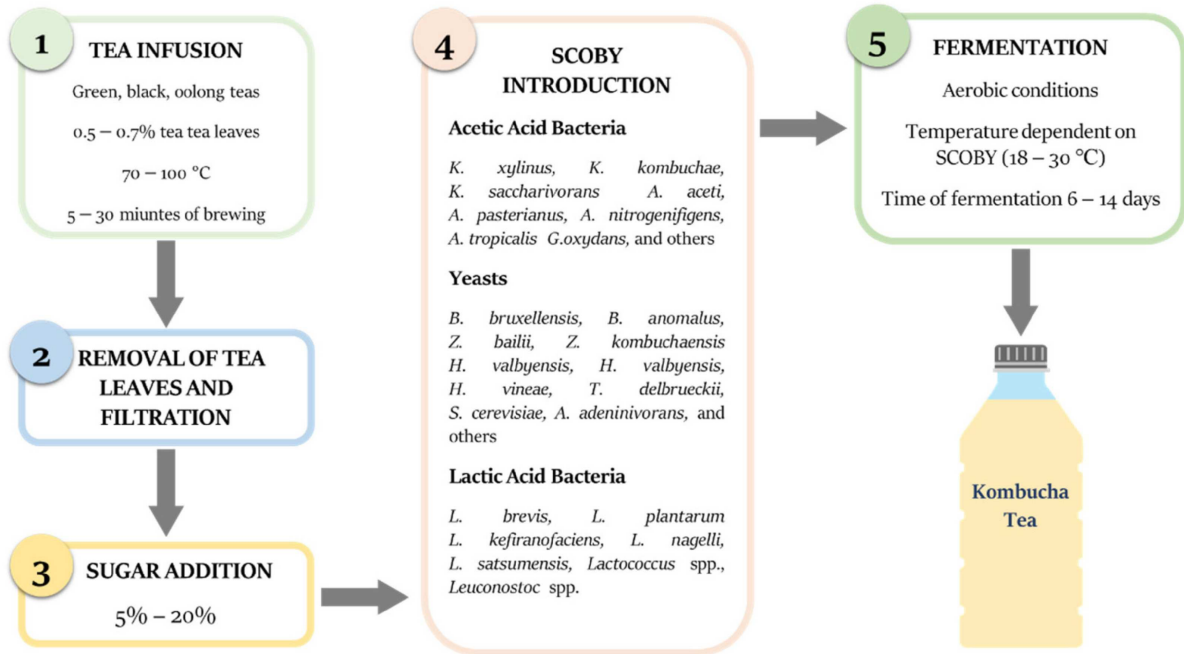


Figure 14: Principales étapes de préparation du kombucha, les données de temps et les concentrations peuvent varier entre les différentes productions. D'après Antolak et al., (2021).

*B. bruxellensis* a aussi été isolée à partir d'une boisson très similaire, le kéfir de fruit (appelé aussi kéfir d'eau), dans laquelle les feuilles de thé sont remplacées par des fruits et l'inoculum microbien (biofilm de levures et bactéries) se présente sous forme de « grains de kéfir » (pour revue : Lynch et al., 2021). *B. bruxellensis* peut y être l'espèce de levure majoritaire, et dans ce cas, les études ont montré qu'elle produisait des alcools supérieurs et des esters (Patel et al., 2022). Cependant, comme dans tout consortium, évaluer spécifiquement l'impact d'une espèce reste complexe, et sera certainement un des enjeux de ces prochaines années.

De manière surprenante, une étude récente menée sur le microbiote intestinal de population rurale et urbaine d'Afrique du Sud a montré que l'espèce *B. bruxellensis* était le biomarqueur dominant chez les individus vivants dans les villes (Kabwe et al., 2020). Les auteurs observent des différences importantes dans l'alimentation de ces deux groupes et concluent que la forte présence de *B. bruxellensis* vient de la part importante de produits fermentés (feta, vin, pain au levain) dans le régime urbain comparé au régime alimentaire rural. L'interprétation de ce résultat reste très évasive et d'autres études sont indispensables. Néanmoins ces résultats suggèrent que la présence de cette levure dans les produits alimentaires fermentés est encore sous-estimée. L'évolution des techniques d'identification non ciblées, de type « omics » par exemple, devrait permettre de mieux estimer l'occurrence de cette levure dans les niches anthropisées.

Méthodes de contrôle et de lutte contre *B. bruxellensis*

*B. bruxellensis* est donc présente dans de nombreux procédés fermentaires, dans lesquels elle provoque des déviations organoleptiques importantes ou des défauts de rendement. Sa détection, son contrôle, et son élimination représentent un enjeu économique important, particulièrement pour la filière œnologique. Les méthodes de détection de *B. bruxellensis* sont nombreuses (pour revue : Tubia et al., 2018) (Tableau 2). Les méthodes de dénombrement sur boîte sont historiquement les plus utilisées (méthodes cultivables), mais leur intérêt est limité par plusieurs facteurs : la faible capacité de croissance de *B. bruxellensis*, le niveau de population faible lors de son occurrence ainsi que la possibilité d'un état Viable-Non-Cultivable (VNC) (pour revue : Ferremi Leali et al., 2022). La mesure de concentration des éthyl-phénols est aussi très répandue, mais leur détection indique que le vin est déjà contaminé et n'est donc pas un moyen adapté à la prévention. Des méthodes utilisant des marqueurs moléculaires (RAPD, AFLP, REA-PFGE, Sau-PCR, PCR) ont aussi été développées, mais elles ne rendent pas compte de la viabilité relative des cellules (Albertin et al., 2014; Ferremi Leali et al., 2022; Vigentini et al., 2013). Enfin, l'évolution récente des méthodes de cytométrie en flux rendent cette technique attractive, bien que les seuils de détection restent élevés (pour revue : (Longin et al., 2017)).

Tableau 2: Principales méthodes utilisées dans la détection de *B. bruxellensis*, avec leurs avantages et inconvénients. D'après (Tubia et al., 2018).

Technique	Avantages	Disadvantages
Direct methods		
Plating	Reliability	
	Low cost	Development/incubation time
	Easy	False positives
Microscopy	Quantitative	VBNC not detected
	Fast	
	VBNC detected	Alive vs. death cells identification
Molecular detection	Easy	Variable morphology
	Quantitative	
	Fast	
Flow cytometry	Sensitivity	Alive vs. death cells identification
	Reliability	Specialized staff
	VBNC detected	Expensive
Indirect methods		Low specificity
	Fast	Specialized staff
	Sensitivity	Expensive
GC-MS		
	Fast	Late detection (after beverages contamination)
	Sensitivity	
	Low cost	
	Easily integrated	

Le dioxyde de soufre (SO<sub>2</sub>) est l'intrant le plus utilisé par les vinificateurs depuis le 18<sup>ème</sup> siècle (Ribéreau-Gayon et al., 2017). En plus de ses capacités antiseptiques à large spectre, il possède des capacités antioxydantes et antioxydasiques. L'efficacité des sulfites dépend principalement du pH du vin (Ribéreau-Gayon et al., 2006). Son utilisation généralisée ces 70 dernières années a fortement

contribué à la diminution des altérations microbiologiques du vin (Bartowsky, 2009; Ferremi Leali et al., 2022). Il a notamment été largement recommandé dans la lutte contre *B. bruxellensis* à la fin du siècle dernier (Du Toit et al., 2005). Cependant, depuis plus d'une dizaine d'années, des études scientifiques font état de souches de *B. bruxellensis* tolérantes et même résistantes à la présence de SO<sub>2</sub> dans l'ensemble des vignobles de la planète (Conterno et al., 2006; Dimopoulou et al., 2019a; Serpaggi et al., 2012; Vigentini et al., 2013). En 2012, sur 41 isolats de domaines australiens, plus de 90% des souches étaient tolérantes à la présence de SO<sub>2</sub> (Curtin et al., 2012). Cette capacité a été assignée à des groupes génétiques spécifiques de *B. bruxellensis*, validée par des expériences de compétition en milieu modèle avec des concentrations croissantes de SO<sub>2</sub> (Avramova et al., 2019, 2018b). De manière intéressante, les groupes génétiques associés à une résistance/tolérance aux sulfites sont très minoritairement détectés (voire absents) dans les vins avant les années 1990 et leur proportion augmente jusqu'à devenir majoritaire de nos jours (Cibrario et al., 2019). Cette résistance/tolérance pourrait donc être liée à la généralisation de l'utilisation des sulfites en œnologie et pourrait être le résultat d'une adaptation à la pratique œnologique, similaire à ce que l'on peut observer chez les souches œnologiques de *S. cerevisiae* (García-Ríos and Guillamón, 2019; Legras et al., 2018; Varela et al., 2018).

L'utilisation des sulfites en œnologie est aujourd'hui de plus en plus remise en question. Près de 1% de la population mondiale présente une sensibilité accrue au SO<sub>2</sub>, provoquant des problèmes d'urticaire, de bronchospasme et autres (Vally and Thompson, 2003). L'évolution des connaissances sur la toxicité du SO<sub>2</sub> et les réglementations qui en ont découlé ont soulevé de l'inquiétude chez les consommateurs, qui plébiscitent la limitation des intrants dans leur alimentation et le développement de procédés plus durables. De plus, en raison du changement climatique, le pH des vins est en constante augmentation, réduisant ainsi l'efficacité antimicrobienne du SO<sub>2</sub> (van Leeuwen and Darriet, 2016). Ainsi, la recherche d'alternatives est une nécessité.

Plusieurs méthodes de contrôle ont ainsi été évaluées sur *B. bruxellensis*, incluant des méthodes chimiques, microbiologiques et physiques (pour revue : Lisanti et al., 2019; Pinto et al., 2020; Tubia et al., 2018). Les principales méthodes chimiques évaluées pour le vin sont l'ajout de SO<sub>2</sub>, de DMDC (Dicarbonate de diméthyle) et l'utilisation de chitosane; et pour le traitement des baies de raisins et des barriques, le traitement à l'ozone ou à l'eau électrolysée (Bellincontro et al., 2017; Castro Marín et al., 2020; Paulin et al., 2020; Renouf et al., 2008). Les principales méthodes de contrôle microbiologique (appelée bioprotection) comprennent : (i) l'utilisation de « toxines killer » produites par *S. cerevisiae* (Mazzucco et al., 2019; Mehlomakulu et al., 2015; Santos et al., 2011), (ii) l'utilisation de souches *starter* industrielles pour réaliser la fermentation rapidement ou (iii) l'utilisation de levures autochtones dans l'objectif ne pas laisser les levures d'altération s'installer (Bergebég et al., 2018, 2017; Branco et al., 2019). Les méthodes physiques les plus pratiquées en œnologie sont la filtration, la flash pasteurisation, l'application de champs électriques pulsés (PEF), l'utilisation de hautes pressions hydrostatiques (HHP) (Lisanti et al., 2019; Pérez-López et al., 2020; Pinto et al., 2020). Cependant, les méthodes chimiques ou microbiologiques ont pour la plupart une efficacité limitée, et/ou sont associées à des problématiques de toxicité et d'impact sur la santé des consommateurs ainsi que sur la qualité du produit. L'utilisation des méthodes physiques nécessite un appareillage important ainsi qu'une consommation énergétique forte et ont bien souvent un fort impact organoleptique sur le produit final. Malgré cela, l'utilisation coordonnée et réfléchie de ces techniques reste prometteuse car ces technologies sont souvent complémentaires et synergiques. Il est donc important de continuer à évaluer leur potentiel, notamment sur *B. bruxellensis*.

## II/Caractéristiques génétiques et phénotypiques de *Brettanomyces bruxellensis*

A la lecture de la littérature concernant *B. bruxellensis*, l'un des aspects les plus frappants est la grande diversité des comportements décrits pour différents isolats et différentes conditions environnementales. Cependant, la prise en compte de la structure des populations et de l'analyse des génomes permet de réconcilier des travaux contradictoires en apparence : certaines variations phénotypiques pourraient être liées à différentes populations au sein de l'espèce ou à certains facteurs environnementaux. Une revue scientifique a été écrite en ce sens lors de cette thèse traitant de la diversité phénotypique et génétique récemment dévoilée. Une attention particulière a été portée sur la signification de cette diversité au niveau écologique. Il s'agit de la première revue, à notre connaissance, sur ces aspects de diversité et d'écologie concernant la levure *B. bruxellensis*. Ce travail a été réalisé grâce aux regards croisés des chercheurs de l'UMR Œnologie et de l'unité GMGM (Génétique moléculaire, génomique, microbiologie) de l'université de Strasbourg et publié début 2022 dans l'édition spéciale "Evolutionary ecology of human associated microbes" de la revue *Molecular Ecology*. Les données supplémentaires de l'article sont présentées dans la partie annexe de la thèse.

# *Brettanomyces bruxellensis*: Overview of the genetic and phenotypic diversity of an anthropized yeast

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## Abstract

Human-associated microorganisms are ideal models to study the impact of environmental changes on species evolution and adaptation because of their small genome, short generation time, and their colonization of contrasting and ever-changing ecological niches. The yeast *Brettanomyces bruxellensis* is a good example of organism facing anthropogenic-driven selective pressures. It is associated with fermentation processes in which it can be considered either as a spoiler (e.g., winemaking, bioethanol production) or as a beneficial microorganism (e.g., production of specific beers, kombucha). In addition to its industrial interests, noteworthy parallels and dichotomies with *Saccharomyces cerevisiae* propelled *B. bruxellensis* as a valuable complementary yeast model. In this review, we emphasize that the broad genetic and phenotypic diversity of this species is only beginning to be uncovered. Population genomic studies have revealed the coexistence of auto- and allotriploidization events with different evolutionary outcomes. The different diploid, autotriploid and allotriploid subpopulations are associated with specific fermented processes, suggesting independent adaptation events to anthropized environments. Phenotypically, *B. bruxellensis* is renowned for its ability to metabolize a wide variety of carbon and nitrogen sources, which may explain its ability to colonize already fermented environments showing low-nutrient contents. Several traits of interest could be related to adaptation to human activities (e.g., nitrate metabolization in bioethanol production, resistance to sulphite treatments in winemaking). However, phenotypic traits are insufficiently studied in view of the great genomic diversity of the species. Future work will have to take into account strains of varied substrates, geographical origins as well as displaying different ploidy levels to improve our understanding of an anthropized yeast's phenotypic landscape.

## KEYWORDS

adaptation, beer, fermentation, hybridization, polyploidy, wine

## 1 | INTRODUCTION

Yeasts are eukaryotic species encountered in most, if not all, earth biomes (Starmer & Lachance, 2011). Yeasts are heterotrophic, they

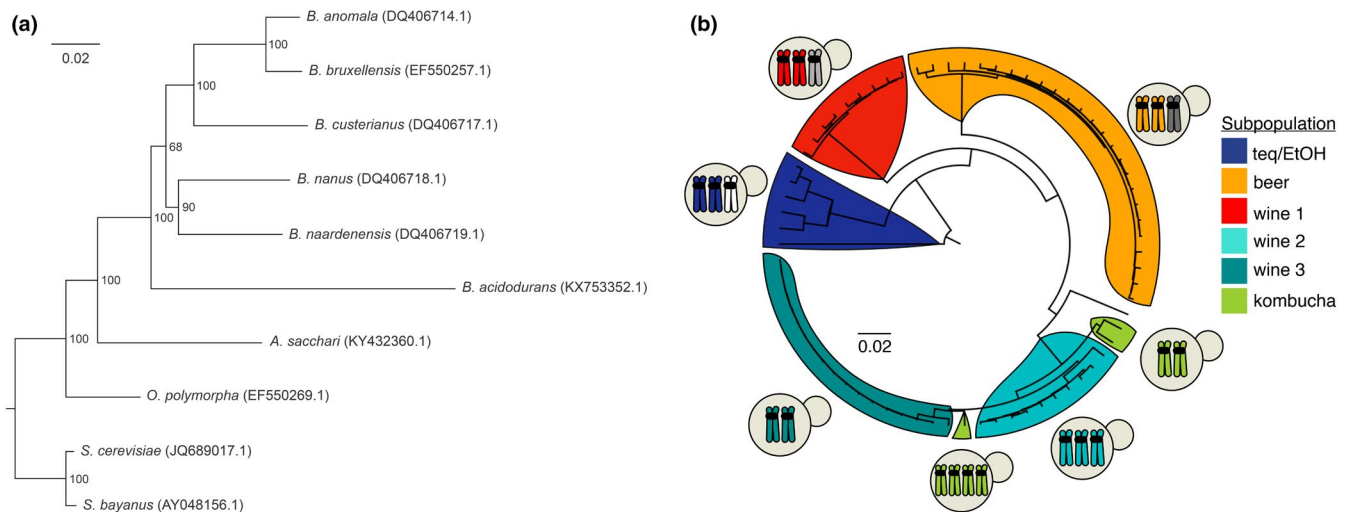
are described as primary decomposers of organic matter, and are particularly associated with the early colonization of nutrient-rich substrates. The ability of some yeast species to efficiently perform alcoholic fermentation, that is, to convert sugar into ethanol, made



it possible to forge close ties with human beings, leading to a tight coevolution between yeast and human (Starmer & Lachance, 2011). Their simple life cycle, their small genomes (10 to 20 Mbp) which are genetically diverse and their multifaceted metabolisms have propelled yeasts as valuable models to study evolutionary genetics and ecology (Gladieux et al., 2014; Hittinger et al., 2015). In addition to the famous yeast model organism *Saccharomyces cerevisiae*, the genus *Brettanomyces* (of which *Dekkera* is a synonym) has received a lot of attention recently, due to its association with various food processes and to its unusual genomic composition. This genus belongs to the *Pichiaceae* family, which is a part of the *Saccharomycotina* subphylum (Kufferath & Van Laer, 1921; Riley et al., 2016). It is composed of six species (*B. nanus*, *B. naardenensis*, *B. bruxellensis*, *B. anomalus*, *B. custerianus* and *B. acidodurans*), with *Allodekкера sacchari* being the closest non-*Brettanomyces* species known to date (Jutakanoke et al., 2017; Péter et al., 2017; Figure 1a). Recently, high quality genomes were obtained for five *Brettanomyces* species, revealing distant species separated in two clades with *B. nanus* and *B. naardenensis* on one hand, and *B. bruxellensis*, *B. anomalus* and *B. custerianus* on the other hand (Roach & Borneman, 2020). The calculation of average nucleotide identity along the genomes between each species pairs, ranging between 60.6% and 77.1%, revealed that *Brettanomyces* species are relatively distant of each other: as a comparison, the most distant species within *Saccharomyces* genus show 79.9% nucleotide identity (*S. cerevisiae* and *S. eubayanus*) and 75.2% within *Metschnikowia* genus (*M. hawaiiiana* and *M. orientalis*), two yeast genus frequently

associated with fermented foods (Lachance et al., 2020; Roach & Borneman, 2020). The five *Brettanomyces* species whose genome is fully sequenced exhibit gene family expansions related to fermentation, such as glucosidase enzymes involved in starch or galactose metabolism, as well as in nitrogen assimilation. In addition, twelve horizontal gene transfer (HGT) events were detected within *Brettanomyces* genus, and may explain the ability of *B. bruxellensis* and *B. anomalus* to utilize sucrose (Roach & Borneman, 2020). Gene expansions and HGTs are well described markers of domesticated subpopulations of *S. cerevisiae* (Gallone et al., 2018; Giannakou et al., 2020; Legras et al., 2018; Peter et al., 2018). The identification of interesting parallels with the evolution of *S. cerevisiae* supported *Brettanomyces* as a valuable yeast model to study adaptation to fermentative environments.

Due to its positive or negative role in different industrial applications, *B. bruxellensis* is the species that has received the most attention. It was first isolated by Claussen in 1904 as being responsible for the special flavour of British beers, and therefore was the first patented microorganism (Claussen, 1904; Steensels et al., 2015). In brewery, *B. bruxellensis* plays a major role for some special beer types such as the Lambics from Belgium, Flanders Red and Brown ales, or the "coolship ales" from the USA (Bokulich et al., 2012; Claussen, 1904; De Roos & De Vuyst, 2019). It contributes to the peculiar flavour of spontaneous fermented beers, bringing additional complexity to their aromatic bouquet. In addition, over the past decades, the growing popularity of craft beers and the rise of microbreweries



**FIGURE 1** Genetic position and population structure of *Brettanomyces bruxellensis*. (a) Neighbour-joining tree of the *Brettanomyces* genus. The distance tree comprises the six *Brettanomyces* species, *Allodekкера sacchari* (the closest sister species to *Brettanomyces* clade), *Ogataea polymorpha*, *Saccharomyces cerevisiae* and *Saccharomyces bayanus* as outgroups. 26S ribosomal RNA gene sequences were used. A global alignment with free end gaps was prepared using Geneious (Prime 2020.2) and the default setting for multiple alignments. A neighbour-joining tree was built with the Tamura-Nei Model of genetic distances. The final tree represents a consensus of 1,000 resampled trees obtained with an extended majority rules method. The consensus supports of the nodes are given in %. Sequences data was downloaded from NCBI. (b) Genetic diversity within *Brettanomyces bruxellensis* species. *Brettanomyces bruxellensis* subpopulations are represented by different colours and named from previous reports (Avramova, Cibrario, et al., 2018; Gounot et al., 2020). The tree was built from whole genome Illumina short-read sequencing of 71 *B. bruxellensis* isolates aligned to the reference genome *B. bruxellensis* (Fournier et al., 2017) and 24,313 genetic variants evenly distributed across the genome (Eberlein et al., 2021). The ploidy level of each population was schematized, two chromosomes represent diploid groups, three chromosomes represent triploids ones. Light grey, dark grey and white chromosomes represent the independent haploid acquired genomes from unknown sister species

led to an increased interest among brewers who use *B. bruxellensis* alone or in coculture with other species. For example, *B. bruxellensis* has been described as a good candidate for beers with a spicy phenolic note (Holt et al., 2018). *Brettanomyces bruxellensis*' scientific literature with a focus on brewery is substantial (Capece et al., 2018; Lentz, 2018; De Roos & De Vuyst, 2019; Serra Colomer et al., 2019; Steensels et al., 2015). *Brettanomyces bruxellensis* is also involved in the elaboration of other spontaneous fermented beverages such as kombucha, kefir, etc. in which its role is mostly considered as positive (Lynch et al., 2021; Tran et al., 2020).

Despite its positive contribution to particular beer flavours and traditional beverages, *B. bruxellensis* is also recognised as a major spoiler for top-selling ale and lager beers due to sensory or turbidity defects (Shimotsu et al., 2015). The same account for wine, where *B. bruxellensis* negative contribution is also widely described, amplified by its resistance to sulphite, one of the common control method historically used (Agnolucci et al., 2017; Avramova et al., 2019; Blomqvist & Passoth, 2015; Malfeito-Ferreira, 2018; Schifferdecker et al., 2014; Suárez et al., 2007). Its presence in wine is associated with the production of volatile molecules (called volatile phenols), associated to unpleasant aromas described as barnyard, horse sweat or burnt plastic (Chatonnet et al., 1992; Kheir et al., 2013). The "Brett" taint negatively affects up to 25%–30% of red wines in the world (Alston et al., 2021; Gerbaux et al., 2000; Romano et al., 2008). *Brettanomyces bruxellensis* is able to penetrate the micropores of the barrels and therefore reused barrels—a frequent practice in oenology—are a recurrent cause of contamination (Cartwright et al., 2018; Fabrizio et al., 2015). In bioethanol production, this species is considered as the main yeast spoiler as it can reduce the ethanol yield and cause large economic losses (Basilio et al., 2008; Bassi et al., 2018; Blomqvist & Passoth, 2015; de Souza Liberal et al., 2007; Radecka et al., 2015; Seo et al., 2020). In addition its beneficial-spoiler duality, another oddity of *B. bruxellensis* is its ability to act as a first fermenter (even starter) yeast in some processes (e.g., craft beer, kombucha, bioethanol) or as a fermentation "finisher" (e.g., wine, Lambic beers, etc.), the actual fermentation usually being carried out by *S. cerevisiae*. This starter-finisher versatility suggests multiple fermentative adaptations that may have arisen from the complex structure of *B. bruxellensis* genome that includes large chromosomal rearrangements, hybridization events, as well as ploidy level variation (Gounot et al., 2020). In particular, a remarkable characteristic of *B. bruxellensis* species resides in the rare coexistence of diploid and steady triploid isolates (Curtin et al., 2012; Piškur et al., 2012).

In this review, we first focus on the genetic and genomic composition of *B. bruxellensis* revealed by population genomic surveys. This genetic diversity is reflected at the phenotypic level, with contrasting behaviours regarding metabolic and life-history traits that have ecological significances. We describe the impact of such diversity on central metabolism characteristics and technological traits, and we discuss how future researches including both genomic and phenotypic approaches may shed lights on the evolutionary history of a human-associated yeast.

## 2 | GENETIC AND GENOMIC DIVERSITY OF *BRETTANOMYCES BRUXELLENSIS*

### 2.1 | Population structure of *Brettanomyces bruxellensis*

A wide variety of molecular tools (e.g., AFLP, RAPD) were developed to assess the genetic diversity of *B. bruxellensis* species (reviewed in Renouf et al., 2007). Most of these studies focused on the intraspecific diversity of small cohorts: between 10 and 100 isolates mainly from the same process (Agnolucci et al., 2009; Conterno et al., 2006; Curtin et al., 2007; Miot-Sertier & Lonvaud-Funel, 2007). Thus, an important step has been taken with the genotyping of more than 1,000 worldwide strains using 12 microsatellite markers (Avramova, Cibrario, et al., 2018). This study provided deep insights into the genetic diversity and ploidy level in several subpopulations of different ecological origin. The population structure was mainly explained by the ploidy level (~47% of the variance), followed by the geographical origin (~5% of the variance) and the fermented product from which the strain had been isolated (~6% of the variance). However, when considering non-wine isolates, the geographical origin explains ~55% of the total variance, indicating that wine isolates are highly disseminated across the world compared to strains isolated from other substrates. Isolates from different origins (such as beer, kombucha, tequila and bioethanol isolates) were mostly clustered into one or two genetic groups, suggesting different adaptation processes to various anthropic environments (Avramova, Cibrario, et al., 2018). This large population study allowed the identification of different genetic subpopulations within *B. bruxellensis*, which were subsequently validated and refined by whole genome sequencing approaches (Eberlein et al., 2021; Gounot et al., 2020; Serra Colomer, Chailyan, et al., 2020; Table S1). At least six subgroups can now be considered, either diploid (two groups) or triploid (four groups; Figure 1b). Among diploid groups, one subpopulation is composed mainly of strains isolated from wine, and is named the wine 3 group (known as G2N3, CBS2499-like or Wine 2n in various publications, Table S1), the other is associated with kombucha, beer and wine isolates, and we named it the kombucha group (known as G2N1, L14165-like, Farmhouse). Regarding the triploid subpopulations, the beer group contains strains from beer and wine (G3N1, AWRI1608-like, Lambic), the two other groups are strongly associated with winemaking and are referred as wine 1 (or G3N2, AWRI1499-like, Wine 3n) and wine 2 groups (or G3N3, L0308-like). The last group, named teq/EtOH group, contains most isolates from bioethanol and tequila process (CBS5212-like, Tequila). Recent genomic data suggested that the teq/EtOH group was not monophyletic, yet more in-depth analyses are necessary to refine precisely the actual number of teq/EtOH groups. Recently still, genome sequencing revealed an autotetraploid strain (Figure 1b), genetically close to a small subpopulation already observed but described as diploid (G2N2, KOM1449-like). More isolates are needed to delineate this subpopulation and its ecological and genomic features. The vast majority (~80%) of wine strains are found in three groups (wine 1 and wine 2 triploid groups,

wine 3 diploid group, Figure 1b), yet some wine isolates can be found occasionally in other groups, with variations depending on the wine producing region (Avramova, Cibrario, et al., 2018; Cibrario et al., 2019). Beer isolates are also distributed in different groups, although to a lesser extent (two main groups). Regarding the other isolation niches (such as kombucha, tequila, bioethanol), additional works and more strains are needed to determine whether they fall within one specific subpopulation, or if they are more disseminated as for winemaking. Dissemination via equipment could play an important role in exchanges within and between ecological niches: craft brewers frequently age beer in wood barrel that previously contained red or white wines (Sanna & Pretti, 2015) and similar practice is common for tequila's ageing (Aguilar-Méndez et al., 2017). *Brettanomyces bruxellensis*' population structure seems to be well-defined, but only anthropic isolates of the species have been described, with no wild strains identified so far.

## 2.2 | Genomic landscape of *Brettanomyces bruxellensis*

Within the *Saccharomycotina* subphylum, *B. bruxellensis* diverged from the model species *S. cerevisiae* between 200 and 300 MY ago, before the whole-genome duplication (WGD) event that appeared

in the *Saccharomyces* lineage 100 MY ago (Fisher et al., 2018; Guo et al., 2016; Wolfe & Shields, 1997). WGD outcomes (e.g., gene duplication, promoter rewiring) were suggested to be involved in the acquisition of the make-accumulate-consume (MAC) strategy in the *Saccharomyces* genus (Thomson et al., 2005). Thus, it seems that both species, *S. cerevisiae* and *B. bruxellensis*, have independently acquired the MAC ability (Rozpędowska et al., 2011). The first partial genome sequence of *B. bruxellensis* strain CBS 2499 showed around 50% of nucleotide identity with *S. cerevisiae* (Woolfit et al., 2007), and more recent studies identified that at least 3,300 orthologous gene families were conserved across both clades (out of 5000 for *B. bruxellensis*) (Cheng et al., 2017).

Over the past decade, several *de novo* assemblies have been published with increasing quality (Table 1), in particular thanks to the combination of long-read sequencing (e.g., Oxford Nanopore sequencing) with short-read sequencing (Fournier et al., 2017). Such high quality assemblies were used as reference genomes for population genomic surveys (Eberlein et al., 2021; Gounot et al., 2020; Serra Colomer, Chailyan, et al., 2020). These population studies revealed that the pangenome is composed of 5,409 ORFs (open reading frames) with 5,106 core and 303 accessory ORFs within the species. Although no significant functional enrichment was found for the set of accessory genes, some of them were shown to be involved in drug and sugar transports (Gounot et al., 2020). Many

TABLE 1 Whole-genome sequencing of *Brettanomyces bruxellensis* isolates

Publication	Isolates ID (or number of isolates)	Niche; origin of isolation	Country/region of isolation	Ploidy <sup>a</sup>
Woolfit et al. (2007)	CBS 2499	Wine	France	ND
Piškur et al. (2012)	CBS 2499	Wine	France	Diploid
Curtin, Borneman, et al. (2012)	AWRI 1499	Wine	Australia	Triploid
Crauwels et al. (2014)	ST05.12/22 = VIB X9085	Beer	Belgium	Diploid
Valdes et al. (2014)	LAMAP 2480	Wine	Chile	ND
Borneman et al. (2014)	AWRI 1608	Wine	Australia	Triploid
	AWRI 1613	Wine	Australia	Diploid
Crauwels et al. (2015)	ST05.12/26 = MUCL 49865	Beer	Belgium	Diploid
	ST05.12/48	Beer	Belgium	Diploid
	ST05.12/53	Beer	Belgium	Triploid
	ST05.12/59 = CBS 6055	Dry ginger ale	United States of America	Triploid
Tiukova et al. (2019)	CBS 11270	Bioethanol	Sweden	Diploid
Olsen et al. (2015)	CBS 2796	Wine	Germany	ND
Fournier et al. (2017)	9 strains	Wine, beer, bioethanol	Europe, Oceania, Africa	Diploid, triploid
	53 strains	Wine, beer, bioethanol, soft drink	Europe, Oceania, Africa, America	Diploid, triploid
Serra Colomer et al. (2020)	64 strains	Beer, bioethanol, wine, kombucha, tequila	Europe, Oceania, Africa, America	Diploid, triploid
Eberlein et al. (2021)	71 strains	Beer, bioethanol, wine, kombucha, tequila	Europe, Oceania, Africa, America	Diploid, triploid

<sup>a</sup> ND, "not determined" by the corresponding publication.

core genes involved in carbon and nitrogen uptake have been identified, consistent with the ability of *B. bruxellensis* to metabolise a wide range of complex nutriment (e.g., xylose, lactose, cellobiose and nitrate).

In *B. bruxellensis*, the nucleotide diversity estimated by the average number of pairwise nucleotide differences,  $\pi$ , is high ( $\pi = 1.2 \times 10^{-2}$ ) compared to *S. cerevisiae* ( $\pi = 3 \times 10^{-3}$ ) (Gounot et al., 2020). In addition, roughly 50% of the available strains of *B. bruxellensis* (exclusively isolated from anthropic niches) are triploids (Avramova, Cibrario, et al., 2018). Polyploidy in industrial yeasts is known to confer robustness and environmental stress resistance (Albertin & Marullo, 2012; Querol & Bond, 2009; Steensels et al., 2021). Interestingly, while triploidy appears to be predominant in the available strains, aneuploidy is rarer in *B. bruxellensis* than in *S. cerevisiae* with 5.6% and 19.1% of aneuploidy isolates, respectively (Gounot et al., 2020; Peter et al., 2018). Genomic studies have shown that the triploid genomes have different genomic architectures arising from intra- and interspecific hybridization events (Borneman et al., 2014; Eberlein et al., 2021; Gounot et al., 2020). In a recent study, the polyploid genomes were reconstructed using different phasing strategies, revealing that each polyploid subpopulation had a unique history (Abou Saada et al., 2021). The different triploid genomes (at least four) are composed of a core diploid *B. bruxellensis* genome and an additional haploid one. These additional genomes are either genetically closely related to the diploid one (with a genetic divergence lower than 1%) or genetically divergent (greater than 3%), indicating auto- as well as allopolyploidization events. Interestingly, the three allopolyploidization events have occurred independently with a specific and unique donor for each of the polyploid subpopulations (Table 1). The closest *Brettanomyces* sister species, *B. anomalus*, shows >23% of genetic dissimilarity with the diploid *B. bruxellensis* genome, excluding the known *Brettanomyces* sister species as possible donors. Large-scale population genomic surveys with a long-read sequencing strategy will help refine precisely the evolutionary history of each subgenome, and any intertwined relationships.

In addition, *B. bruxellensis* genomes show different levels of loss of heterozygosity (LOH). LOH events are a source of genomic rearrangements and can contribute to the rapid onset of phenotypic diversity (Dutta et al., 2021; Sampaio et al., 2020; Smukowski Heil et al., 2017). LOH has gained attention for its frequent association with fitness, adaptation, polyploid stabilization, or even pathogenesis in yeasts but also in other organisms such as the oomycete *Phytophthora capsica*, hybrids of the cultivated rice *Oryza* or the *Cobitis* fish species (Beekman & Ene, 2020; Forche et al., 2011; Janko et al., 2021; Lamour et al., 2012; Morales & Dujon, 2012; Todd et al., 2019; Wang et al., 1999). Compared to *S. cerevisiae* where LOH represents 50% of the genome, *B. bruxellensis* presents a low level of LOH, which is variable across subpopulations (Gounot et al., 2020; Peter et al., 2018). In the diploid isolates (wine 3 and kombucha groups), the LOH regions represent 13% of the entire genome. In the triploid subpopulations, different scenarios were observed. For

subpopulations that have undergone an allopolyploidization event, a higher proportion of the genome is under LOH in the beer and wine 1 groups (Figure 1b) with 26.6% and 22.3%, respectively (Eberlein et al., 2021). However, this fraction mainly concerns the acquired haploid genome. By contrast, the genomes of the teq/EtOH group are less impacted by LOH events (10.5%), which mainly involve the core diploid genome from *B. bruxellensis*. For the autotriploid population, LOH was also highlighted and conserved across six sequenced strains, resulting in the presence of only two haplotypes while three were expected (Eberlein et al., 2021). The conservation of a given haplotype over the others may indicate specific selection pressures on the alleles present in such LOH regions. Future studies will have to focus on the gene content of these LOH regions to identify possible genetic signatures of adaptation to anthropized environments.

Regarding the copy number variation (CNV), genes affected by CNV harbour functions related to drug transporters, nitrogen assimilation or ethanol production (Borneman et al., 2014; Crauwels et al., 2014; Curtin, Borneman, et al., 2012; Gounot et al., 2020). Ploidy level and CNV could play an important role in the adaptation to the ecological niche, where multiple copies of a particular gene or gene family can be beneficial in the new environment. For example, in *S. cerevisiae* yeast, beer isolates have more increased copies of genes involved in maltose uptake and breakdown are amplified (Gallone et al., 2016; Gladieux et al., 2014). In the pathogenic *C. albicans*, CNV are associated to a significant fitness benefit to antifungal drugs (Todd et al., 2019). Within allotriploid isolates, the diploid core genome was more prone to duplication events than the acquired haploid genome (Gounot et al., 2020). Further studies will be needed to determine if CNVs are actually associated with increased adaptation to specific ecological niche, as suggested for nitrate assimilation in bioethanol production process (Galafassi, Capusoni, et al., 2013). Indeed, genes involved in the nitrogen pathway have been independently lost in several diploid isolates within different subpopulations, indicating differential selective pressure (Gounot et al., 2020). Genomic rearrangements affecting genes associated with traits of ecological interests have also been reported. For example, genes coding beta-glucosidase activity are lost in beer isolates but conserved in wine isolates (Crauwels et al., 2017). This enzymatic activity could be useful to consume efficiently peculiar carbon sources found in wines aged in oak barrels, due to the liberation of specific wood polysaccharides. These variations were related to different aroma production, indicating technological interest in addition to ecological significance (Serra Colomer, Funch, et al., 2020). Variations were also observed for maltose-related genes, although it was not possible to associate a phenotype to a specific genotype (Crauwels et al., 2015; Serra Colomer, Chailyan, et al., 2020). Concerning horizontal gene transfer (HGT) in *B. bruxellensis*, three events from bacteria were recently highlighted, of which one encompassing an invertase-coding gene (from *Asaia* bacteria). This enzymatic activity is speculated to have conferred the ability to hydrolyse sucrose (Roach & Borneman, 2020). Horizontal gene transfers are drivers of adaptive evolution in eukaryotes and may have contributed to adaptation to high-sugar environments (Gladieux et al., 2014; Schönknecht et al., 2014). The

prevalence of these HGTs in the different subpopulations has yet to be described, and it will be interesting to assess their possible involvement in the adaptation of niches.

In addition to the nuclear genome, genetic diversity has also been described for *B. bruxellensis*' mitochondrial genome. The mitogenome is large and variable in size (between 75 and 100 kb), compact with introns and intergenic sequences (Eberlein et al., 2021; Procházka et al., 2010). By contrast to *S. cerevisiae*, the mitochondrial genome contains NADH dehydrogenase subunit genes, which allow the recycling of NAD in presence of oxygen during the fermentation process (Procházka et al., 2010). Across the different genetic subpopulations, the synteny of the mitogenome is well conserved with the exception of the teq/EtOH group for which a large inversion event and increased size (up to 100 kb) due to high intron content in the COB and COX1 genes were evidenced (Eberlein et al., 2021). The intron content is known to be variable within *Saccharomyces* genus, especially for COX1 and COB genes and was shown to be a marker of hybridization events (especially COX1 introns) (De Chiara et al., 2020; Prasai et al., 2017). In terms of nucleotide diversity, the genetic subgroups are highly homogeneous. The teq/EtOH group is more distant, with a genetic variation of 2% to 3% with the reference mitogenome. In *Ascomycota* species, the mitochondria inheritance is biparental, implying initial heteroplasmy in case of hybridization events. The outcome of the different mitotypes can be variable: (i) one or the other might be retained stochastically, (ii) one or the other might be retained due to selective pressures (e.g., to purge nucleocytoplasmic incompatibility or because of higher fitness of one mitotype), (iii) a chimeric mitotype can emerge from the two parental ones due to recombination events (Albertin et al., 2013; De Chiara et al., 2020; Lee et al., 2008). Here, the well-conserved mitotypes across the populations of diploid and polyploidy isolates argue in favour of the selection hypothesis without exchange. By contrast, the teq/EtOH group might have acquired a chimeric mitochondrial DNA, or the mitotype from the donor of the acquired haploid genome. Further studies will be needed to understand the possible impact of these mitochondrial genomes on the phenotypic diversity.

The exploration of the diversity of *B. bruxellensis* genomes has just started. Population genomic surveys unveil a complex genome architecture, with a strong involvement of polyploidy and hybridization events and mechanisms generating intraspecific variation (e.g., CNVs, LOH). LOH events appear to have radically shaped the genomic landscape of *B. bruxellensis* polyploids. This phenomenon is widely observed in polyploid yeasts and is an essential source of interindividual variation in predominantly asexual species (Peter et al., 2018; Steensels et al., 2021). Subsequent large-scale whole-genome sequencing will help to identify and understand the forces that shape the evolution of *B. bruxellensis* genomes, especially in the context of ecological divergence and industrial adaptation. However, genomic approaches alone will not be sufficient to formally demonstrate the relationship between the genomic and the phenotypic diversification of the species. Large-scale phenotyping approaches are therefore necessary to clarify the link between genetic diversity and adaptation to anthropic fermentation environments.

### 3 | PHENOTYPIC DIVERSITY OF *BRETTANOMYCES BRUXELLENSIS*

Although molecular tools bring priceless information to understand the origin of the species, their formation and diversification in ever-changing environments, the physiological abilities and metabolic features of the organisms can also be used to seek a deeper comprehension of their niche space use.

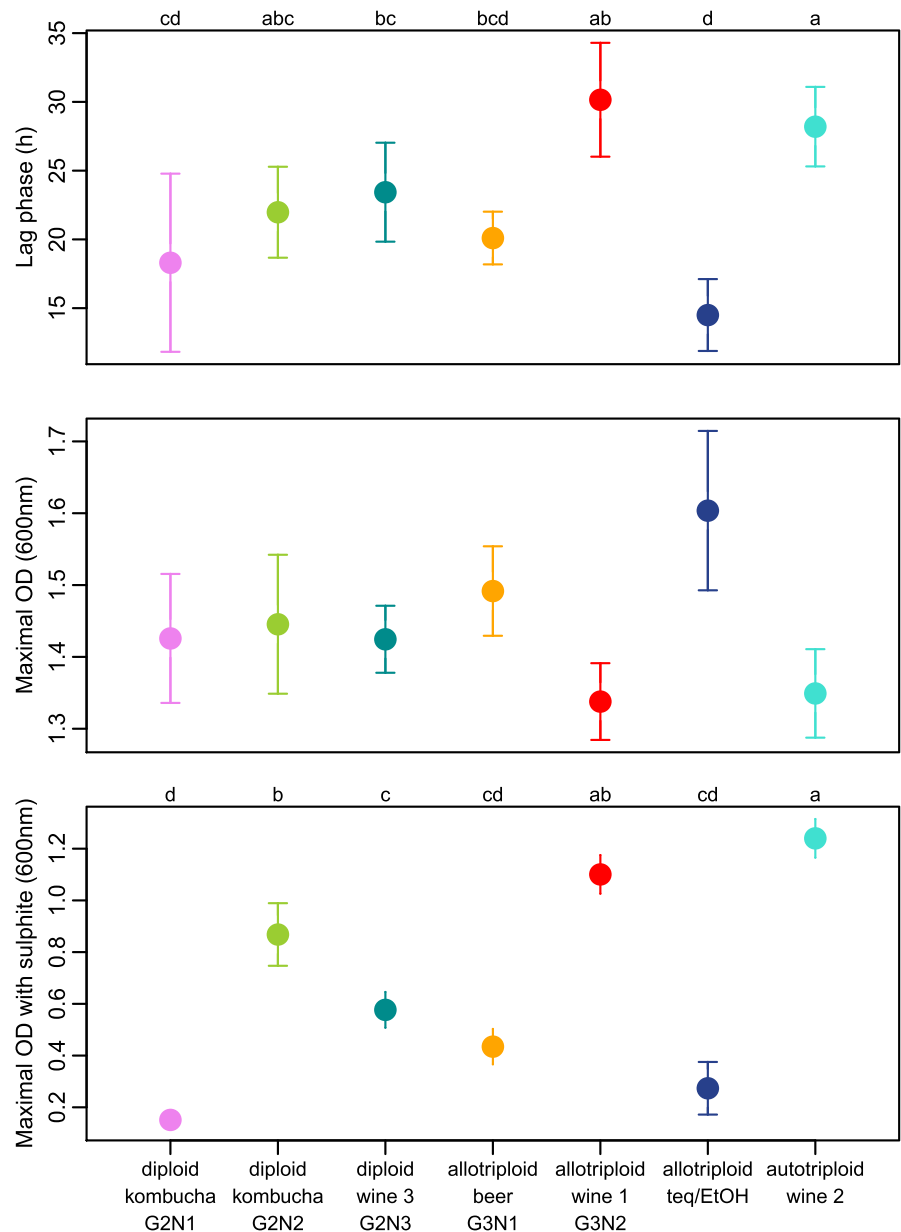
#### 3.1 | Intraspecific diversity of central metabolism and nutrient requirements

The nutritional requirements of *B. bruxellensis* are less described than those of model species such as *S. cerevisiae*. Nevertheless, *B. bruxellensis* exhibits atypical characteristics related to the metabolism of carbon, oxygen, nitrogen and other nutrients, that may explain its ability to colonise harsh environments described as "apocalyptic" (Smith & Divol, 2016). The main phenotypic characteristics of *B. bruxellensis* have already been reviewed (de Barros Pita et al., 2019; Blomqvist & Passoth, 2015; Schifferdecker et al., 2014; Serra Colomer et al., 2019; Smith & Divol, 2016; Steensels et al., 2015). However, phenotypic traits have been little studied in the light of genetic diversification. *Brettanomyces bruxellensis* is characterized by growth variability which is partly related to ecological origin and/or to the genetic group (Louw et al., 2016; da Silva et al., 2019). As an example, bioethanol isolates grow faster compared to wine isolates on seven carbon sources (sucrose, cellobiose, maltose, lactose, glucose, fructose and galactose) (da Silva et al., 2019) and the variability of growth was highlighted for other genetic groups as well (Figure 2).

Generally, central regulatory mechanisms like Glucose Catabolite Repression GCR (Leite et al., 2012; da Silva et al., 2019) and Nitrogen Catabolite Repression NCR (de Barros Pita et al., 2011; de Barros Pita & Tiukova, 2013; Cajueiro et al., 2017; Galafassi, Capusoni, et al., 2013) seem to be less strict in *B. bruxellensis* compared to *S. cerevisiae* (i.e., nonglucose and nonammonium sources can be metabolized even in presence of high glucose/ammonium concentration). Diversity was observed in GCR, with bioethanol isolates of *B. bruxellensis* being less susceptible to GCR than wine isolates (Blomqvist et al., 2010; da Silva et al., 2019). There is no data available to our knowledge for isolates from other substrates, yet this feature needs more research as it may have significant ecological and technological implications: the simultaneous use of different carbon and nitrogen sources could increase the efficiency of absorption in low-nutrient media and be associated with higher fitness in specific environments.

The most striking characteristic of *B. bruxellensis* is its ability to use a wide range of carbon sources as shown by large-scale phenotypic analyses (Cibrario, Miot-Sertier, et al., 2020; Conterno et al., 2006; Crauwels et al., 2015; da Silva et al., 2019; Galafassi et al., 2011; Smith & Divol, 2018). However, the carbon utilisation varies among isolates: a survey on the assimilation of 190 different carbon sources on seven strains clearly showed a variability between isolates, that could be related to different processes (Crauwels

**FIGURE 2** Growth parameters for different subpopulations of *Brettanomyces bruxellensis*. Genetic groups as described by previous reports (Avramova, Cibrario, et al., 2018; Gounot et al., 2020). Growth data was taken from Avramova, Vallet-Courbin, et al. (2018). The growth parameters correspond to the lag phase (hour), maximum OD (600 nm) without or with sulphite (0.6 mg/L of molecular SO<sub>2</sub>). For each genetic group, mean values are represented by a circle, and error bars correspond to standard error. Top letters represent significance groups as defined by Kruskal-Wallis test when significant ( $p$ -value < .05).  $p$ -values were .0054, .11 and  $5.4 \times 10^{-10}$  for lag phase, maximum OD without or with sulphite, respectively



et al., 2015). By contrast to wine isolates, most beer strains were not able to consume galactose, some  $\beta$ -disaccharides (cellobiose and gentiobiose) or some  $\beta$ -substituted monosaccharides (arbutin and  $\beta$ -methyl-D-glucoside) (Crauwels et al., 2015; Serra Colomer, Funch, et al., 2020). This observation could be explained by a variation of beta-glucosidase and alpha-glucosidase activity between the genetic groups (Serra Colomer, Funch, et al., 2020). Different environmental selective pressures could be responsible for this feature (Serra Colomer, Funch, et al., 2020). However, further work involving more than seven isolates is needed, including strains representative of all described subpopulations to properly assess the relationship between carbon assimilation and process.

Like *S. cerevisiae*, *B. bruxellensis* is able to ferment sugars into ethanol even in the presence of oxygen, a phenomenon called the Crabtree effect, which supports the "Make-Accumulate-Consume" (MAC) strategy (Rhind et al., 2011; Smith & Divol, 2016). The

evolutionary and metabolic significances of the Crabtree effect remain highly discussed, although a simplistic viewpoint suggests that metabolic shifts (from respiration to fermentation and back again) are energetically costly and time-consuming (Pfeiffer & Morley, 2014). Furthermore, *B. bruxellensis* is an acetogenic yeast which produces large amounts of acetic acid in addition to ethanol under aerobic conditions. This production varies considerably between strains (up to 10-fold) (Freer, 2002; Freer et al., 2003) and could have ecological implication since acetic acid release in the environments is described as a useful feature to outcompete other microorganisms (Rozpędowska et al., 2011) or to lure/deter flies involved in yeast dissemination (Dzialo et al., 2017). Regarding oxygen impact, conflicting results have been highlighted, with some studies connecting oxygen input to increased or decreased growth (Aguilar Uscanga et al., 2003; da Silva et al., 2019; Smith & Divol, 2018). The conservation of the NADH dehydrogenase subunit genes in the mitogenome

suggests specific need of oxygen during fermentation of *B. bruxellensis* (Procházka et al., 2010). The oxygen requirement is barely deciphered in *B. bruxellensis* and should be the subject of deeper studies in the near future, particularly to determine its possible impact on niche colonization.

In addition to peculiarities regarding carbon and oxygen metabolism, *B. bruxellensis* has atypical nitrogen and vitamins requirements, which may explain its ability to colonize certain niches. In general, *B. bruxellensis* seems to have higher nitrogen needs than *S. cerevisiae* (Leite et al., 2012), and *B. bruxellensis* is able to grow on nitrate as sole nitrogen sources (Borneman et al., 2014; Conterno et al., 2006). This could be advantageous in wine or in bioethanol environment where nitrate is present and not consumed by the first *S. cerevisiae* fermenter (de Barros Pita et al., 2011; Cajueiro et al., 2017). Pena-Moreno et al. even reported that the presence of nitrate boosted ethanol production and growth for some bioethanol strains (Morales-de la Peña et al., 2019). Nitrate consumption capacity varies depending on the ecological origin of isolation (Borneman et al., 2014; Crauwels et al., 2015; da Silva et al., 2019). The nitrate assimilation cluster (NIT cluster) was found to be probably involved in the observed phenotypic variation: it encompasses genes encoding nitrate transporter, nitrate reductase, nitrite reductase and two related transcription factors. It has evolved differently between strains and ecological groups. While NIT cluster was duplicated in a wine diploid isolate capable of assimilating nitrate (belonging to the wine 3 subgroup) and it was partially deleted or subjected to gene conversion in beer isolates unable to assimilate nitrate (belonging to the beer subpopulation) (Borneman et al., 2014; Gounot et al., 2020). In a wine triploid isolate able to assimilate nitrate (from the wine 1 group), three haplotypes are present. To sum up, selective pressures in different ecological niches may have allowed different evolutionary trajectories, although researches involving more representative strains are needed to have the full picture. Regarding vitamins requirements, *B. bruxellensis* was initially defined as an auxo-autotroph (Peynaud & Domercq, 1956) but recent results indicate that biotin is the only vitamin which strongly affects the biotic capacity of *B. bruxellensis* in the long term (von Cosmos & Edwards, 2016). Thiamine requirements appear to depend on the strain and on the presence of ethanol in the growth medium (von Cosmos & Edwards, 2016).

### 3.2 | Traits of technological interest

Another notable characteristic of *B. bruxellensis* species is its ability to convert hydroxycinnamic acids (HCAs) into volatile phenols. HCA metabolism may have an important impact from an ecological viewpoint: HCA have antimicrobial properties, and the ability to convert HAC into less toxic compounds could promote yeast growth (Richard et al., 2015). In addition, HAC are important dietary antioxidants for flies. However, flies are not able to detect directly HCA and use ethyl phenols as an indirect indicator of their presence (Dweck et al., 2015). Flies interaction with yeasts have consequences on their dissemination and the possibility to colonize new

environments, and volatile phenols could be a key factor mediating yeast-insect interactions (Stefanini, 2018). In addition to their ecological importance, volatile phenols are considered as off-flavours in oenology (for review: Suárez et al., 2007; Wedral et al., 2010). The term "volatile phenols" generally includes 4-ethylphenol (4-EP), 4-ethylguaiaicol (4-EG) and 4-ethylcatechol (4-EC) and their vinyl forms, 4-vinylphenol (4-VP), 4-vinylguaiaicol (4-VG), 4-vinylcatechol (4-VC). These are produced through the conversion of hydroxycinnamic acids (HCAs): p-coumaric acid, ferulic acid and caffeic acid, respectively. The production of volatile phenols by *B. bruxellensis* strains is variable and was extensively described in oenological conditions (Agnolucci et al., 2009, 2010; Conterno et al., 2006; Di Toro et al., 2015; Hixson et al., 2012; Madsen et al., 2017; Romano et al., 2017; Vigentini et al., 2008; Zepeda-Mendoza et al., 2018). Most studies involved end-point analyses, which makes it difficult to distinguish between the intrinsic capacities of the strains and their modulation by external parameters. Furthermore, the respective importance of the different factors governing such variability is poorly described: a few authors showed that volatile phenol production was both strain-dependent and wine matrix-dependent (Chandra et al., 2016; Cibrario, Miot-Sertier, et al., 2020; Dias, Dias, et al., 2003; Zhu et al., 2012). The niche of isolation could also play a role and brewing isolates have a more efficient metabolism toward ferulic acid (leading to 4-EG) than p-coumaric acid compared to wine isolates (Lentz & Harris, 2015). Some wine and soft drink isolates possess a duplication of the vinyl phenol reductase gene, which is absent in beer isolates (Crauwels et al., 2017). However, while all these studies confirmed the existence of intraspecific variation, their impact on the production of volatile phenols and their possible ecological significance remains to be properly assessed.

*Brettanomyces bruxellensis* show important intraspecific variability regarding the production of other aroma-active molecules such as esters, fatty acids, terpenes, phenolic compounds, N-heterocycle, (see *Brettanomyces* aroma wheel, Joseph et al., 2017; Serra Colomer, Funch, et al., 2020; Tyrawa et al., 2019). Although some environmental factors may impact ester production (e.g., ethanol and p-coumaric acid concentrations Conterno et al., 2013; Joseph et al., 2007), esterase activity is also strain-dependent (Holt et al., 2018; Spaepen & Verachtert, 1982; Steensels et al., 2015; Verstrepen et al., 2003). The intraspecific variation in the production of esters is mirrored at the aromatic level and impacts fruitiness, which is particularly important for Lambic beer style (Van Oevelen et al., 1977). The activity of some alpha-glucosidase and beta-glucosidase enzymes may be involved in the release of aromatic compounds like terpenes (Crauwels et al., 2014; Daenen et al., 2007; Serra Colomer, Funch, et al., 2020; Vervoort et al., 2016). N heterocycles can be produced by *B. bruxellensis* in beverages (mostly in wine or beer) (for review see Snowdon et al., 2006). These molecules are responsible for the "mousy" off-flavour, which causes rejection by the consumers. The metabolic pathway is not fully understood, but the amino-acid content and the oxygen availability in fermented beverages are key parameters for their production (Grbin & Henschke, 2000; Grbin et al., 2007). The state of art is somewhat inconsistent, which may be

explained by the difficulty to properly measure such compounds and their seemingly random occurrence in fermented beverages. From an ecological aspect, all these aromatic compounds could play key roles in natural environments, including signalling and communication with other organisms, and/or attractants for animals, particularly insects (Dzialo et al., 2017).

*Brettanomyces bruxellensis* shows an incredible ability to persist over long time in some industrial environments (Cibrario, Miot-Sertier, et al., 2020). Such capacity of persistence could be linked to the various physiological states of the cells, beside the classical free-living (planktonic) state. Indeed, *B. bruxellensis* is able to form biofilm, pseudo-mycelium, chlamyospore-like structure and viable but nonculturable (VBNC) cells. While many aspects remains unknown or contradictory, a physiological change between these forms could be induced by stress conditions related to the presence of some chemical compounds and/or to nutrient limitation (Uscanga et al., 2000). Intraspecific diversity was observed in the ability to enter or exit the VBNC state (Capozzi et al., 2016; Longin et al., 2016) as well as to form pseudohyphae (Martyniak et al., 2017) or biofilm (Joseph et al., 2007). Recently, two concomitant studies (Dimopoulou, Renault, et al., 2019; Lebleux et al., 2020) have suggested that the ability to produce biofilms depends on the genetic groups. Although only a limited number of strains were tested, the beer group showed robustness regarding biofilm formation in glucose-rich or glucose-limited media. For other genetic groups, production of biofilm was more dependent on environmental characteristics, suggesting different regulatory mechanisms (Dimopoulou, Renault, et al., 2019). These differences were also partly mirrored by the biochemical and physicochemical properties of the surface of the strains (Dimopoulou, Renault, et al., 2019). Finally, whether *B. bruxellensis* is actually able to sporulate and to undergo a sexual cycle remains a mystery. Early description of ascospore-like forms by Van der Walt (1964) prompted the definition of a teleomorphic stage (i.e., the sexual reproductive stage of a fungal species in mycology). The teleomorph was named *Dekkera bruxellensis* (*B. bruxellensis* being the anamorph counterpart, that reproduces asexually). Since then, ascospores formation was scarcely described (de Souza Liberal et al., 2007), and most authors consider *B. bruxellensis* as lacking an effective sexual cycle (Hellborg & Piškur, 2009). In particular, the existence of a meiosis should be associated with triploid instability, which is not evidenced at the genomic level. The impact of polyploidy and hybridization on the ability to switch from one form to another (planktonic, sessile, biofilm, pseudomycelium) remains to be elucidated. From an ecological viewpoint, the possibility to adopt various lifestyles and switch from one to another cellular morphologies may facilitate niches adaptation and drastic environment changes, as it the case for dimorphic fungi whose yeast-hyphae switch is recognized as an essential adaptation for host colonization and pathogenicity (Boyce & Andrianopoulos, 2015).

As one of the main worldwide wine spoilers, winemakers try to prevent and/or control *Brettanomyces* contamination through several methods such as the use of filtration, application of ozone, high pressure, ultrasound, ultraviolet irradiation, pulsed electric fields,

chitosan or the addition of sulphite (Table S2). So far, most studies evaluating the efficiency of these different applications have included a very small subset of strains (Table S2) with questionable representativeness, especially for wine isolates that are found into different genetic groups. As a result, conflicting conclusions have been recorded: for example, chitosan treatments have sometimes been described as very effective (Bağder Elmacı et al., 2015) or moderately effective against *B. bruxellensis* (Petrova et al., 2016). This incongruity has been partly resolved, a strain-dependent sensitivity to chitosan was demonstrated (Paulin et al., 2020). In future studies, it would be interesting to define a standardized method to evaluate the efficiency of antimicrobial treatments against *B. bruxellensis* including a panel of isolates representative of the genetic diversity of the species.

In winemaking, the most common treatment is the addition of sulphur dioxide (Barata et al., 2008). High intraspecific diversity in sulphite tolerance was repeatedly demonstrated in several studies (Avramova, Cibrario, et al., 2018; Dimopoulou, Hatzikamari, et al., 2019; Galafassi, Toscano, et al., 2013). On a cohort of 100 isolates from winemaking, three phenotypic groups were defined: sensitive (slowed growth), tolerant (delayed growth) and resistant (no impact on growth) (Vigentini et al., 2013). A recent study was able to connect sulphur sensitivity to the genetic groups, with two triploid groups (wine 1 and wine 2) containing most tolerant/resistant isolates (Avramova, Cibrario, et al., 2018). Competition experiments between sensitive/tolerant isolates under increasing concentration of sulphite showed specific adaptation of isolates from wine 1 allotriploid group to high SO<sub>2</sub> environments (Avramova et al., 2019). Different *SSU1* haplotypes (*SSU1* encoding sulphite efflux pump) related to variable SO<sub>2</sub> tolerance were characterized (Valdetara et al., 2020; Varela et al., 2019, 2020). It revealed that gene dosage effect (the number of *SSU1* haplotype) as well as the *SSU1* regulation could be at least partially involved in SO<sub>2</sub> tolerance (Varela et al., 2019). In a recent experiment, Bartel et al. (2021) conducted a laboratory experimental evolution in presence of sulphur dioxide concentrations, and evidenced adaptive evolution in different genetic backgrounds targeting partly *SSU1* (Bartel et al., 2021). A genotyping study showed that the two genetic groups (wine 1 and wine 2) containing the most tolerant/resistant isolates of *B. bruxellensis* were scarcely isolated before 1990 and that their proportion had increased steadily since then, possibly with the increase of sulphur use in oenology (Cibrario et al., 2019). Sulphur tolerance/resistance in *B. bruxellensis* could thus be the result of anthropization and adaptation to winemaking environments as for wine strains of *S. cerevisiae* (Gallone et al., 2016; García-Ríos & Guillamón, 2019; Kaewkod et al., 2019). The fact that sulphite tolerant/resistant phenotypes are present in two different genetic clusters (one autotriploids, one allotriploid) may indicate independent acquisition and possibly different adaptive mechanisms. How much and how the change in ploidy contributes to the resistant/tolerant phenotype remains to be resolved in future studies.

In conclusion, although a high number of surveys investigated various *B. bruxellensis* traits with fundamental and applied interest, many of these published studies used a small subset of strains usually



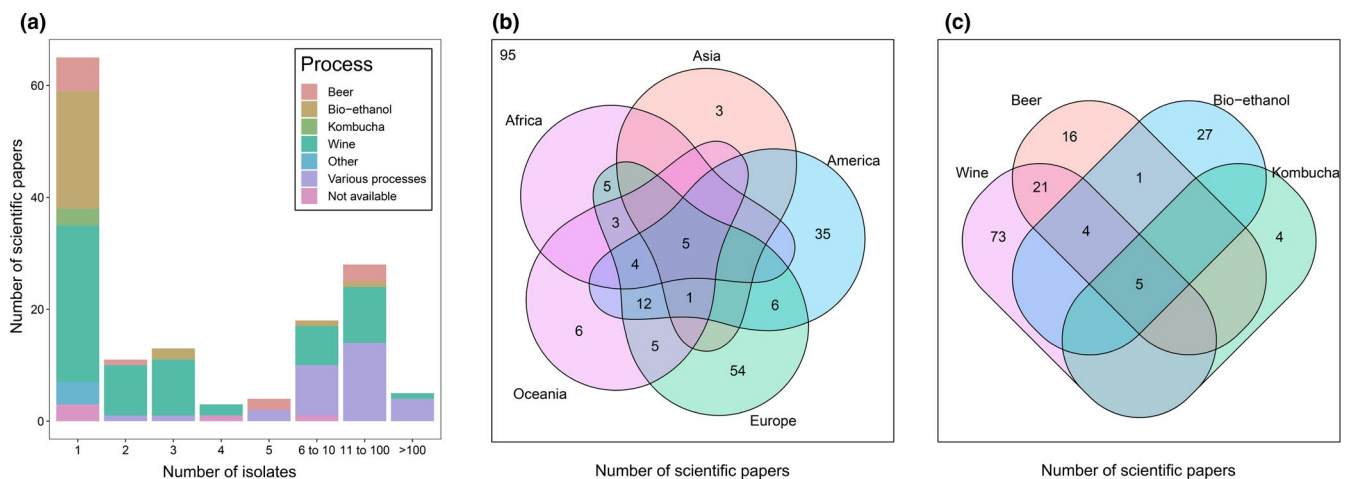
not representative of the genetic diversity of the species (Figure 3, Table S3). *Brettanomyces bruxellensis* literature is scattered with incongruences that could be directly related to intraspecific diversity and the lack of representability of the isolates tested. It highlights the necessity, for future research, to properly take into consideration strains from different ploidy levels, with varied hybridization backgrounds (auto- and allotriploids), isolated from various substrates and geographical origins and distributed within the different genetic populations described.

## 4 | DISCUSSION AND PERSPECTIVES

### 4.1 | *Brettanomyces bruxellensis*, the fermentation finisher

*Brettanomyces bruxellensis* has attracted increasing attention recently due to its involvement in industrial processes and its unusual genomic composition. *Saccharomyces cerevisiae* is one of the most intensively studied eukaryotic models, at the fundamental level in molecular, cellular and ecology biology, but also from an applied point of view as it is widely used as a fermentation starter for several human processes (Boone, 2014; Chambers & Pretorius, 2010; Goddard & Greig, 2015; Peter & Schacherer, 2016). Interestingly, while *B. bruxellensis* species is associated with similar anthropic processes (e.g., oenology, brewery), it is particularly recognized for its competence to colonize already fermented environments (such as wine or beer rather than grape must or wort) (Schifferdecker et al., 2014). *Brettanomyces bruxellensis* is thus a perfect model of fermentation finisher, a complementary counterpoint to *S. cerevisiae* model of fermentation starter, sharing the same industrial niches, but not with the same temporality. Interestingly, no isolates of *B. bruxellensis* were identified outside human related processes so far. Several reasons may explain the nondetection of wild isolates. First,

*B. bruxellensis* is a slow-growing yeast species, so it may be easily outcompeted by other yeast species whenever culturable methods are used (Agnolucci et al., 2017). Another possibility is that *B. bruxellensis* could be part of biofilm communities in natural environments—albeit at low-abundance and/or in viable but not cultivable (VBNC) state preventing its isolation. Indeed, 40%–80% of cells on Earth live and persist in multispecies communities (bacteria, archaea, eukaryotes, etc.) forming biofilms, for example, aggregated structures frequently enclosed into matrixes of extracellular polymeric substances (Flemming et al., 2016; Flemming & Wuertz, 2019). Growth in polymicrobial biofilms is relatively protected from environmental variations, and could improve survival in hostile environments (Flemming & Wuertz, 2019). The identification of low-abundance or VBNC species within complex communities remains a challenge, prompting the use of metagenomics, culturomics or reverse genomics approaches (Martellacci et al., 2019; Ryu et al., 2021). *Brettanomyces bruxellensis* has abilities to form VBNC or to be involved in microbial consortia such as kombucha's SCOBY (Symbiotic Community of Bacteria and Yeast) (Savary et al., 2021). These characteristics make it plausible the hypothesis of natural biofilms as one of their wild environments, although no definite evidences are described so far. In addition to biofilm, natural fermentations are another putative example of ecosystems that could foster the growth of wild *B. bruxellensis*. Many insects, mammals or birds store foods (fruits, grains, meat, etc.) for times of less plentiful sustenance. Food storage is prone to microbial growth or spoilage, and natural fermentations occurred long before humans, and long before human-directed fermentations (Carrigan et al., 2015; Post & Urban, 1993; Ruxton et al., 2014; Wiens et al., 2008). These naturally-occurring fermentations are poorly described, but they probably display similar characteristics to the anthropic environments of *B. bruxellensis*: presence of ethanol and organic acids, wide variety of ever evolving sugar, nitrogen and other nutrients contents, oxygen availability, succession of complex microbial communities, etc. In such natural fermentations, *B. bruxellensis*



**FIGURE 3** Representativity and diversity of *Brettanomyces bruxellensis* collections studied in the literature. (a) Histogram of the number of isolates of *Brettanomyces bruxellensis* used in the studies referenced in Table S3, with their process/substrate of isolation. (b) Venn diagram of the number of studies including isolates of *B. bruxellensis* from various geographical origins. (c) Venn diagram of the number of studies including isolates of *B. bruxellensis* from various process origins

could play second fiddle and colonize the environments after more efficiently growing and fermenting microorganisms. Also, in natural environments, the role of wild-fermentation finisher would be congruent with the scavenging abilities of *B. bruxellensis* (Smith & Divol, 2016). However, this hypothesis remains purely speculative: the literature is scarce regarding the microbial communities associated with food caching or hoarding and so are the available isolates from these niches (Herrera et al., 1997; Post & Urban, 1993). Obtaining and studying natural isolates would be an ideal option, but even in the absence of data on their wild counterparts, future studies both at the genomic and phenotypic levels will help in outlining the characteristics of the natural reservoirs of *B. bruxellensis*. In particular, study of the central metabolism and the molecular mechanisms (HGT, LOH, CNV, etc.) driving their evolution will help to determine which metabolic functions are conserved, lost or gained and their possible involvement into adaptations to anthropic environments. For example, determining whether maltose metabolism or beta-glucosidase activity were acquired before *B. bruxellensis*' anthropic associations may give clues concerning its possible natural niches (i.e., crops- or wood-related). Future works should explore more thoroughly all these aspects (central metabolism, VBNC and biofilm abilities, etc.) to gain more insights into a fermentation-related microorganism and to improve our possibilities of controlling the species in various processes.

## 4.2 | Exploring the relationships between polyploidy/hybridization and adaptation to anthropized environments

Genomic analyses unveiled a genetic diversity of *B. bruxellensis* related to specific substrates. Diploid isolates coexist with triploid ones of hybrid origins, and several subpopulations of diploid and triploid individuals are described. Some of these groups are related to specific substrate origins (wine, beer, kombucha, tequila) and display adaptive traits related to their ecological niche (e.g., sulphite tolerance/resistance in winemaking, ability to metabolize nitrate in bioethanol production, maltose utilization in beer process). In industrial processes, where stress is omnipresent, polyploidy and aneuploidy are recurrent events in domesticated populations of *S. cerevisiae* (Peter et al., 2018; Querol & Bond, 2009; Steensels et al., 2021). In *B. bruxellensis*, the level of aneuploidy (approximately 5%) is surprisingly low considering: (i) the proportion of aneuploidy in other yeast species (19% in *S. cerevisiae*, 15% and 33% in the pathogenic yeasts *Cryptococcus neoformans* and *Candida albicans*, respectively [Gounot et al., 2020; Peter et al., 2018; Rhodes et al., 2017; Scopel et al., 2021; Selmecki et al., 2006]) and (ii) the proportion of triploids in *B. bruxellensis* (>50%) which are known to evolve quickly toward aneuploidy and diploidy in *Saccharomyces* species (Avramova, Cibrario, et al., 2018; Gerstein et al., 2008; Todd et al., 2017). The phylogeny and evolutionary history of *B. bruxellensis* species are particularly complex to reconstruct because of the intertwined events of polyploidization and hybridization (Linder et al., 2003). New

approaches will be needed to unravel its complex genetic architecture and to elucidate the precise relationship between genomic evolution and the actual adaptation to anthropized environments. The evolution of many species (plant, animal, microorganism) is strongly shaped by human activities. When anthropogenic-driven transformation is purposefully associated with improved attributes, the species are labelled as domesticated, as in *S. cerevisiae* for which several independent domestication events were evidenced (Gallone et al., 2018; Giannakou et al., 2020; Steensels et al., 2019). In the case of *B. bruxellensis*, the situation seems to be more complicated: the fact that some beer isolates are used by brewers for their improved ability to metabolize specific sugars may correspond to domestication. Conversely, adaptation to winemaking environments through acquisition of sulphite resistance is probably an unintentional consequence of anthropogenic influence. Thus, for wine groups, the term "domestication" does not apply to what seems to be an adaptive evolution at the expense of humans. A few works already provided evidence of various beneficial or detrimental traits associated with the different anthropized environments colonized by *B. bruxellensis*. The underlying molecular mechanisms remain to be explored more thoroughly, in particular the role played by LOH, HGT, CNV and other mechanisms in the evolution and adaptation of the species. How polyploidization and hybridization actually impacted the evolutionary routes of *B. bruxellensis* is still an open question. Future directions should take advantage of this model yeast to examine closely the influence of anthropogenic activities on the species and their genomic, phenotypic and adaptive consequences, whether positive or negative for humans.

The description of both auto- and allotriploid groups raises the question of their origin. The recent genomic approaches, and particularly phasing methods, allowed the precise description of the genomic content of these triploids that harbour a core diploid genome added with an additional haploid one of various intra- and interspecific origins (Abou Saada et al., 2021). Triploid formation in *B. bruxellensis* happened at least four times, suggesting the successive occurrence of a not-so-rare event of polyploid formation, followed by a not-so-rare establishment mechanism (selection or random genetic drift). Regarding the possible mechanisms of polyploid formation, accidental miss-repartition of chromosomes during mitosis can lead to unbalanced number of chromosomes in daughter cells (Todd et al., 2017; Wertheimer et al., 2016). However, this pathway would account only for the formation of autotriploids (not allotriploids), furthermore without heterozygosity increase, which is described in *B. bruxellensis* wine 2 autotriploid group. In addition, the presence of a complete haploid set of chromosomes tends to invalidate the hypothesis of mitosis mishaps, which would more frequently lead to aneuploids rather than polyploids. Autopolyploidy can also occur via endoreduplication—replication of the whole nuclear genome in absence of mitosis—a phenomenon frequently described in plants (Harari et al., 2018; Sugimoto-Shirasu & Roberts, 2003). However, endoreduplication accounts only for even ploidy levels, not for odd (triploid) ones, and is not associated with increased heterozygosity levels. The production of unreduced

gametes, followed by intra- or interspecific hybridization, is another route of polyploid formation frequently described in plants and animals (Otto & Whitton, 2000). The absence of evidence of sexual cycle in *B. bruxellensis* makes this mechanism less likely. Protoplast (spheroplast) fusion could be an interesting hypothesis: protoplast formation could occur after cell wall digestion in insect guts and subsequent protoplasts fusion could allow intra- and interspecific hybridization of nonsexual species (Steensels et al., 2014). To date, protoplast fusion is the most likely hypothesis of auto- and allotriploid formation, yet the literature lacks of formal evidence and future genomic and ecological analyses may shed lights on the possible routes of polyploid formation in *B. bruxellensis*. In addition to polyploidization/hybridization mechanism, the prominence of *B. bruxellensis*' triploids indicates that triploid formation was followed by the successful establishment of these lineages, through either neutral genetic drift or natural/artificial selection. It seems highly unlikely that random genetic drift could account alone for the presence of triploid lineages in *B. bruxellensis*: firstly, polyploidy is related to energetic and resource costs that, far from being neutral, should lead to its counterselection (Comai, 2005; Neiman et al., 2013). Second, at least four auto- and allotriploid events have led to the independent establishment of triploid lineages, a high number for a purportedly stochastic phenomenon. Third, some of these triploid lineages show (or are suspected to show) higher fitness in their environments of predilection, such as sulphite resistance for the wine 1 and wine 2 groups, the possible metabolization of maltose and other complex sugars for the beer group, or increased growth and the ability to metabolize nitrate for teq/EtOH group (Avramova, Vallet-Courbin, et al., 2018; Crauwels et al., 2015; Galafassi, Capusoni, et al., 2013; Serra Colomer, Chailyan, et al., 2020). All these elements suggest that polyploidization in *B. bruxellensis* is not neutral from an evolutionary viewpoint, although more genomic and phenotypic studies are needed to deepen our understanding of the evolutionary fates of these polyploids compared to their diploid counterparts.

Finally, the large genetic differences (up to 3%) recorded between subpopulations raise the question of the actual number of species within this clade (Eberlein et al., 2021). No sexual cycle was formally recorded to date and no gene flow between *B. bruxellensis* subpopulations was ever described, suggesting independent evolution within each clade, and speciation in progress. Future large-scale genomic and phenotypic analyses will help determine whether we should still consider *B. bruxellensis* as a single but complex species with diverse subpopulations, or whether we should redefine *B. bruxellensis* as a complex of single species.

### 4.3 | *Brettanomyces bruxellensis*, a yeast model to study the relationship between polyploidy, hybridization and adaptation to human-related environments

Polyploidy and hybridization, two frequently associated events, have long been described as key evolutionary mechanisms underlying radiation and adaptation in many clades of plants, animals or fungi

(Gregory & Mable, 2005; Otto & Whitton, 2000; Todd et al., 2017; Van De Peer et al., 2017). In flowering plants, where the impact of polyploidy is the most studied, polyploidy and hybridization are frequently associated with phenotypic diversification, trait innovation, increased fitness, invasiveness abilities, adaptation to harsh environments and domestication (Ainouche & Wendel, 2014; Soltis & Soltis, 2009). Historically less studied in microorganisms, polyploidy and hybridization are now well established as driving evolutionary forces in yeast, Chromalveolata, but also in prokaryotes, including Bacteria and Archaea (Albertin & Marullo, 2012; Marcet-Houben & Gabaldón, 2015; Soppa, 2022). Yeast usually enables the development of systems biology approaches, and *B. bruxellensis* triploids may allow the investigation of the fates of genes duplicated by whole genome duplication and their impact on phenotypic traits. In addition, the close association between various human processes and different ploidy/hybridization status makes of *B. bruxellensis* a valuable model to decipher the evolutionary mechanisms involved in the adaptation to anthropized niches. The study of the diploid and triploid populations of *B. bruxellensis* may provide valuable insights into the ecological and evolutionary significance of natural polyploidy.

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#### CONFLICT OF INTEREST

The authors declare that they have no competing interests.

#### AUTHOR CONTRIBUTIONS

Jules Harrouard, Chris Eberlein, Joseph Schacherer and Warren Albertin conceived the original outline of the review, with substantial inputs from all authors. All authors screened the literature and selected the subset of publications to be included. Jules Harrouard, Chris Eberlein, Joseph Schacherer and Warren Albertin drafted a first version. Marguerite Dols-Lafargue assisted more specifically drafting the central metabolism section, Isabelle Masneuf-Pomarede and Cécile Miot-Sertie the aroma/biofilm/technological part, and Patricia Ballestra assisted with the aroma section. Jules Harrouard, Chris Eberlein and Warren Albertin contributed to the figures. All authors extensively revised the manuscript.

#### DATA AVAILABILITY STATEMENT

Phenotypic data files (Figure 2): Avramova, Cibrario, et al. (2018) <https://doi.org/10.3389/fmicb.2018.01260>. Genomic data (Figure 1b): Eberlein et al. (2021) <https://doi.org/10.1101/gr.275380.121>.

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## SUPPORTING INFORMATION

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### III/ Evolution, adaptation et anthropisation

#### Définition des concepts

L'Evolution n'est pas une marche linéaire vers le *progrès*, il s'agit d'une série d'évènements (bien souvent chaotiques) menant à des individus plus ou moins adaptés à un environnement donné (Buskirk et al., 2020). Dans le travail fondateur de Darwin, *On the Origin of Species* (1859), Charles Darwin a fourni un cadre qui a permis de comprendre sélection, diversification et extinction grâce à l'application de trois concepts : variation, hérédité et sélection. L'évolution est donc un processus de transformation d'une espèce au cours du temps *via* un ensemble de variations génétiques et phénotypiques. L'adaptation évolutive représente les changements héréditaires permettant l'augmentation de la valeur adaptative de la population dans un environnement donné et peut être définie comme la somme des changements morphologiques, physiques et comportementaux permettant aux individus la persistance des populations et des espèces dans un environnement (Grenier et al., 2016).

Le terme anglo-saxon '*fitness*', utilisé dans la littérature scientifique est difficile à définir clairement, et difficile à traduire en français. Il comporte deux pendants : la valeur sélective et la valeur adaptative. La valeur sélective est « la contribution, en nombre d'individus, à la génération suivante d'un individu » et va donc dépendre du succès reproducteur de l'individu (Barthélémy, 2020). La valeur adaptative d'un individu dépend de l'écart entre le phénotype d'un individu dans un environnement et le phénotype optimal de l'espèce dans cet environnement (efficacité d'un phénotype). Les deux notions peuvent évoluer dans le même sens, c'est-à-dire que l'individu le mieux adapté à un environnement peut y produire le plus de descendance. L'inverse peut également être possible, où un individu « maladapté » à un environnement peut produire beaucoup de descendants durant le temps de sa survie. Cependant, par choix et pour simplifier, le terme de valeur adaptative sera utilisé dans ce manuscrit.

Le phénotype dépend de trois facteurs, les facteurs génétiques (G), l'environnement (E), et les interactions de ces deux facteurs (GxE). Les facteurs génétiques correspondent à la part innée, héréditaire du phénotype et vont donc être étroitement liés à la sélection génétique. L'expression phénotypique d'un génotype dans différents environnements (la réponse phénotypique à des conditions environnementales différentes) s'appelle « norme de réaction » (Schlichting and Pigliucci, 1998). Tous les génotypes ont une norme de réaction, mais seuls ceux qui ont une norme de réaction variant en fonction de l'environnement sont dit « plastiques » (Barthélémy, 2020). Si la norme de réaction des génotypes plastiques, est différente d'un génotype à un autre, alors il y a interaction entre les génotypes et l'environnement (Figure 15). La plasticité phénotypique est donc la capacité d'un génotype (un individu) d'exprimer différents phénotypes dans différents environnements (Pigliucci, 2005; Przybylo et al., 2000; Wennersten and Forsman, 2012). Il s'agit finalement d'une palette de réponses phénotypiques à des stimuli de l'environnement (Pfennig, 2021a).

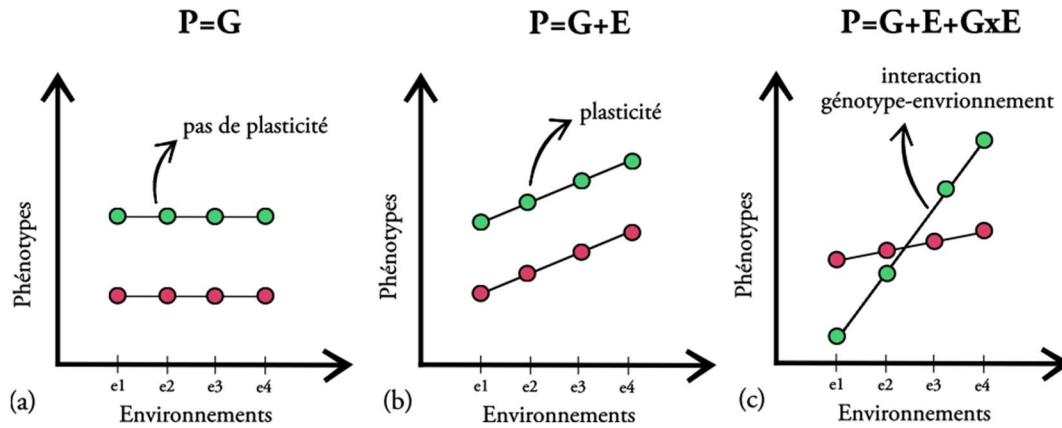


Figure 15: Représentation de deux génotypes dans différents environnements (normes de réactions). Sur le premier panel, les deux génotypes ne présentent aucune variation du phénotype dans les différents environnements, indiquant que les génotypes ne sont pas plastiques. Le phénotype ( $P$ ) dépend donc uniquement du génotype ( $G$ ). Dans le second panel, les phénotypes varient selon l'environnement, les deux génotypes sont donc plastiques. Le phénotype ( $P$ ) dépend du génotype ( $G$ ) ainsi que de l'environnement ( $E$ ). Dans le dernier panel, les deux phénotypes varient de manière différente d'un génotype à l'autre. Les génotypes sont plastiques mais il y a aussi une interaction spécifique de l'environnement en fonction du génotype ( $G \times E$ ). D'après Barthélémy, (2020).

L'adaptation évolutive est permise par deux mécanismes pouvant agir en synergie ou en opposition : la sélection génétique et la plasticité phénotypique, qui seront détaillés ci-dessous (Grenier et al., 2016). L'adaptation évolutive est un processus qui nécessite du temps (plusieurs générations), elle est guidée à la fois par des changements moléculaires et génétiques mais aussi par les facteurs environnementaux reçus par les individus. Si différentes populations d'une espèce expérimentent de façon continue des pressions de sélections due à des environnements distincts, ces populations vont évoluer vers une adaptation spécifique aux différents environnements. Le terme alors utilisé par la biologie évolutive est adaptation locale ('local adaptation') d'une population à un environnement.

### Sélection génétique

La sélection génétique se traduit par l'augmentation dans une population de la fréquence d'un génotype offrant une valeur adaptative supérieure. Elle permet l'établissement et l'augmentation de la fréquence d'allèles favorables, à la défaveur des allèles défavorables, et aura pour conséquence la réduction de la diversité génétique. Ce phénomène a lieu sur un temps long (plusieurs générations). La sélection génétique affecte également la fréquence des allèles neutres par le phénomène d'auto-stop (les régions proches ségrégent avec les gènes ou *loci* sous sélection). Au niveau phénotypique, les conséquences de la sélection génétique sont un changement de la valeur moyenne du caractère ou de sa variance. Il existe trois types de sélection (Figure 16): (i) la sélection directionnelle qui a pour conséquence la diminution ou l'augmentation du caractère phénotypique, (ii) la sélection stabilisante qui a pour conséquence la réduction de la variance du caractère dans la population, (iii) et la sélection disruptive avec la sélection des individus extrêmes et la mise en place d'une distribution bimodale du caractère dans la population (Grenier et al., 2016).

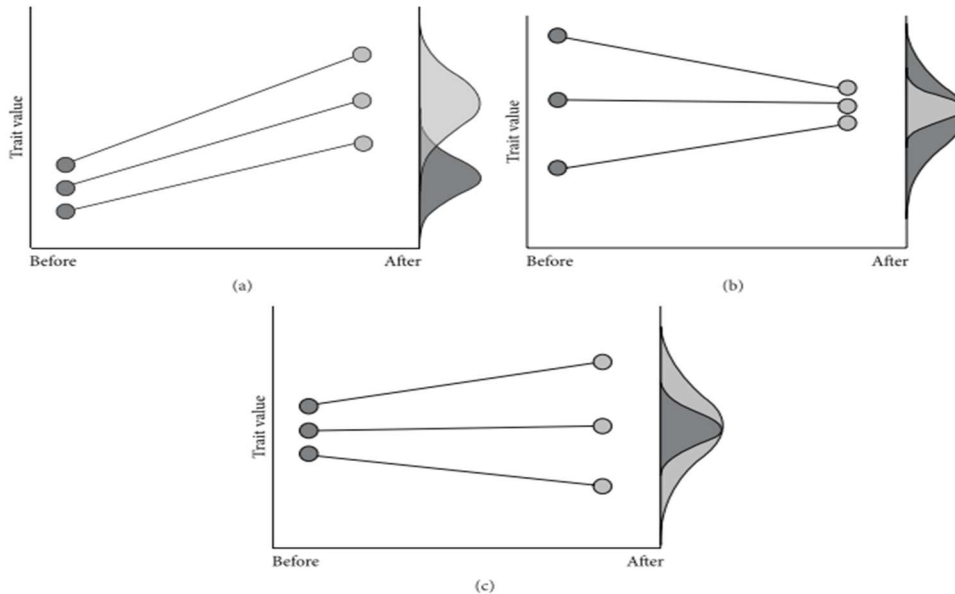


Figure 16: Changements de la variance ou de la moyenne des caractères phénotypiques d'une population en fonction de la sélection génétique. Le panel (a) représente une sélection directionnelle, le caractère phénotypique de la population est sélectionné pour une augmentation de la moyenne du caractère. Le panel (b) représente une sélection stabilisante qui a pour conséquence une réduction de la variance du caractère. Le panel (c) représente une sélection disruptive du caractère résultant dans l'augmentation de la variance de celui-ci sans variation de la moyenne. D'après Grenier et al., (2016).

#### La plasticité phénotypique : une adaptation réversible à l'échelle de l'individu

“Arguably, one of plasticity’s most important roles is to buy time for populations to adapt evolutionarily, i.e., to undergo heritable changes that increase fitness and thus persistence in the new environment.” (Diamond and Martin, 2021)

La plasticité phénotypique est la capacité d'un génotype de présenter différents phénotypes dans différents environnements (pour revue : Pfennig, 2021a). Les changements de phénotype liés à la plasticité phénotypique agissent sur des temps plus courts que la sélection génétique, à l'échelle de l'individu. Un individu avec un caractère phénotypique plastique aura un phénotype défini par les signaux qu'il percevra de l'environnement ainsi que par les effets stochastiques au niveau individuel. Ce changement de phénotype peut être réversible au sein du même individu mais aussi conservé sur plusieurs générations (Figure 17) (Chevin et al., 2013; Crispo, 2007; Zan and Carlborg, 2020). La plasticité peut conférer un avantage évolutif en permettant à un organisme de répondre immédiatement à des variations environnementales (Pfennig, 2021b). Son impact est parfois avancé pour être la première étape dans l'adaptation évolutive d'une espèce à une nouvelle niche écologique, permettant la survie des individus au changement d'environnement, pour que la sélection génétique ait lieu ('plasticity-first', 'Buying Time') (Baldwin, 1896; Diamond and Martin, 2021; Levis and Pfennig, 2016; Perry et al., 2018).

Les causes de la plasticité phénotypique sont encore assez méconnues, car la plasticité phénotypique a été historiquement sous-estimée voire ignorée dans la plupart des études d'adaptation évolutive



(Pfennig, 2021b; Yadav et al., 2016). Les approches moléculaires de ces dernières années ont montré que les conditions environnementales participent à la régulation des voies génétiques (Sultan, 2021). Des études de génétique quantitative (Quantitative Trait Loci, QTLs) ont montré que les causes pouvaient être des interactions gène-environnement, gène-gène-environnement (épistasie dépendant de l'environnement) et des modifications épigénétiques (Forsberg and Carlborg, 2017; Peltier et al., 2018; Zan and Carlborg, 2020). Cependant, lorsque qu'il s'agit de la modification d'une séquence nucléotidique, c'est-à-dire une mutation, alors cette plasticité phénotypique est soumise à l'action de la sélection génétique. Les deux mécanismes sont imbriqués et il est difficile (voir impossible) de délimiter précisément leur importance propre.

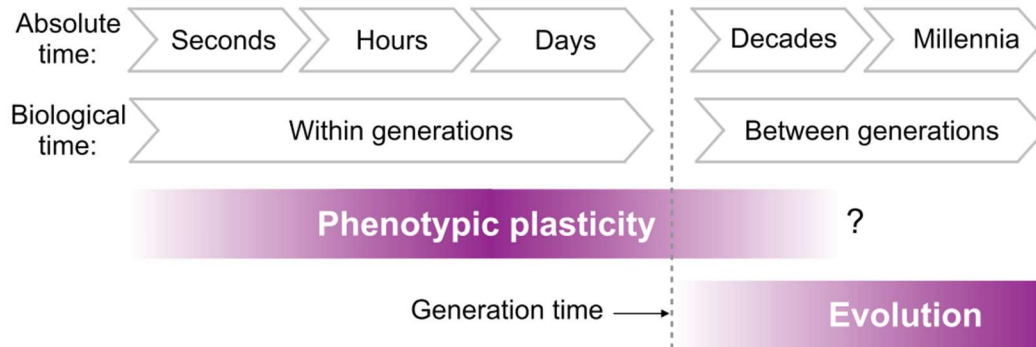


Figure 17: Echelle de temps, phénotypes et plasticité phénotypique ou évolution (adaptation évolutive). D'après Pfennig, (2021b).

La plasticité phénotypique a de nombreuses applications pratiques et peut éclairer des questionnements qui sont très actuels. La plasticité des individus sera de première importance dans leur valeur adaptative à l'heure des changements environnementaux rapides et certainement encore sous-estimés de l'anthropocène. L'optimisation des rendements agricoles par l'uniformisation des semences pourrait supprimer la plasticité phénotypique des plantes cultivées pour obtenir des rendements stables quelles que soient les conditions pédoclimatiques (la même approche peut être conduite sur les animaux d'élevage) (Pfennig, 2021b), ce qui semble être à contrecourant avec la nécessaire plasticité pour réussir à faire face aux changements environnementaux induit par l'anthropocène.

#### *Contraintes de la plasticité phénotypique et de la sélection génétique*

La plasticité phénotypique peut amener l'expression de phénotypes adaptés d'un génotype non adapté, ce qui réduit les pressions de sélection de ce génotype permettant sa persistance (Diamond and Martin, 2021). D'un autre côté, la plasticité phénotypique peut aussi amener l'expression de phénotypes *maladaptés* à un environnement. La plasticité phénotypique possède des contraintes regroupée sous deux termes : (i) un coût dans l'expression d'un phénotype résultant de la perte de valeur adaptative par rapport à un phénotype acquis par sélection génétique (maintenance des mécanismes de plasticité par exemple); (ii) une limite dans l'expression d'un phénotype suffisamment adapté. Si ces contraintes sont suffisamment fortes, la perte de plasticité d'un caractère peut être une étape permettant l'adaptation évolutive de l'espèce (Grenier et al., 2016).

Dans ces conditions, la stabilité d'un phénotype quel que soit l'environnement est appelée « canalisation » ou « robustesse », elle peut découler de la sélection génétique (Crispo, 2007; Grenier

et al., 2016; Kitano, 2004; Lively, 1986). Cette canalisation est en revanche désavantageuse dans un nouvel environnement, car le phénotype ne variera plus suffisamment aux stimuli de l'environnement et pourra être *maladapté* (Figure 18) (diminution de la valeur adaptative). Les deux phénomènes (plasticité, sélection) sont donc constamment en balance selon que les changements procurent des avantages à court ou long terme. En effet, la survie d'une espèce dépend de sa capacité à présenter le phénotype le plus adapté possible dans un environnement donné, tout en conservant la capacité à survivre et se reproduire si l'environnement vient à changer (Chevin et al., 2013). La sélection génétique et la plasticité phénotypique contribuent donc conjointement à la valeur adaptative et la diversité génétique de l'espèce.

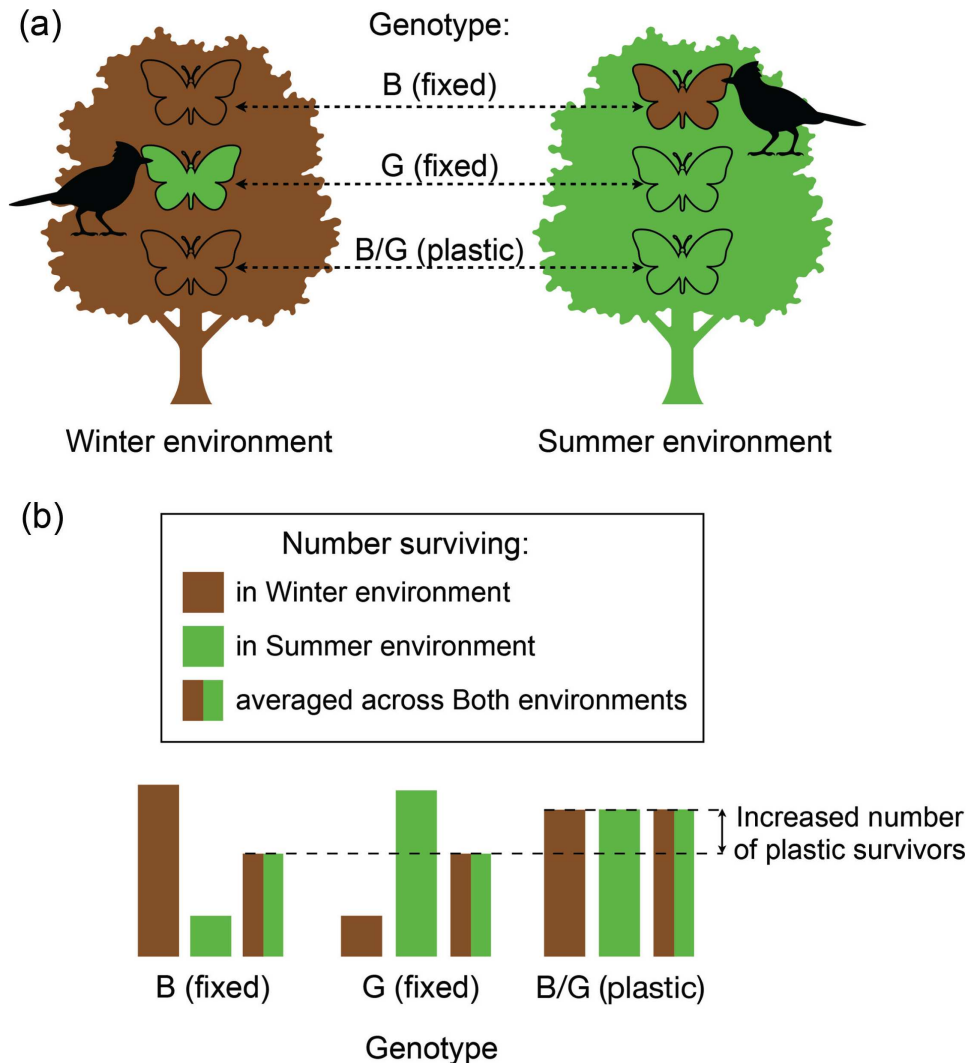


Figure 18: Exemple d'un apport de la plasticité phénotypique dans la valeur adaptative. La capacité d'exprimer des phénotypes variables et adaptés peut offrir un réel avantage dans des environnements changeants par rapport à un phénotype canalisé. D'après Pfennig, (2021a).

En conclusion, la sélection génétique et la plasticité phénotypique ont un impact important sur l'adaptation évolutive et peuvent être vues comme deux mécanismes temporellement distincts l'un de l'autre, mais étroitement liés dans la définition de la valeur adaptative d'un individu.

*L'adaptation à des milieux anthropisés : le cas particulier de la domestication*

L'adaptation génétique d'espèces à des milieux anthropisés a été mise en évidence depuis des dizaines d'années dans la littérature scientifique (Otto, 2018). La forme la plus extrême de cette adaptation est très certainement la domestication. La domestication est définie comme la sélection d'organisme par et pour l'être humain, de façon consciente ou non. Les caractères d'intérêt technologique sélectionnés par l'être humain varient d'une espèce à l'autre : rendement en grain pour le maïs, capacité d'apprivoisement du chien, capacité fermentaire pour la levure *S. cerevisiae*, etc. La domestication peut avoir comme effet une réduction de la valeur adaptative (e.g. capacité de survie et/ou de reproduction notamment dans milieu naturel d'origine), rendant l'organisme dépendant de l'être humain pour sa survie. Les premiers signes de domestication remontent pour la plupart à la révolution Néolithique (10 000 ans avant notre ère), avec la naissance de l'agriculture et la sédentarisation, à l'exception de la domestication du chien qui remonte au Paléolithique (15 à 33 000 ans) (Wayne and Vonholdt, 2012). La domestication est un cas particulier d'adaptation : la sélection y est généralement absolue, souvent récente. Comparée à l'adaptation à un milieu naturel, elle laisse des traces plus facilement identifiables dans les génomes et les phénotypes, ce qui augmente l'intérêt de son étude. Si la domestication des animaux et des végétaux s'est faite de façon volontaire et consciente (sélection par l'être humain des individus à propager), la domestication des micro-organismes, elle, s'est faite de manière non intentionnelle : les procédés technologiques utilisant des microorganismes (fermentation notamment) sont en effet très antérieurs aux travaux ayant permis la découverte et le rôle des microbes dans ces mêmes procédés. De plus la domestication des microorganismes diffère de celle des animaux ou végétaux car elle n'a pas été réalisée au niveau de l'individu mais d'une population (clonale ou non) (pour revue : Dupont et al., 2017; Gladieux et al., 2014; Marsit et al., 2017; Steensels et al., 2019). Le *backslipping* est l'une des pratiques qui a permis la domestication de plusieurs micro-organismes par transfert d'une partie d'un milieu déjà fermenté vers un milieu à fermenter. Chez les micro-organismes, 5 caractéristiques sont fréquemment associées aux populations domestiquées et pourraient servir de critères de domestication: (i) la différenciation génétique entre population(s) domestiquée(s) et population(s) sauvage(s); (ii) une dépendance et une adaptation aux environnements anthropisés (les niches anthropisées sont en effet souvent éloignées des niches naturelles); (iii) une perte de valeur adaptative de l'espèce dans la niche naturelle due à la perte de gènes qui ne sont plus soumis à sélection (par exemple perte de la capacité d'assimilation de sucres complexes par exemple ou '*genome decay*' qui peut suivre le relâchement de la pression de sélection); (iv) réduction de la diversité génétique (sous l'effet de la sélection intensive, de goulot d'étranglement et de dérive génétique); (v) mélange entre des sous-populations génétiquement éloignées, indiquant la présence d'une dispersion associée à l'être humain (Steensels et al., 2019). Les mécanismes évolutifs sous-jacents sont nombreux : changement nucléotidique (Single Nucleotide polymorphism, SNPs); variation du nombre de copies d'un gène (Copy Number Variation, CNVs); translocations; transferts horizontaux de gènes (Horizontal Gene Transfert, HGTs); hybridations; polyploïdie (Gerstein and Sharp, 2021; Marsit et al., 2017; Steensels et al., 2019; Stelkens and Bendixsen, 2022).

Les levures sont des modèles biologiques idéaux pour réaliser des études génomiques et phénotypiques comparatives : leur manipulation est facile, la multiplication clonale est possible, elles présentent un cycle de vie court, un génome peu complexe pour des eucaryotes, de larges collections d'individus (Avramova et al., 2018a; Peter et al., 2018). *S. cerevisiae*, le principal modèle d'étude chez les levures, est l'une des espèces les plus étudiées sur ces sujets. Cette levure est retrouvée dans de nombreuses niches écologiques (saké, vin, bière, pain, bioéthanol, fromage, etc.), avec des pressions environnementales variables mais généralement non permissives pour de nombreux autres micro-

organismes (quantité importante d'éthanol, un métabolite jouant un rôle antimicrobien majeur (Legras et al., 2018)). La phylogénie de *S. cerevisiae* a permis d'identifier la géographie et la niche écologique comme facteurs structurant la diversité génétique (Liti et al., 2009; Peter et al., 2018). Les variations se retrouvent aussi au niveau phénotypique : les populations de cette espèce sont en effet très spécialisées (Legras et al., 2018).

#### Adaptation et domestication de *S. cerevisiae* et comparaison avec *B. bruxellensis*

Les signaux d'adaptation et de domestication chez *S. cerevisiae* ont attiré beaucoup d'attention récemment, et ont fait l'objet de revues très complètes (Dupont et al., 2017; Gallone et al., 2018; Giannakou et al., 2020; Marsit et al., 2017; Steensels et al., 2019). L'objectif de cette section n'est pas de faire une liste exhaustive des signaux d'adaptation et de domestication déjà identifiés, mais de relever ceux pour lesquels des parallèles intéressants peuvent être faits avec *B. bruxellensis*.

#### Hybridation et polyploïdisation

*S. cerevisiae* partage une partie de ses niches écologiques avec d'autres espèces de *Saccharomyces* (la bière, le vin, le cidre, les milieux naturels...). La proximité physique et génétique de ces différentes espèces permet l'apparition d'évènements hybridation dans ces différentes niches (Blein-Nicolas et al., 2015; De Barros Lopes et al., 2002; Querol and Bond, 2009). L'hybridation a pour conséquence majeure la combinaison de génomes plus ou moins divergents (hybridation interspécifique ou intraspécifique), parfois associée à l'augmentation de la ploïdie (Albertin and Marullo, 2012). Dans la majorité des cas, ces deux évènements sont concomitants et sont donc étudiés conjointement.

La formation d'hybrides aboutit à des individus qui présentent des difficultés à réaliser une méiose (barrière post-zygotique), mais pourront se multiplier végétativement (Lee et al., 2008; Morales and Dujon, 2012; Rancati and Pavelka, 2013). Ces hybrides sont particulièrement représentés dans les environnements anthropiques (Arroyo-López et al., 2009; Gangl et al., 2009 et pour revue : Bendixsen et al., 2021; Gabaldón, 2020; Steensels et al., 2021). Par rapport aux souches parentales, les hybrides peuvent présenter plusieurs avantages, comme une résistance accrue aux stress, ainsi que des performances fermentaires augmentées par rapport aux souches parentales. Ce phénomène est appelé « vigueur hybride » ou « hétérosis » (Blein-Nicolas et al., 2015). L'évènement d'hybridation peut être suivie d'une perte d'hétérozygotie (Loss Of Heterozygosity, LOH) par recombinaison (Stelkens and Bendixsen, 2022). Ce phénomène peut s'accompagner de l'augmentation de certains caractères grâce à la sélection des évènements de remplacement des allèles maladaptés par des allèles plus adaptés (conversion génique) (Smukowski Heil et al., 2017; Steensels et al., 2021). Cette perte d'hétérozygotie conduit parfois à la stabilisation génomique de l'hybride (Rancati and Pavelka, 2013; Steensels et al., 2021; Wertheimer et al., 2016). L'hybridation et/ou la polyploïdie peuvent aussi être à l'origine d'une anomalie du nombre de chromosomes appelée aneuploïdie, créée par des problèmes de ségrégation lors de divisions mitotiques ou méiotiques (jusqu'à 44% des génomes hybrides interspécifiques (Bendixsen et al., 2022)). *S. cerevisiae* est particulièrement tolérante à cet état instable qui permet des changements importants dans le dosage génique, ainsi que des modifications dans les niveaux transcriptionnels (Krogerus et al., 2016 et pour revue : Gilchrist and Stelkens, 2019). L'hybridation et/ou la polyploïdie sont donc des mécanismes majeurs de l'évolution et de la spéciation chez les levures. Un doublement du génome (Whole-Genome Duplication, WGD), probablement accompagné d'un évènement d'hybridation interspécifique (allotétraploïdisation) aurait permis

l'acquisition du mode de vie "make-accumulate-consume" chez *Saccharomyces* spp. (consommation rapide des sucres pour la production d'éthanol et consommation de l'éthanol), il y a environ 100 millions d'années (Guo et al., 2016; Marcet-Houben and Gabaldón, 2015; Wolfe, 2015). Un autre évènement d'hybridation interspécifique connu est celui de *S. pastorianus*, levure majeure dans la fermentation de la bière à basse température, qui serait un allotétraploïde (issu de l'hybridation entre *S. cerevisiae* et *S. eubayanus*) en cours de diploïdisation (Libkind et al., 2011; Okuno et al., 2015). La capacité de fermentation à basse température, l'utilisation efficace de sucres complexes du wort aurait été acquises lors de cet évènement d'hybridation (Monerawela and Bond, 2018). L'augmentation de la ploïdie peut s'accompagner d'une augmentation immédiate de la valeur adaptative, et sur le long terme, la polyploïdisation peut permettre l'accumulation de mutations récessives habituellement délétères (Fisher et al., 2018; Gilchrist and Stelkens, 2019). Par exemple, les individus autopolyploïdes sont très représentés dans les souches de panification (40% de la population) qui montrent une phase de latence de fermentation réduite dans ce milieu-là (Albertin et al., 2009; Bigey et al., 2021).

L'hybridation impacte le génome nucléaire, mais aussi le génome mitochondrial. Les mitochondries sont des organelles dynamiques, mobiles qui peuvent fusionner et se diviser. Chez les levures, l'hérédité mitochondriale est biparentale, issue des deux parents. Un zygote nouvellement formé est hétéroplasmique : il possède les deux ADN parentaux qui peuvent recombinaison et former de nouveaux mitotypes. Un seul mitotype est généralement conservé au cours des divisions cellulaires, conduisant à de l'homoplasmie (les mitochondries d'une cellule partagent la même information génétique) (Solieri, 2010). Le mitotype peut impacter des phénotypes comme les capacités de croissance et de respiration, ou la capacité de résistance à des stress oxydatifs (Albertin et al., 2013; Picazo et al., 2015). Une récente étude sur 353 génomes mitochondriaux de *S. cerevisiae* a montré une diversité nucléotidique supérieure dans les régions codantes du génome mitochondrial comparé au génome nucléaire. Cette diversité est plus marquée chez les populations domestiquées que chez les populations naturelles, ce qui suggère une évolution plus rapide du génome mitochondrial chez les populations domestiquées (De Chiara et al., 2020). L'utilisation de *S. cerevisiae* dans des procédés principalement fermentaires pourrait être associée à une pression de sélection plus faible du génome mitochondrial (particulièrement associé à la respiration), ce qui pourrait expliquer la plus grande diversité mitochondriale observée (Albertin et al., 2013; De Chiara et al., 2020). De plus, la phylogénie mitochondriale de ces individus ne correspond que peu à la phylogénie nucléaire, phénomène probablement dû à des hybridations fréquentes (De Chiara et al., 2020). Le mitotype semble aussi jouer un rôle majeur dans l'adaptation de *Saccharomyces* sp. à des températures différentes (thermotolérance). Les hybrides entre *S. cerevisiae* (espèce mésophile) et *S. uvarum* (espèce psychrophile) présentent une sélection du génome mitochondrial de *S. cerevisiae* dans leur descendance à 20-24°C et celui de *S. uvarum* à 10°C (Hewitt et al., 2020). La valeur sélective d'un hybride peut donc dépendre de son mitotype et de son environnement.

#### *Transferts génétiques horizontaux (HGTs) et translocations chromosomiques*

L'adaptation est aussi possible par transferts horizontaux de gènes (HGTs), un mécanisme permettant l'acquisition rapide de nouvelles fonctions bien connu chez les bactéries et les archées, mais historiquement sous-estimé chez les eucaryotes (pour revue : Gonçalves and Gonçalves, 2022; Van Etten and Bhattacharya, 2020). Ces transferts ne font pas appels à des évènements d'hybridation et

concernent des espèces parfois très éloignées génétiquement (Harrison et al., 2022). Ainsi, le cluster de gènes *GAL*, composé de 7 gènes et qui permet l'hydrolyse du galactose chez de nombreuses espèces de levures aurait été acquis chez *S. cerevisiae* par HGT, avec des variations génétiques au sein des populations (Harrison et al., 2022; Peter et al., 2018). Trois grandes régions génomiques (région A, B, C, impliquant 39 gènes au total) ont été identifiées comme HGTs acquises à partir de *Torulaspota microellipsoides*, et sont présentes uniquement chez les souches de *S. cerevisiae* de vin. Une de ces régions (région C), acquise il y a environ 2000 ans, contient deux gènes (*FOT*) dupliqués qui codent des transporteurs d'oligopeptides (Marsit et al., 2015 et pour revue : Becerra-Rodríguez et al., 2020). La présence de ces gènes apporte un avantage compétitif aux souches de vin lors de la fermentation en facilitant l'assimilation d'oligopeptides (sources d'azotes). Cette activité métabolique a pour effet secondaire la stimulation de la production de composés volatils désirables (alcools supérieurs et esters), caractère d'intérêt technologique, sous sélection humaine (Marsit et al., 2015). Les transferts horizontaux de gènes sont aussi très documentés chez d'autres micro-organismes inféodés à des niches anthropisées, comme plusieurs espèces de *Penicillium* (*roqueforti*, *camemberti*, *salamii*, *nalgiovense*) (Cheeseman et al., 2014; Dumas et al., 2020; Lo et al., 2022; Ropars et al., 2017). Ces événements soulignent l'importance de ce mécanisme dans l'adaptation évolutive aux niches anthropisées et la domestication des micro-organismes.

Un marqueur bien connu de domestication chez *S. cerevisiae* implique un événement de translocation chromosomique. Il s'agit de l'acquisition de la résistance au SO<sub>2</sub> par les souches du vin, qui a pu être observée et réobtenue au laboratoire. L'évènement en cause permet la relocation du gène *SSU1* (codant un transporteur permettant d'exporter les sulfites hors de la cellule) en amont d'un promoteur fort, avec pour conséquence une augmentation de la transcription de ce gène (Pérez-Ortín et al., 2002). La pression de sélection pour le phénotype « tolérance aux sulfites » est tellement forte en condition œnologique que plusieurs événements distincts de translocations ont été identifiés, indiquant une évolution convergente (Zimmer et al., 2014). Du fait de la pression de sélection, 88% des souches œnologiques présentent au moins une translocation (sur 3 réarrangements chromosomiques identifiés : XV-t-XVI (translocation), VIII-t-XVI (translocation) et inv-XVI (inversion)) en amont du gène *SSU1* présent sur le chromosome XVI de *S. cerevisiae* (Marullo et al., 2020). Les populations non œnologiques ne présentent pas ces translocations (Peter et al., 2018).

#### Variations structurelles locales et variations nucléotidiques

Les variations structurelles locales comprennent les délétions, insertions, duplications et inversions (pour revue : Gorkovskiy and Verstrepen, 2021). La variation du dosage génique (Copy Number Variation, CNVs) peut moduler la valeur adaptative d'un individu. Ainsi, chez les souches de *S. cerevisiae* retrouvées en œnologie, la résistance au cuivre (contenu dans la bouillie bordelaise) est associée à l'augmentation du nombre de copie de *CUP1* (>18) (gène codant une métallothionéine capable de fixer les ions cuivre toxiques) (Adamo et al., 2012). Cette variation du nombre de copies a aussi été observée en laboratoire et est présente dans des souches sauvages, indiquant une probable évolution convergente (Gerstein et al., 2015; Marsit et al., 2017). La famille des gènes *MAL* (*MALT* (transporteurs du maltose & dérivés), *MALS* (maltases), *MALR* (mécanismes de régulations)) est soumise à de nombreuses fluctuations, tant au niveau de leur position chromosomique que de leur nombre de copies (Giannakou et al., 2020). Ces gènes permettent l'assimilation des fragments d'amidon issus des céréales (maltose et maltotriose). Les souches de *S. cerevisiae* utilisées en brasserie et panification présentent de nombreuses duplications (6-15) de ces gènes, et ce génotype est associé

à une assimilation efficace de ces sucres (Bigey et al., 2021; Gallone et al., 2018; Gonçalves et al., 2016; Lahue et al., 2020).

Les variations nucléotidiques uniques (single nucleotide polymorphisms SNPs) sont les mutations les plus fréquentes dans les génomes des levures (Marsit et al., 2017). Un ensemble de 284 variations nucléotidiques a récemment été associé à la modulation des caractères biotechnologiques dans une collection de 1011 individus (Peltier et al., 2019). Ainsi, par exemple : une variation dans la séquence du gène *MAL11* (codant un transporteur) spécifique des souches de bière rend ce transporteur plus affiné pour les malto-oligosaccharides présents dans le moût de bière (*wort*) (Gallone et al., 2016; Giannakou et al., 2020). Des changements nucléotidiques ont aussi été observés dans un gène codant un transporteur de glucose Hxt3p chez les souches de levure de voile : les mutations élargissent la sélectivité du transporteur au fructose, principal sucre résiduel de la fermentation (Coi et al., 2017). La domestication de *S. cerevisiae* a aussi modifié les arômes produits par celle-ci : la perte de fonction des gènes *PAD1* et *FDC1* est associée à une diminution de la production 4-VG (4-vinylguaiacol) chez la majorité des souches issues de la bière et du saké (Figure 19) (Gallone et al., 2018; Mukai et al., 2010)

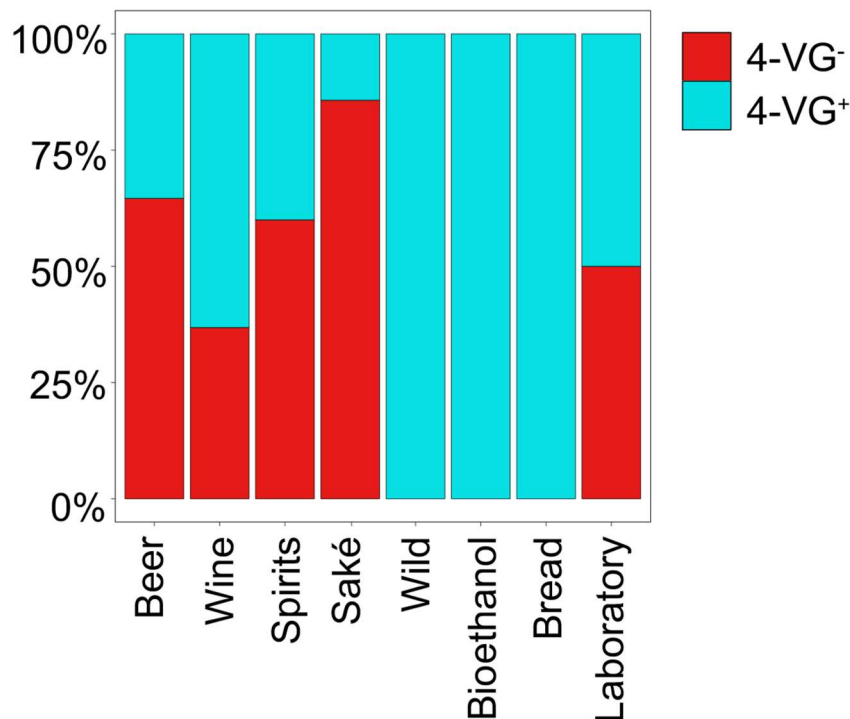


Figure 19: Pourcentage de souches capables de produire des 4-vinylguaiacol (bleu) en fonction de de leur origine. D'après Gallone et al., (2016).

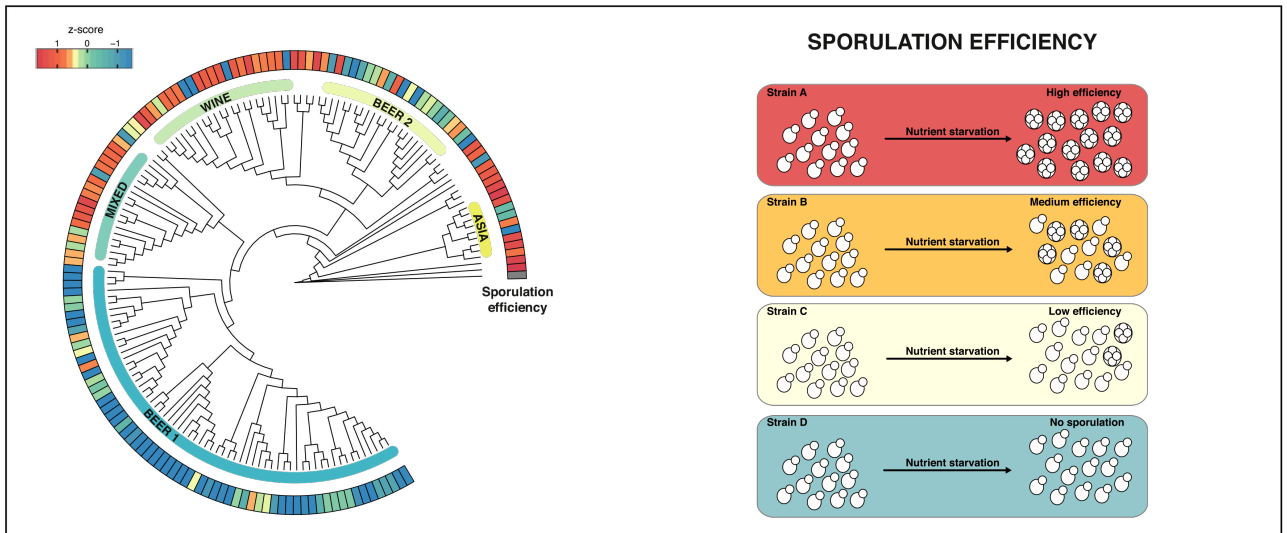
#### Cas de la plasticité phénotypique chez les populations domestiquées de *S. cerevisiae*

*S. cerevisiae* est capable de passer d'une forme unicellulaire à une forme multicellulaire non différenciée, selon l'environnement dans lequel elle se trouve. Ainsi, le voile est une forme multicellulaire adoptée par *S. cerevisiae*, selon un mécanisme de protection coopérative vis-à-vis d'un

environnement stressant, par élongation cellulaire (formation de pseudohyphes) (Alexandre, 2013). Cette forme permet aux cellules de se trouver à l'interface air-liquide d'un milieu, où la respiration de l'éthanol pourra être réalisée, en fin de fermentation alcoolique par exemple. La formation de pseudohyphes augmente par ailleurs la capacité d'invaginer des surfaces poreuses et est aussi impliquée dans la formation de biofilm (Gimeno et al., 1992). Le gène *FLO11* est le gène central de ce changement de morphologie, et sa régulation dépend de facteurs environnementaux (privation d'azote ou de sources de carbone) (pour revue : Bouyx et al., 2021). Des régulations épigénétiques ont aussi été mises en évidence et permettraient une hétérogénéité phénotypique qui augmenterait la vitesse de réponse aux modifications environnementales au sein de la population (Rowlands et al., 2019). Les souches de voile du vin (souches retrouvées après la fermentation alcoolique du vin) présentent plusieurs mutations dans les régions promotrices de *FLO11*, renforçant sa plasticité et l'hétérogénéité au sein de la population qui s'adapte ainsi rapidement à des variations environnementales (Bouyx et al., 2021; Coi et al., 2017).

La domestication des souches de *S. cerevisiae* serait plus avancée chez les souches de bière que chez les souches de vin (Steensels et al., 2019). La fermentation des céréales en bières peut être réalisée sur l'ensemble de l'année (stockage des céréales possible). Ceci permet la réalisation de fermentations alcooliques plus régulières dans l'année et donc le développement des populations microbiennes plus fréquemment (Gallone et al., 2018). Au contraire, pour le vin, la phase de fermentation n'est possible qu'une fois par an (après vendange), et les organismes doivent survivre le reste de l'année. La forme sexuée de *S. cerevisiae* (par sporulation) a été associée à l'augmentation du taux de survie dans un milieu carencé (Briza et al., 1990). Une diminution importante de l'efficacité de sporulation (44% souches ne peuvent sporuler) ou une perte de viabilité des spores (80% des souches qui peuvent sporuler montrent une létalité des spores formées) ont été observées en milieu carencé chez les souches de bière par rapport aux souches issues d'autres matrices fermentées (Figure 20) (Gallone et al., 2016). Une perte de la résistance à la privation de nutriments a aussi été observée pour ces mêmes souches. Il semble que la domestication des souches de *S. cerevisiae* de bière a altéré leur capacité à s'adapter à un changement d'environnement, réduisant leur valeur adaptative hors de l'industrie brassicole (Gallone et al., 2018; Marsit et al., 2017). Des études supplémentaires sont nécessaires pour déterminer si la perte de la capacité de sporulation est associée à une perte des mécanismes propres de sporulation ou si les individus ne sont plus capables d'intégrer les signaux environnementaux.





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Figure 20: Efficacité de la sporulation des différentes populations de *S. cerevisiae*, les souches du sous-groupe BEER1 montrent une réduction de leur capacité de sporulation sous privation de nutriments. D'après Gallone et al., (2018).

### Éléments concernant l'espèce *B. bruxellensis*

Les études scientifiques concernant l'espèce *B. bruxellensis* ont été principalement menées dans un but technologique (méthode d'élimination, utilisation dans la bière, impact dans le vin). Les connaissances que nous en avons sont assez parcellaires et ont été tirées d'études mettant en jeu un petit nombre de souches, généralement peu représentatives de la diversité génétique de l'espèce. Dans la littérature, les résultats en apparence contradictoires sont nombreux et il est difficile de savoir si ces contradictions s'expliquent par l'étude de souches différentes et/ou par des conditions expérimentales différentes. Très peu de travaux se sont intéressés à la variation phénotypique de l'espèce *B. bruxellensis* dans différents milieux à large échelle : l'un des objectifs de cette thèse est donc de combler au moins en partie ce manque.

La récente étude de génétique des populations réalisée par Avramova et al. a permis de mettre en lumière une population structurée par deux facteurs principaux, la ploïdie et la niche d'isolement, ce qui suggère une adaptation aux environnements anthropisés (Avramova et al., 2018a). Cette structure de population a ensuite été confirmée par le séquençage à grande échelle d'individus représentatifs de la diversité (Eberlein et al., 2021; Gounot et al., 2020; Serra Colomer et al., 2020a). Ces approches mettent en évidence des variations génétiques importantes entre les différentes populations, et l'existence chez *B. bruxellensis* de mécanismes évolutifs similaires à ceux décrits chez *S. cerevisiae*. Cependant, les liens entre variation génétique, variation phénotypique et adaptation aux milieux anthropisés restent encore hypothétiques. Dans cette partie, les particularités génétiques et phénotypiques de l'espèce *B. bruxellensis* et leur intérêt évolutif seront discutés et confrontés (dans la mesure du possible) à ce qui est décrit chez *S. cerevisiae*.

### Hybridation et polyploïdisation

L'hybridation et la polyploïdisation ont joué un rôle majeur dans l'adaptation de *S. cerevisiae* aux procédés biotechnologiques. Chez *B. bruxellensis*, plus de 50% des isolats sont polyploïdes, suite à un

évènement d'hybridation soit intraspécifique (individus autotriploïdes) soit interspécifique (allotriploïdes). Trois évènements d'hybridations interspécifiques indépendants avec une espèce parente de *B. bruxellensis* ont été identifiés jusqu'à présent et concernent les population *wine 1*, *beer* et *teq/EtOH* (Figure 21) (Borneman et al., 2014; C. D. Curtin et al., 2012; Eberlein et al., 2021). Les individus allotriploïdes possèdent un *core* génome diploïde appartenant à *B. bruxellensis* et un génome haploïde supplémentaire divergent du génome diploïde ( $\approx 3\%$  de divergence nucléotidique) (Eberlein et al., 2021). Les espèces donneuses des génomes haploïdes sont pour l'heure inconnues, mais les autres espèces du genre *Brettanomyces* (*B. anomalus*, *B. custersianus*, *B. nanus*, *B. naardenensis*, *B. acidodurans*) ont été exclues car trop divergentes génétiquement.

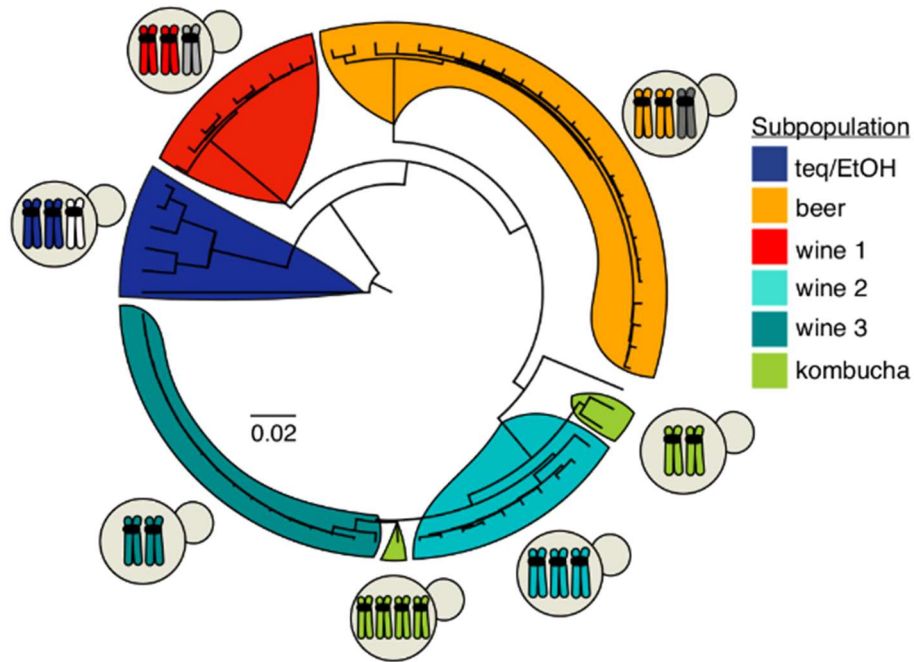


Figure 21: Diversité génétique au sein de l'espèce *B. bruxellensis*. Le niveau de ploïdie est schématisé. Les chromosomes en gris et blanc représentent les génomes supplémentaires acquis ne faisant pas partie de l'espèce *B. bruxellensis*. D'après (Harrouard et al., 2022).

Comme chez *S. cerevisiae*, les génomes allotriploïdes montrent de grands évènements de pertes d'hétérozygotie (LOH). Ces évènements sont différents d'une population à l'autre (position génomique, taille), mais très conservés au sein des populations suggérant des évènements uniques. Dans les régions concernées, le génome conservé peut être le génome core-diploïde comme le génome supplémentaire haploïde (Figure 22) (Eberlein et al., 2021). Cependant, contrairement à ce qui est observé chez *S. cerevisiae*, la fréquence d'aneuploïdie est très faible notamment au regard de la fréquence de la ploïdie. L'état triploïde semble donc stable chez cette espèce et les phénomènes qui entraînent un déséquilibre du nombre de chromosomes (aneuploïdie) seraient moins actifs que chez *S. cerevisiae*.

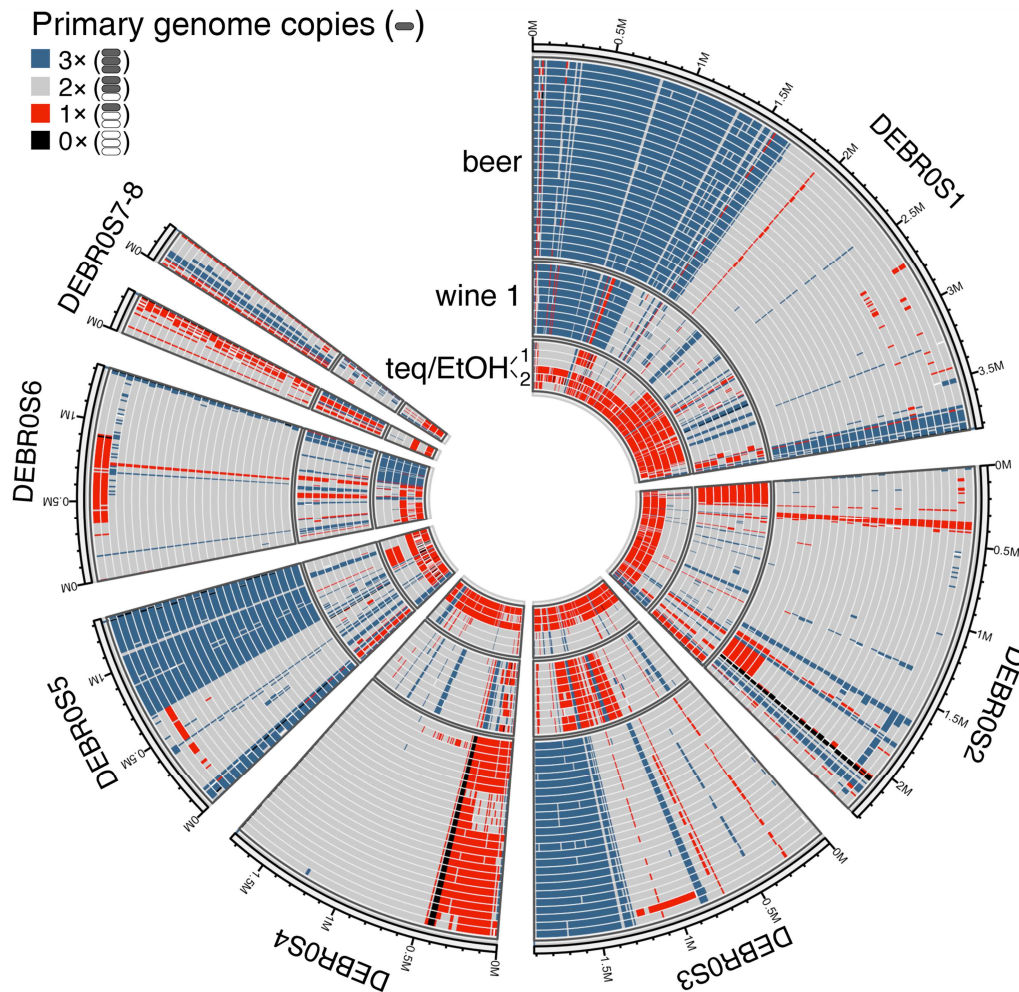


Figure 22: Génomes alignés des trois populations allotripléides de *B. bruxellensis*. Les régions où les trois haplotypes présents sont issus du core-diploïde génome, en gris pour les régions où le core-génome est présent sur deux copies, en rouge lors qu'il n'y a qu'un haplotype du core-génome et en noir lorsqu'il n'y a plus de copie du core-génome. D'après Eberlein et al., (2021).

En plus des trois populations allotripléides, une autre population a été identifiée comme autotripléide (*wine 2*). Les individus de cette population possèdent un génome diploïde associé à un génome haploïde peu divergent (>1%), suggérant un évènement d'hybridation intra-spécifique (Eberlein et al., 2021).

Malgré l'existence de plusieurs évènements d'hybridation, le pan-génome de *B. bruxellensis* est relativement restreint comparé à celui de *S. cerevisiae* (Gounot et al., 2020). Concernant les données phénotypiques, les groupes allotripléide *wine 1* et autotripléide *wine 2* présentent une tolérance à la présence de SO<sub>2</sub> plus importante que les autres groupes (Avramova et al., 2019, 2018b). Cependant, il ne semble pas que cette capacité soit directement liée à l'hybridation interspécifique chez le groupe *wine 1*. En effet, l'haplotype du gène *SSU1* (principal acteur de la résistance aux sulfites chez *B. bruxellensis*) apporté par le génome supplémentaire est moins efficace que ceux du core génome diploïde (Bartel et al., 2021; Varela et al., 2019). L'étude de l'impact phénotypique de ces évènements d'hybridation ne fait que débiter. Une attention particulière devrait être apportée aux régions génomiques soumises à la perte d'hétérozygotie chez les individus polyploïdes et pourrait permettre l'identification de gènes clés dans l'adaptation évolutive de l'espèce.

*B. bruxellensis* possède un génome mitochondrial très particulier, car il s'agit de la seule espèce «petite» positive, anaérobie facultative et Crabtree positive qui possède le complexe I de la chaîne respiratoire (Procházka et al., 2010). Cette particularité permet un recyclage du NADH en présence d'oxygène, et ainsi de contrebalancer le déséquilibre redox provoqué par la fabrication d'acide acétique. L'étude des génomes mitochondriaux des hybrides de *B. bruxellensis* a montré que dans deux des populations allotriploïdes, le mitotype conservé est identique à celui des populations diploïdes (Eberlein et al., 2021). A l'inverse, le groupe *teq/EtOH* présente un mitotype peu homogène et différent de celui des autres populations (Eberlein et al., 2021). Il serait intéressant de préciser l'impact phénotypique éventuel de ces différents mitotypes, notamment en présence de quantité variables d'oxygène et de différentes pressions de sélection retrouvées dans les niches occupées par les isolats *teq/EtOH*.

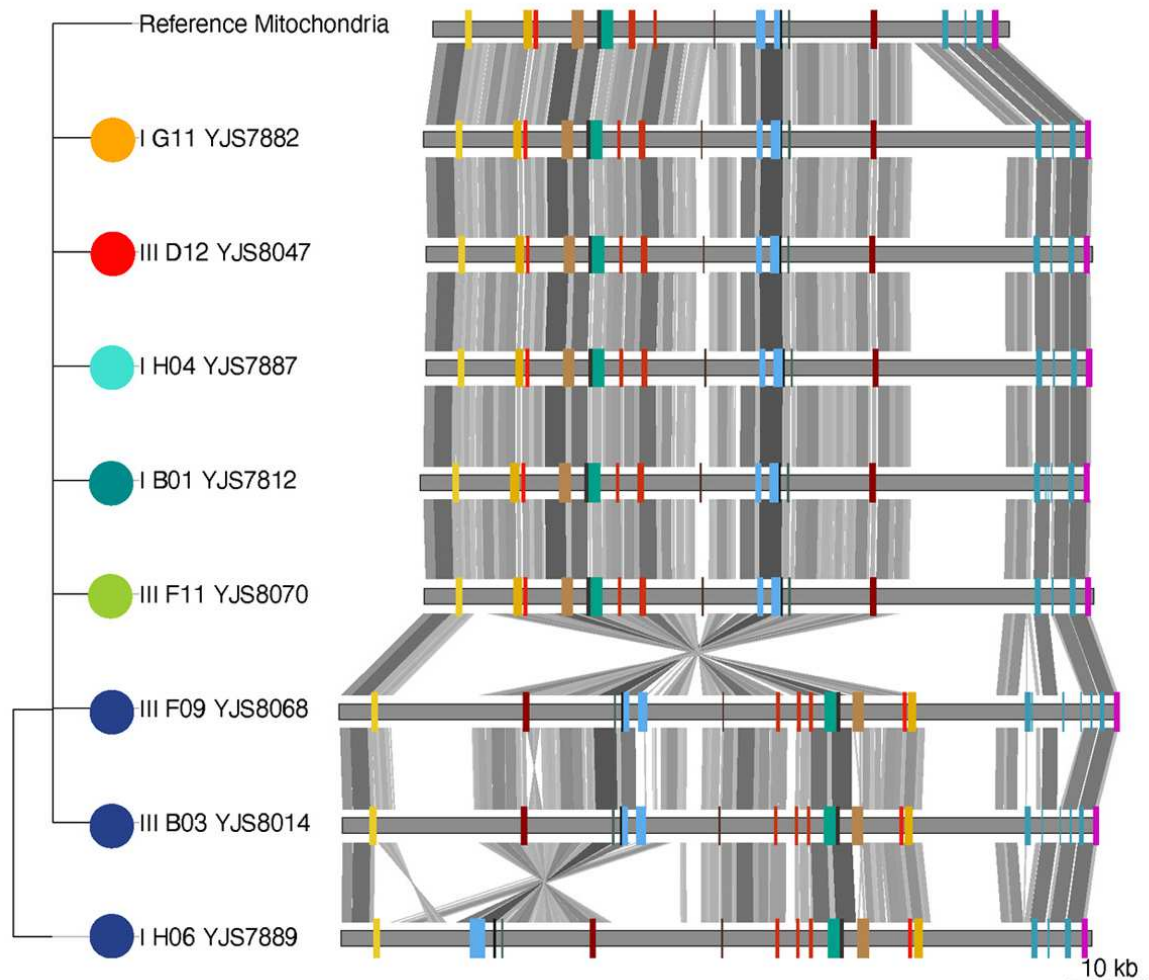


Figure 23: Synténie des génomes mitochondriaux chez *B. bruxellensis*. D'après Eberlein et al., (2021).

### Transferts génétiques horizontaux (HGTs) et translocations chromosomiques

Trois événements de HGTs ont été récemment identifiés chez *B. bruxellensis* (Roach and Borneman, 2020). L'un des loci les plus intéressants contient le gène *GH32* codant une  $\beta$ -fructofuranosidase qui permettrait l'hydrolyse du saccharose en glucose + fructose chez *B. bruxellensis*. Le locus acquis est d'origine bactérienne (genre *Asaia*) et est inséré dans une région subtélomérique. Ce transfert

horizontal a aussi été identifié chez *B. anomalus*. Il est donc probable que ce transfert génétique soit antérieur à la séparation des deux espèces. Un autre évènement de HGT a permis l'acquisition d'un gène codant une protéine de capsule (Capsule biosynthesis protein CapA), provenant d'une bactérie *Enterobacteriales* (gram-). Ces protéines de surface joueraient un rôle de protection, et seraient impliquées dans la formation de biofilms et/ou la virulence (Mmatli et al., 2020). *B. bruxellensis* partage ce transfert avec *B. anomalus*, *B. custersianus*, *B. nanus*. Le dernier transfert horizontal identifié a permis l'acquisition d'un gène qui code une protéine d'oxydo-réduction (NAD(P)-dependent oxidoreductase) provenant d'une bactérie du genre *Clostridium*. Les bactéries de ce genre sont diverses mais connues pour leur métabolisme anaérobie strict ainsi qu'une production d'acidité importante (acide acétique principalement) (Dürre, 2016). Ce gène est aussi présent chez *B. anomalus* (Roach and Borneman, 2020). Ces trois évènements de HGT ont été identifiés chez une seule souche de *B. bruxellensis* ; leur prévalence dans la population de *B. bruxellensis* et leur impact phénotypique restent encore à évaluer pour mieux évaluer leur contribution à l'adaptation aux procédés anthropisés. Le séquençage de plusieurs isolats de *B. bruxellensis* pourrait aussi permettre l'identification de nouveaux HGTs, potentiellement spécifiques à une/des populations génétiques, et d'identifier des nouvelles signatures potentielles d'adaptation.

A ce jour et à notre connaissance, aucun évènement de translocation chromosomique n'a été décrit. Ceci pourrait être un biais méthodologique ou lié à une limitation technique, vue la difficulté d'identifier ces évènements lors de séquençages (particulièrement en utilisant une technologie *short read*) dans un contexte d'individus allopolyploïdes. Il est également possible que le phénomène de translocation soit peu présent chez *B. bruxellensis*, pour des raisons qui restent inconnues.

#### *Variations structurelles locales et variations nucléotidiques*

La présence de CNVs chez *B. bruxellensis* a été démontrée dans plusieurs études (Borneman et al., 2014; Crauwels et al., 2014; Eberlein et al., 2021; Gounot et al., 2020; Roach and Borneman, 2020; Serra Colomer et al., 2020a). Ces CNVs sont plus fréquents chez les souches allotriploïdes par rapport aux souches diploïdes (881 contre 169 respectivement), indiquant une variabilité génomique plus importante chez les hybrides interspécifiques (Gounot et al., 2020). Bien que des différences de nombre de copies de gène aient été identifiées dans la famille des gènes *MAL*, aucune étude n'est parvenue à assigner les génotypes à des phénotypes pertinents (consommation des malto-oligosaccharides de la bière), ce qui suggère l'existence de régulations complexes (Crauwels et al., 2017; Serra Colomer et al., 2020b). Plusieurs études ont aussi montré une variation du nombre de copies de trois gènes responsables de l'assimilation des nitrates (*NIT* cluster) avec la perte de ce cluster chez certaines souches de bières. Cependant, les études étaient menées avec de petits nombres de souches. Des analyses plus ambitieuses avec plus de souches venant de diverses origines seraient nécessaires, pour comprendre la distribution de cette variation dans les populations génétiques (Borneman et al., 2014; Crauwels et al., 2014; Serra Colomer et al., 2020b). Une duplication du gène *VPR*, associé à la production d'éthyl-phénols, est présente chez les souches de vin et de boissons non alcoolisées (5 isolats) et absente chez les souches de bière (3 isolats). Ce génotype serait associé à une réduction des quantités produites d'éthyl-phénol produit chez les isolats de bière (Crauwels et al., 2017), réduction non retrouvée sur des études menées avec un plus grand nombre de souches (Cibrario et al 2020). Des variations nucléotidiques ont aussi été identifiées dans le gène *SSU1* (Varela et al 2019). Les mutations de ce gène portées par la population *wine 1* permettent une résistance accrue aux sulfites, grâce à une transcription importante ainsi qu'à une efficacité supérieure de la

protéine Ssu1p dans l'export des sulfites (Valdetara et al., 2020; Varela et al., 2019). Une récente étude d'évolution expérimentale sur *B. bruxellensis* a permis de mettre en évidence l'importance de la transcription de ce gène, montrant que les clones évolués expérimentalement présentaient une augmentation du nombre de copies pour ce gène (Bartel et al., 2021).

#### *Plasticité phénotypique chez B. bruxellensis ?*

La capacité de *B. bruxellensis* à former du biofilm, à invaginer la gélose mais aussi potentiellement des joints plastiques (observation personnelle), ainsi qu'à développer un voile en surface lors de la production de bières par fermentation spontanée pose évidemment la question des mécanismes moléculaires impliqués. Les souches de bière semblent enclines à former des biofilms quelle que soit la quantité de sucres présente, alors que les souches issues d'autres matrices fermentées présentent des phénotypes plus plastiques aux changements environnementaux (Dimopoulou et al., 2019b). Comprendre ces mécanismes de régulation représente un réel challenge, tant pour comprendre les mécanismes d'adaptation que pour améliorer le contrôle de *B. bruxellensis* dans les procédés fermentaires. Une thèse est actuellement en cours sur ce sujet au sein de l'unité UMR œnologie (Paul Le Montagner).

## Conclusion

La levure *B. bruxellensis* est présente dans de nombreuses niches anthropisées, dans lesquelles cette espèce est perçue comme problématique (vin, bière, bioéthanol) ou bénéfique (bières de spécialités, kombucha, kéfir). La diversité génétique de *B. bruxellensis* est importante. Au travers de l'exemple de la domestication de *S. cerevisiae*, plusieurs évènements moléculaires (hybridation, polyploïdisation, réarrangements génomiques, variations génétiques et nucléotidiques) et des caractéristiques phénotypiques ont été mis en avant et discutés. Bien que les connaissances scientifiques que nous avons de l'espèce *B. bruxellensis* ne soient pas aussi avancées, plusieurs évènements moléculaires (plusieurs évènements d'hybridations, proportion de la polyploïdie dans la population, LOH, CNVs, SNVs) ainsi que des phénotypes d'intérêts ont été soulignés (résistance aux sulfites, formation de biofilm), et pourraient être reliés à l'adaptation évolutive de *B. bruxellensis* dans les différents procédés anthropisés dans lesquels on la retrouve. Cependant, bien que *B. bruxellensis* partage de nombreux environnements communs et présente des similitudes de signatures (génétiques et phénotypiques) avec *S. cerevisiae*, la temporalité de son développement (début de fermentation vs fin de fermentation), ainsi que la perception que l'être humain en a (auxiliaire technologique vs contaminant) sont bien différents. Les pressions de sélection menant à l'adaptation évolutive de *S. cerevisiae* et de *B. bruxellensis* sont donc certainement différentes. Si, dans les bières de spécialité et le kombucha l'ensemencement volontaire de culture pure et le *backslipping* peuvent permettre la sélection de caractères technologiques, les souches isolées comme agents d'altération (vin, bière et bioéthanol) ne sont probablement pas sélectionnées du fait d'activités bénéfiques à l'être humain mais plutôt pour leur capacité à se développer dans diverses matrices comme second colonisateur (ce qui nécessite une plus grande versatilité que celle requise chez un premier colonisateur) et leur capacité de dissémination/dispersion. La capacité à attirer des insectes via des molécules odorantes pourraient leur permettre de gagner de nouveaux biotopes une fois les milieux épuisés. Le fait que *B. bruxellensis* soit la seule espèce de levure à produire des quantités importantes d'éthyl phénols et que toutes les souches étudiées aient cette capacité soulève en effet la question : pourquoi l'évolution a-t-elle systématiquement conservé ce métabolisme si spécifique ?

Pour identifier des signes d'adaptation évolutive, l'acquisition de données génétiques et phénotypiques relatives à un large nombre d'individus appartenant aux différentes populations est nécessaire, ceci permettant des approches comparatives entre les populations. Dans ce cadre, un des objectifs principaux de cette thèse est d'étudier la diversité phénotypique de *B. bruxellensis* sur un grand nombre d'individus. La première question sera de savoir si les populations génétiques récemment identifiées (Avramova et al., 2018a) présentent des phénotypes distincts suivant les environnements, ou bien si les phénotypes ne sont pas en lien avec la structure génétique de la population. De cela, découleront plusieurs questions : si les différences phénotypiques sont liées aux populations, y a-t-il des caractères d'espèces (caractères communs à l'espèce) ? Le cas échéant lesquels et sont-ils variables d'un individu à l'autre ? Y a-t-il des caractères spécifiques à des populations ? Si oui, lesquels et sont-ils en lien avec des caractères pouvant sous-tendre l'adaptation de *B. bruxellensis* à des environnements anthropisés ? Si l'on regarde la distribution des différents phénotypes au travers de la population, est-il possible de reconstituer un phénotype 'ancestral', commun aux populations génétiques, et ainsi proposer des scénarios d'évolution écologique ? Dans l'objectif de répondre à ces questions, une première étude phénotypique sera réalisée dans différents environnements anthropisés habituellement colonisés par cette espèce (vin, moût de raisin, bière,

wort et moût de kombucha) (Chapitre 2). Plusieurs caractères seront mesurés, sous forme de cinétique (fermentaire et de croissance) ou de mesure en fin d'expérience (dosages de composés d'intérêts, approche de cytométrie en flux...). Une seconde étude phénotypique sera aussi réalisée dans des milieux synthétiques utilisés au laboratoire, avec différentes sources d'azotes (Chapitre 3).

Sur un plan plus appliqué, des travaux antérieurs ont montré des variations importantes de réponse entre les populations en fonction des traitements antimicrobiens (Avramova et al., 2018b). Ce qui amène à la question suivante : la variabilité phénotypique de *B. bruxellensis* impacte-t-elle l'efficacité de procédés innovants pour l'œnologie ? Une partie du temps et des travaux de cette thèse ont été consacrés à l'évaluation de technologies innovantes pour lutter contre des contaminations microbiennes et particulièrement contre *B. bruxellensis* dans le milieu œnologique (Chapitre 4).



## Chapitre 2 : Adaptation de la levure *Brettanomyces bruxellensis* aux procédés fermentaires

Remarque :

Cette partie correspond aux principaux travaux menés lors de cette thèse. Les données acquises sont denses et diverses. Certaines parties nécessitent davantage d'analyses, principalement les données des composés volatiles. Un retard important a été pris pour le démarrage de ces travaux (1 an), avec la livraison tardive du cytomètre de flux et les contraintes liées à la pandémie. Cependant les analyses déjà menées permettent la rédaction de résultats (plus ou moins préliminaires) et de leur discussion sous la forme d'un article scientifique. Contexte de l'étude :

L'espèce *B. bruxellensis* a été souvent décrite comme ayant une importante variabilité phénotypique d'une souche à l'autre. Des travaux récents de séquençages ont permis l'identification de populations génétiques distinctes au sein de l'espèce, associées à des niches spécifiques d'isolement (Avramova and Masneuf-Pomarede, 2017; Eberlein et al., 2021). La question est de savoir si les populations régulièrement isolées à des niches fermentaires spécifiques présentent des signatures phénotypiques d'adaptations divergentes à ces mêmes niches. Pour cela, 5 milieux ont été choisis, un vin commercial, un moût de raisin, une bière commerciale, un moût de bière et un moût de kombucha. Un panel de 151 souches a été utilisé pour sa représentativité génétique de l'espèce, appartenant aux 6 populations précédemment identifiées (Harrouard et al., 2022). Un nombre important de phénotypes ont été mesurés et comparés entre les 5 milieux ainsi qu'entre les différentes populations. Les premières analyses des résultats obtenus ont permis d'affiner, en accord avec les données de génomique (laboratoire GMG Strasbourg), le nombre de populations considérées. Ainsi, les populations *wine 3* et *kombucha* ont été divisées (*wine 3-1* et *wine 3-2*, *kombucha-1* et *kombucha-admixed* respectivement) et donc 8 populations ont été considérées.

# Phenotypic landscape of the spoilage yeast *Brettanomyces bruxellensis* reveals variable adaptation patterns to fermentation processes

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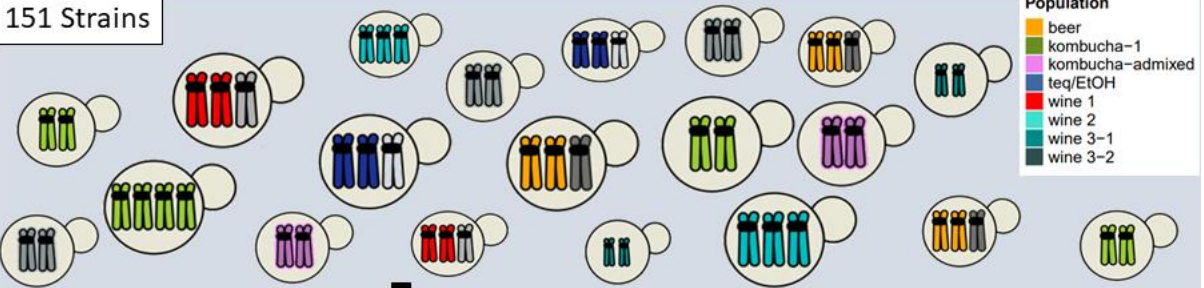
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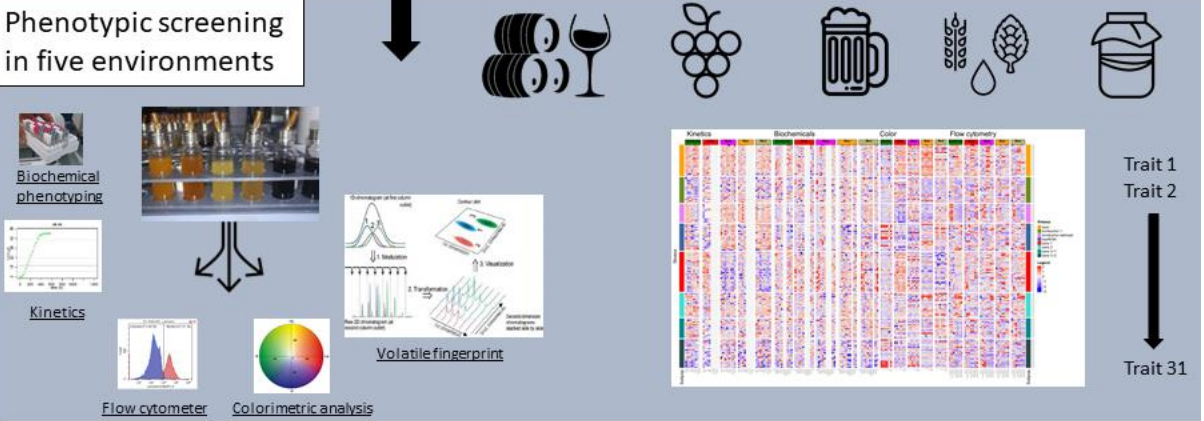
<sup>6</sup>Tonnellerie Seguin Moreau, Cognac France, France

# Graphical Abstract

151 Strains

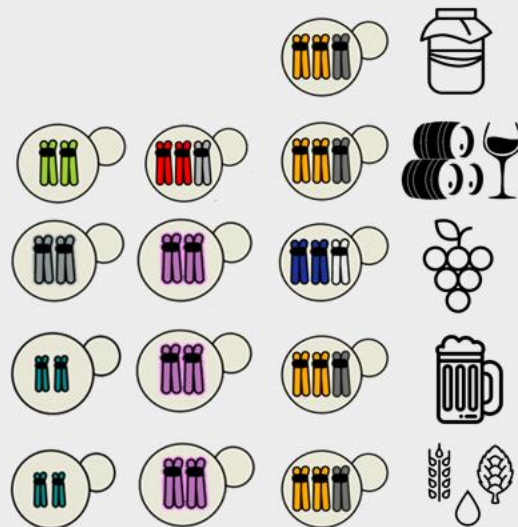


Phenotypic screening in five environments



## Phenotypic variation according to the genetic population

- ✓ Growth comparison
- ✓ Fermentation analysis
- ✓ Metabolites assays
- ✓ Phenotypic variation and plasticity
- ✓ Adaptation measure ("Local VS Foreign")
- ✓ Measure of the impact of population on phenotypes
- ✓ *B. bruxellensis* volatile fingerprint



# Abstract

The budding yeast *Brettanomyces bruxellensis* has been found associated with human activities. On the one hand, this microorganism is considered as the major yeast for microbiological defects in wine. Its development also represents a major problem for the industrial production of beer and bioethanol. On the other hand, this yeast is also recognized for its positive impact on the production of beverages such as kombucha, kefir as well as certain specialty beers. Recent genetic and genomic investigations have revealed the coexistence of auto- and allotriploidization events with different evolutionary outcomes, that led to a diploid-triploid complex species. While the phenotypic landscape of *B. bruxellensis* is often described with high strain variability, it was scarcely investigated in the light of the population structure of the species. This study aimed to mirror the population structure of *B. bruxellensis* with phenotypic behaviours. We performed a large-scale phenotyping study of 151 strains, representative of the genetic diversity, in five environments associated to *B. bruxellensis* growth (wine, must, beer, wort and kombucha). Several traits were assessed, such as the growth and fermentation ability, specific metabolic behaviours, life-history parameters and color impacts. The phenotypic impact of the genetic populations was estimated. Phenotypic variations were mainly affected by the environment, followed by the population, with an important level of strain specificity. Local adaptation, phenotypic plasticity and homogeneity of the different genetic populations were measured through these five media. These data revealed that the different populations are phenotypically divergent and show adaptive signatures. Some populations are highly specialized to specific media (such as wine) and associated to reduce fitness in other environments. Unexpectedly, most populations were able to efficiently consume maltose and maltotriose in brewery media with variations at strain level. This could indicate that maltose/maltotriose consumption is a species trait that was lost independently in specific strains and populations, probably because of relaxed selective pressure. Metabolomics investigation of the volatile fingerprint of a subset of strains was also conducted. More than 400 molecules were affected by *B. bruxellensis* growth and several metabolic pathways were enriched or depleted. This large-scale phenotyping study confirms that anthropized environments have shaped *B. bruxellensis* diversity.

## Introduction

Since the Neolithic period (12,000 years ago), plants and animals have been exposed to anthropized environments, shaped by Humans, which can lead to local adaptation and ultimately to the domestication of a species (Hulme-Beaman et al. 2016; Larson, Fuller 2014). Adaptation to anthropized environments is of interest to study due to the large agricultural and economic impacts, and to explore adaptive divergence mechanisms of traits that can be identifiable from natural populations. Adaptive divergence can cause a decrease in fitness in the original environment, which can be highlighted with comparative studies with the natural population (Marsit, Dequin 2015). Adaptation and domestication of plants and animals species have been intensively studied since Darwin in the middle of 19<sup>th</sup> century

(Darwin 1868), somehow, microbes have been disregarded. Microorganisms have been used unconsciously for millennia for their ability to perform fermentation. Back-slopping (e.g. the addition of a small portion of a previous batch of fermented food to start the new batch of fermentation) has been the main microorganism propagation method used which allowed several microbial generations (Gallone et al. 2018; Whittington, Dagher, Bruno-Bárcena 2019). Microbial fermentations result in an increased biological safety and conservation, improved digestibility, enriched flavour and sometimes euphoriant impact on consumers (Selosse 2022; Şanlıer, Gökçen, Sezgin 2019). The advance of genome sequencing and the genomic area allowed studying domesticated species used in industry such as *Saccharomyces cerevisiae* yeast; the food related *Penicillium* mould; or lactic acid bacteria (*Lactococcus*) (for review: Steensels et al. 2019; Dupont et al. 2017; Marsit et al. 2017).

*Brettanomyces bruxellensis* is a yeast associated with different anthropized niches. This species acts either as a spoilage microorganism in winemaking, brewing and production of bioethanol, or as a valuable technological auxiliary in kombucha and kefir preparations or in specific beers styles (Serra Colomer, Funch, Forster 2019; Suzuki 2020; Malfeito-Ferreira 2018; de Barros Pita et al. 2019; Tran et al. 2020). *B. bruxellensis* lifestyle seems to be adapted to low nutrients availability (both carbon and azote sources) with high ethanol and low oxygen content, and its development generally happens after *S. cerevisiae* growth. Such environments have been described as 'apocalyptic' (Smith, Divol 2016). This yeast is able to metabolize large variety of carbon sources (glucose, fructose, sucrose, cellobiose, maltose, galactose, lactose) and particular nitrogen sources, such as nitrates or amino acids (Da Silva et al. 2019; Cajueiro et al. 2017; Crauwels et al. 2015; Conterno et al. 2006; Smith, Divol 2018). Recent population genetic study revealed a diploid-triploid complex species (57% of the isolates being triploids), with at least three distinct interspecific and one intraspecific hybridizations. Beside ploidy level, the isolation environment shapes the structure of the population (Avramova, Cibrario, et al. 2018). These results were confirmed by large genomic surveys which highlighted different evolutionary outcomes for populations (Gounot et al. 2020; Serra Colomer et al. 2020; Eberlein et al. 2021, for review: Harrouard et al. 2022). Large loss of heterozygosity (LOH) events have shaped allotriploid genomes, maintaining either the diploid core-genome or the supplemental haploid one (Eberlein et al. 2021). Genomic data have underlined potential signatures of adaptation to anthropized environments. However large-scale phenotyping of the different populations is needed to determine if the genetic diversity is actually shaped by selection and adaptation to anthropized niche or rather by neutral divergence caused by isolation due to reduced (or lost) sexual cycle and physical distance. One first relevant clue underpinning adaptive divergence concerns resistance/tolerance to sulphite addition, SO<sub>2</sub> being the major wine preservative. At the phenotypic level, two genetic populations present a relative resistance to SO<sub>2</sub> concentration

found in red wine, namely the *wine 1* allotriploid and *wine 2* autotriploid groups (Avramova, Vallet-Courbin, et al. 2018). Like in *S. cerevisiae*, the gene *SSU1* was identified to have a major role in sulphite resistance (Bartel et al. 2021; Varela et al. 2019). Molecular mechanisms were evidenced for the *wine 1* population, characterised by increased transcription of *SSU1* gene with nucleotide variations associated to a better efflux of the Ssu1p pump (Varela et al. 2019; Bartel et al. 2021; Valdetara et al. 2020).

The phenotypic behaviours of *B. bruxellensis* in the literature are described as diverse, with a high isolate specificity leading sometimes to contradictory results (Conterno et al. 2006; Crauwels et al. 2017; Harrouard et al. 2022). Nevertheless, phenotypic traits have been poorly investigated in the light of the recently unveiled population structure. This study aims to explore the phenotypic diversity of *B. bruxellensis* in connection with the recent genetic data. Five distinct environments with ecological relevance were used for phenotypic comparison of 151 strains: a commercial red wine and a grape must, a commercial beer and a beer must (wort), and a home-made kombucha must. Growth kinetics were monitored and fermentation kinetics of must, wort and kombucha were measured. At the end of the experiment, end-point analyses of life-history traits (by flow cytometry), specific metabolites of interest and color analyses were performed. The impact of environment (E), genetic (G) and genetic by environment (GxE) interactions were estimated on 31 parameters. Within genetic and genetic by environment factors, the impact of the genetic populations was estimated. The existence of local adaptation, plasticity and variations of the populations were tested across the five tested environments. Growth fitness was assessed using comparative growth with a few *S. cerevisiae* strains as challengers. Finally, the volatile fingerprint of 16 strains was investigated using a comprehensive two-dimensional gas chromatography (GCxGC) combined with time-of-flight mass spectrometry (TOF-MS).

## Material and Methods:

### a) Yeast strains and culture media used

Two yeast species were used in this study, *Brettanomyces bruxellensis* (n=151) and *Saccharomyces cerevisiae* (n=5, with 3 wine and 2 beer strains) (Table 1). Yeasts were propagated on solid YPD (Yeast extract 1%, Peptone 1%, Dextrose 2% and Agar 2%). The phenotypic screening was carried out in five different media. A commercial and pasteurized organic grape must (Merlot) was purchased from Famille Cassy Laurent (Morizès, Bordeaux, France). The commercial wine was

obtained from Chateau Larrouquey (Graves, Bordeaux, France). The wort was bought from Mangrove Jack's (MJ Traditional Series Pale Ale Pouch) (Auckland, New Zealand) and prepared at the laboratory following the instructions of the manufacturer. The commercial beer was bought from Heineken. Finally, a kombucha-wort was prepared using organic Sensha green tea leaves (6 g/L) infused at 80°C for 1 hour in a mix of tap water (25%) and osmosed water (75%). Organic sucrose was added after the infusion (80 g/L). The main characteristics of the five media are presented in Table 2. The main parameters used in this study are presented in Table 3.

Table 1: Short list of the tested strains with their population belonging

Species	Population	Number of strain	of Strain
<i>B. bruxellensis</i>	beer	22	YJS7816; YJS7817; YJS7818; YJS8081; YJS8657; YJS8229; YJS8010; YJS7966; YJS7895; YJS8048; YJS7868; YJS7810; YJS7926; YJS8251; YJS7944; YJS7813; YJS8089; YJS8072; YJS7995; YJS5397; YJS5400; YJS5454
	kombucha-1	18	YJS8285; YJS8013; YJS8070; YJS7909; YJS7910; YJS7921; YJS7923; YJS7947; YJS7831; YJS8104; YJS7879; YJS5310; YJS5340; YJS5344; YJS5363; YJS5368; YJS5407; YJS5420
	kombucha-admixed	13	YJS8090; YJS7958; YJS7889; YJS5301; YJS533; YJS5349; YJS5384; YJS5398; YJS5402; YJS5406; YJS5413; YJS5417; YJS5431
	teq/EtOH	19	YJS8003; YJS8004; YJS7829; YJS7828; YJS7800; YJS7805; YJS7839; YJS7860; YJS7885; YJS8068; YJS7821; YJS7819; YJS7820; YJS7890; YJS8028; YJS7952; YJS8014; YJS7953; YJS7954
	wine 1	28	YJS7803; YJS8082; YJS8091; YJS8092; YJS8757; YJS7948; YJS8758; YJS7886; YJS7900; YJS8095; YJS7903; YJS7905; YJS7902; YJS7980; YJS7827; YJS7838; YJS7972; YJS7881; YJS7884; YJS5408; YJS5434; YJS5445; YJS5459; YJS5469; YJS5473; YJS5476; YJS5478; YJS5487
	wine 2	17	YJS7804; YJS7801; YJS8045; YJS8063; YJS7807; YJS7989; YJS8187; YJS7894; YJS7809; YJS7891; YJS7853; YJS7824; YJS782; YJS7811; YJS7812; YJS8000; YJS5382
	wine 3-1	14	YJS7802; YJS7967; YJS7955; YJS7874; YJS7844; YJS7973; YJS7848; YJS7849; YJS7862; YJS7911; YJS7888; YJS7806; YJS5392; YJS5449
	wine 3-2	20	YJS7875; YJS7856; YJS7990; YJS5302; YJS5319; YJS5320; YJS5345; YJS5347; YJS5357; YJS5373; YJS5385; YJS5422; YJS5426; YJS5440; YJS5447; YJS5453; YJS5458; YJS5461; YJS5479; YJS5485
<i>S. cerevisiae</i>	S.c	5	F15; FX10; Windsor; Y11858; Y1301

Table 2: Main characteristics of the five media used for this study. HPLC and enzymatic assays were performed 6 times independently

Environment	Sugar	Ethanol	Malic Acid	Acetic Acid	Glycerol	SO <sub>2</sub>	pH	Duration of experiment
<b>Kombucha</b>	80 g/L (Sucrose)	0	0	0	0	0	6.5	740 h
<b>Wine</b>	<2 g/L (Glucose+Fructose)	12,50%	0.02 g/L	0.7 g/L	6 g/L	<80mg/L	3.58	1049 h
<b>Must</b>	250 g/L (Glucose+Fructose)	0	3 g/L	0.15 g/L	0	0	3.36	ranging from 900 to 2100 hours
<b>Beer</b>	1.2 g/L (Glucose+Fructose) 1.7g/L (Maltose) 2.6g/L (Maltotriose)	5%	0	0	0.7 g/L	0	4.28	550 h
<b>Wort</b>	45.5 g/L (Glucose+Fructose) 45g/L (Maltose) 4g/L (Maltotriose)	0	0	0	0	0	5.1	ranging from 500 to 1400 hours

Table 3: table of the 31 parameters investigated in this study, with their significances.

Parameters					
Class	Name	Significance	Calculation	Unit	Method
<b>Kinetics</b>	<b>ODmax</b>	Maximal optical density measured during growth	Calculated from the equation of diauxic growth (Liquori et al.,1981)	OD600nm	Optical density
	<b>OD.LP</b>	Growth lag-phase	Duration before optical density reaches 0.2	Hours	Optical density
	<b>OD.r</b>	Maximal growth rate	Calculated from the equation of diauxic growth (Liquori et al.,1981)	Division per hour	Optical density
	<b>t90OD</b>	Time for which the optical density reached 90% of the ODmax	Calculated from the equation of diauxic growth (Liquori et al.,1981)	Hours	Optical density
	<b>CO2max</b>	CO <sub>2</sub> released during all the fermentation		g/L	Weight loss
	<b>CO2.LP</b>	Fermentation lag-phase	Using loess fit, when CO <sub>2</sub> release reaches 2g/L	hours	Weight loss
	<b>CO2.r</b>	Maximal fermentation rate	Using loess fit	g/L/hour	Weight loss
	<b>t95CO2</b>	Time when the fermentation reached 95% of CO2max	Using loess fit, when CO <sub>2</sub> release reaches 95% of the CO2max	hours	Weight loss
<b>Biochemicals</b>	<b>SO<sub>2</sub></b>	SO <sub>2</sub> production	Concentration in the sample minus the concentration in the negative control (blank)	mg/L	Enzymatic assay
	<b>MalicAcid</b>	Malic acid production	Concentration in the sample minus the concentration in the negative control (blank), expressed in mg/L	g/L	Enzymatic assay
	<b>AceticAcid</b>	Acetic acid production	Concentration in the sample minus the concentration in the negative control (blank), expressed in g/L	g/L	Enzymatic assay
	<b>Glycerol</b>	Glycerol production	Concentration in the sample minus the concentration in the negative control (blank), expressed in g/L	g/L	Enzymatic assay
	<b>Glucose_Fructose</b>	Glucose and fructose consumption	Concentration in the sample minus the concentration in the negative control (blank), expressed in g/L	g/L	HPLC measurement
	<b>Maltose</b>	Maltose consumption	Concentration in the sample minus the concentration in the negative control (blank), expressed in g/L	g/L	HPLC measurement
	<b>Maltotriose</b>	Maltotriose consumption	Concentration in the sample minus the concentration in the negative control (blank), expressed in g/L	g/L	HPLC measurement
	<b>Sucrose</b>	Sucrose consumption	Concentration in the sample minus the concentration in the negative control (blank), expressed in g/L	g/L	HPLC measurement



	Ethanol	Ethanol production	Concentration in the sample minus the concentration in the negative control (blank), expressed in g/L	g/L	HPLC measurement
	<b>pH</b>	pH variation			
<b>Color</b>	<b>L</b>	Corresponds to the perceptual lightness (clarity)	Described by OIV, samples measured were subtracted from the negative control		Optical densities
	<b>a</b>	Values between green and red colors	Described by OIV, samples measured were subtracted from the negative control		Optical densities
	<b>b</b>	Values between blue and yellow colors	Described by OIV, samples measured were subtracted from the negative control		Optical densities
	<b>Int</b>	Corresponds to the intensity of the color	Described by OIV, samples measured were subtracted from the negative control		Optical densities
	<b>Ton</b>	Corresponds to the tonality of the color	Described by OIV, samples measured were subtracted from the negative control		Optical densities
	<b>delta</b>	$\Delta E^*$ Total color difference value between the medium control and the sample	as the square root of the sum of squared differences for L*, a* and b*		Optical density
<b>Flow Cytometry</b>	<b>alive_pct_1</b>	Percentage of viable cells before inoculation in the media	Flow cytometry	percentage	Flow Cytometry
	<b>Yeast_Events_2</b>	Number of total cells count at the end of the experiment	Flow cytometry	cell/ $\mu$ L	Flow Cytometry
	<b>alive_pct_2</b>	Percentage of viable cells at the end of the experiment	Flow cytometry	percentage	Flow Cytometry
	<b>alive_FSC_2</b>	Mean of the Forward Scatter (proxi for cell size) of the viable cells at the end of the experiment	Flow cytometry		Flow Cytometry
	<b>alive_SSC_2</b>	Mean Size Scatter (proxi for cell granulosity) of the viable cells at the end of experiment	Flow cytometry		Flow Cytometry
	<b>dead_FSC_2</b>	Mean Forward Scatter (proxi for cell size) of the dead cells at the end of the experiment	Flow cytometry		Flow Cytometry
	<b>dead_SSC_2</b>	Mean Size Scatter (proxi for cell granulosity) of the dead cells at the end of experiment	Flow cytometry		Flow Cytometry

### b) Growth and fermentation kinetic monitoring

Pre-cultures: For grape must, kombucha and wort, strains were firstly grown at 24°C for 48h in the corresponding medium diluted in MiliQ water with 50:50 ratio. For wine and beer, strains were acclimated following two steps: first in 50-50 ratio (wine or beer : must or wort) of the corresponding medium for 48h then in 90-10 ratio (wine or beer: must or wort) for five days at 24°C. After acclimation, cell concentration was measured using flow Cytometry (Cytoflex, Beckman Coulter Inc, Brea, California USA).

Before inoculation, each medium was filtered (nitrate cellulose 0.45  $\mu$ m, Millipore, France). Growth and fermentation kinetics were monitored as previously described by Peltier et al. (2018). Briefly, yeast cultures were conducted at 24°C in 20 mL screw vials (Thermo Fisher Scientific, Bordeaux, France) with 12.5 mL of medium. Hypodermic needles (G 26-0.45x13 mm, Terumo, Shibuya, Tokyo, Japan) were inserted through the septum to allow CO<sub>2</sub> release. In each medium, each strain was inoculated at 2.5x10<sup>5</sup>.mL<sup>-1</sup> viable cells, as determined by flow cytometry. Fermentation and growth took place at 24°C in shaken vials by using an orbital shaker (SSL1, Stuart, Vernon Hills, IL, USA) at 165 rpm. Growth

kinetics were estimated with regular (1-2 times per day) optical density measures at 600 nm for beer, wort and kombucha and 610 nm for wine and must (XD 7000 (VIS), Lovibond, USA). Fermentation kinetics were estimated by monitoring manually (1-2 times per day) the weight loss caused by CO<sub>2</sub> release using a precision balance with automatic weight recording (LabX system, Mettler Toledo, Viroflay, France). The amount of CO<sub>2</sub> released according to time was fitted to local polynomial regression, allowing the estimation of the time necessary to reach the maximum CO<sub>2</sub> produced (Peltier et al. 2018). The experiment duration depended on the medium. For wort and must media, for which the CO<sub>2</sub> release was clearly measured, the experiment was stopped when CO<sub>2</sub> release decreased to 0.015 g/L/h with a maximal time of 2100 (+/-24) hours for must and 1400 (+/-24) hours for wort. For wine, kombucha and beer, the experiment duration was similar for all tested strains, 1049 (+/-24), 740 (+/-24) and 550 (+/-24) hours respectively.

### c) Flow cytometry

Several flow cytometry measures were made during the experiment. For wine and beer media, three measurements were done, the first one at the end of the first acclimation, the second at the end of the second acclimation, and the last one at the end of the experiment. Two analyses were done for kombucha, wort and must, one after the acclimation and the other at the end of the experiment. Fifty µL of the homogenised culture with 3 µL of propidium iodide (PI) were diluted in 1 mL final in Mcllvaine buffer (sodium phosphate dibasique 0.2 M, citric acid 0.1 M) and was analysed by flow cytometry (Zimmer et al. 2014). Fifty thousand events were recorded per experiment. The viability of the cells was estimated using 488nm excitation (40mW argon laser), with a 610/20nm long bandpass filter to detect PI fluorescence (dead cells).

### d) Color analysis

After centrifugation (5 min at 3,500g), the supernatant was measured using spectrometer at six wavelengths (420, 450, 520, 570, 620, 630 nm) (FLUOstar Omega, MNGLabtech, France), with a 1/5 dilution with MiliQ water only for wine samples, which was taken into account for CIELAB color space. The Optical spectrum was then integrated, and

multiplied by the CIE standard illuminant D65 and by the CIE 1931 color space to calculate L\*a\*b\* parameters (referred as the CIELAB color space). L\* defines lightness, a\* gives an estimate of the red/green value and b\* the yellow/blue value. ΔE\* (named delta) estimates the total color difference value of the medium with and without yeast growth. It is calculated as the square root of the sum of squared differences for L\*, a\* and b\*. Intensity was calculated following Glories's method (sum of absorbance at 420, 520 and 620 nm), while tonality (Hue) was expressed as the quotient between absorbance at 420 and 520 nm (Glories 1984).

### e) Enzymatic and HPLC assays

At the end of the experiment, a sample volume of 800 µL was manually transferred in Micronics tubes (Novazine, Lyon, France, ref: MP32033L) and stored at -20°C. Acetic acid, malic acid, SO<sub>2</sub>, glycerol, glucose and fructose were measured by semi-automated enzymatic assays (K-ACETGK, K-GCROLGK, K-LMAL-116A, K-TSULPH, Megazyme, Bray, Ireland) following the instructions of the manufacturer at the metabolomics platform of Bordeaux (<https://metabolome.cgfb.u-bordeaux.fr/>) in duplicates for each sample and the mean was used (Peltier et al. 2018). Other compounds such as ethanol, maltose, maltotriose and simple sugars (glucose and fructose) were determined by HPLC-RI (Waters 2414 refractive Index Detector, Waters, Milford, Massachusetts, USA) separated by C18 column (Pursuit 5 C18 300 x 4.6mm, Agilent Technologies, Santa Clara, California, USA) with a flow rate of 0.5mL.min<sup>-1</sup> of MiliQ water as solvent.

### f) Volatolomics approach

Samples were prepared as followed: 3 mL of media sample were diluted in 3 mL of MiliQ water and 3 g/L of ammonium sulphate were added.

Samples were analysed by comprehensive gas chromatography (GCxGC) using a Pegasus BT 4D (Leco, Saint Joseph, USA). The inlet temperature was 240 °C and the inlet mode was splitless with a 1 min purge (50ml/min) Separation was achieved with two columns. The primary column (first dimension) was a DB-5 MS (5%-phenyl-methylpolysiloxane, 50 m x 0.25 mm x 0.25 µm, from Agilent J&W, CA, USA) and the second column (second dimension) was an Rxi-17Sil (50% phenyl/50% dimethylpolysiloxane, 1.2 m x 0.15

mm × 0.15 μm, Restek, Bellfonte, PA). The first column was held at 50°C for 1 min, 3.5°C/min until 205°C during 1 min and then ramped to 280°C and held for 3 min. The second column was with set at 86°C for 8.5 min, 3.5°C/min until 220°C and then 50°C/min until 285°C for 2 min. Helium was used as gas vector with a constant flow of 1 mL/min. The modulator was offset by 10°C relative to primary column. The modulation period was 4 seconds (1.2 s cold, 0.8 s hot). The mass spectrometer was operated using electron ionization (EI) at 70 eV. The ion source temperature was set at 250°C. Spectra were collected from 45 to 450 m/z with a frequency of 200Hz. The instrument was controlled by Leco ChromaTOF software version 5.51. The total analysis run time was 58 min excluding cool down.

Data was processed using LECO's ChromaTOF software to integrate peaks and to identify them based on library searching (NIST2017, FFNSC 2nd edition) and IRL comparison (ΔIRLexp/IRLdata base<30). Signals having a S/N>50 were selected. All tentatively identified chemicals had a minimum match score of 750 out of 1000. All compounds having a similar CAS with a similar retention time (RT1 ± 60 s, RT2 ± 1.5 s) were considered as a same compound. The computer processing time was approximately 3 hours/sample. The chromaTOF output of the automated detection (.csv file) is a list of deconvoluted peaks with among other informations, the 1D, 2D RT, S/N, chemical name, CAS number and similarity value.

The volatolomic data was built as followed: for identified compounds, we computed the sum of each compound per sample (4 media \* (16 strains + 1 control) \* 3 repetitions = 204 samples). Identified compounds containing silicon or arsenic (column leakage) were excluded. For unidentified compounds, we matched compounds having similar time retention (RT1 +/- 30 s, RT2 +/- 0.5 s) and similar XIC (extracted-ion chromatogram, xic +/- 0.5 ppm), and as for identified compounds, we computed the sum of each unknown compound per sample. Only compounds that were quantified in at least 2 upon 3 repetitions for at least one modality were conserved. In the end, 10,616 molecules were quantified, of which 4,988 were identified. KEGG database was then used to detect over- or under-representation of KEGG pathway maps after *B. bruxellensis* growth

(<https://www.genome.jp/kegg/pathway.html>).

Finally, the main chemical families identified (ester, alcohol, etc.) were also tested for over- or under-representation after *B. bruxellensis* growth.

### g) Adaptation identification

*Signature of adaptation:* Adaptation signatures were assessed using the `Local vs. foreign` (LF) parameters defined in Blanquart et al. (2013). The fitness of one population was compared to the mean fitness of all the other populations in a given environment. `Local vs. foreign` indice was defined as the mean value of the population in a given (local) environment, minus average value of all other populations (foreign) in the same environment. Measurement of the LF value was performed for each population in the five environments, and significance was assessed by 1000 bootstraps. Some parameters were estimated to be adaptive when increased (ODmax, OD.r, CO2max, CO2.r, Glucose\_Fructose, Maltose, Maltotriose, Sucrose, alive\_pct\_1, Yeast\_Events\_2, alive\_pct\_2), some as "maladaptive" when increased (OD.LP, t90OD, CO2.LP, t95CO2) and the others as "undetermined" (SO<sub>2</sub>, MalicAcid, AceticAcid, Glycerol, Ethanol, pH, L, a, b, Int, Ton, delta, alive\_FSC\_2, alive\_SSC\_2, dead\_FSC\_2, dead\_SSC\_2).

### h) Comparative growth and fermentation analysis

Growth experimental points were fitted with a diauxic growth model as described by (Liquori et al. 1981).

$$y(t) = y_{\alpha}x_{\alpha} + y_{\beta}x_{\beta}$$

Where:

$$y_{\alpha} = \frac{1 - \exp^{-t/\tau_1^{\alpha}}}{1 - \exp^{-t/\tau_1^{\alpha}} + 1 - \exp^{-t/\tau_2^{\alpha}}}$$

$$y_{\beta} = \frac{1 - \exp^{-t/\tau_1^{\beta}}}{1 - \exp^{-t/\tau_1^{\beta}} + 1 - \exp^{-t/\tau_2^{\beta}}}$$

$$x_{\alpha} + x_{\beta} = 1$$

This model was chosen because some growth curves showed typical biphasic shape with two distinct sigmoidal branches. The diauxic model fitted the experimental data satisfactorily, both for biphasic and monophasic curves.

Comparative growth: Pairwise comparison (each strain versus the other 150 strains) was realised, to each environments, *in silico*. The average fitted growth of the biological triplicates was used for each strain *per* medium combination. Then for each pairwise comparison, the fitted kinetics were used to calculate the growth achievement over time period. At the end of the time period (2100 hours for must, 1400h for wort, 1050h for wine, 740h for kombucha and 550h for beer), we computed the percentage of growth associated to each strain (from 0 to 100%). For graphical representation, for each pairwise comparison, the fittest strain was used to produce a color ramp, from white color when the comparison ended - as it started - in 50:50 ratio between the initial strains, to the full intensity color when one strain fully outcompeted the other (0:100 ratio).

*Fermentation kinetics*: fermentation kinetics were analysed using K-means clustering (cutRepeatedKmeans function; *ClassDiscovery* R package). From 2 to 15 clusters were tested, and the optimal number of clusters (4) was determined visually.

#### i) Plasticity and intra population variances

*Strain plasticity*: Strain individual plasticity were measured using ANOVA analysis (model 3). The variance associated to environments was quantify for each strain when environment factor was significant. The variance was normalized using z-score normalization, and only variances, which were upper than the mean variance plus one standard deviation were represented.

*Intra population variances*: Variance of each population in the different media were compared with the Fligner-Killeen test. The populations were classified in significate groups. Only the extreme two group of significance were kept for the figure (the group which encompasses the population(s) with the lowest variance (blue) and the group with the population(s) with the highest variance (red).

#### j) Statistical analysis

All statistical and graphical analyses were carried out using R homemade scripts. Heatmap representations were build using *ComplexHeatmap* package (Gu, Eils, Schlesner 2016), and other graphical representation

were built with *ggplot2* package. Statistical analyses were conducted using *agricolae* package.

*ANOVA analyses*: were performed as followed:

1) model 1:

$$P_{jm} = \mu + G_j + E_m + (GxE)_{jm} + \varepsilon_{jm}$$

$P$  stands for the value of the tested traits (31 traits) for the combination of  $j$  (1 to 151) strains and  $m$  (1 to 5) media;  $\mu$  stands for the mean value of the trait  $P$ ;  $G$  stands for the genotype effect;  $E$  stands for environment effect;  $GxE$  stands for the interaction between  $G$  and  $E$  factors; and  $\varepsilon$  for residuals of the model.

2) model 2:

$$P_{ijm} = \mu + S(G)_{ij} + G_j + E_m + (GxE)_{jm} + (S(G)xE)_{ijm} + \varepsilon_{ijm}$$

$P$  stands for the value of the tested traits (31 traits) for the combination of  $j$  (1 to 151) strains in  $i$  (1 to 8) populations and  $m$  (1 to 5) media;  $\mu$  stands for the mean value of the trait  $P$ ;  $G$  stands for the genotype effect;  $S(G)$  stands for the population effect;  $E$  stands for environment effect;  $GxE$  stands for the interaction between  $G$  and  $E$  factors;  $S(G)xE$  stands for the interaction between  $S(G)$  and  $E$  factors; and  $\varepsilon$  for residuals of the model.

3) model 3:

$$P_m = \mu + E_m + \varepsilon_m$$

$P$  stands for the value of the tested traits (31 traits) for the combination of  $m$  (1 to 5) media;  $\mu$  stands for the mean value of the trait  $P$ ;  $E$  stands for environment effect; and  $\varepsilon$  for residuals of the model.

4) model 4:

$$P_j = \mu + G_j + \varepsilon_j$$

$P$  stands for the value of the tested traits (31 traits) for the combination of  $j$  strains (belonging to the same population);  $\mu$  stands for the mean value of the trait  $P$ ;  $G$  stands for the genotype effect; and  $\varepsilon$  for residuals of the model.

5) model 5:

$$P_{xm} = \mu + T_x + E_m + (TxE)_{xm} + \varepsilon_{xm}$$

$P$  stands for the value of the tested flow cytometry parameters for the combination of  $x$  (1 to 2) ploidy levels and  $m$  (1 to 5) media;  $\mu$  stands for the mean value of the trait  $P$ ;  $T$  stands for the ploidy effect;  $E$  stands for environment effect;  $TxE$  stands for the interaction between  $T$  and  $E$  factors; and  $\varepsilon$  for residuals of the model.

6) model 6:

$$P_{ij} = \mu + \alpha ODmax + \beta Acetic + S(G)_{ij} + G_j + \varepsilon_{ij}$$

$P$  stands for the value of the tested traits in a given medium for the combination of  $j$  (1 to 151) strains in  $i$  (1 to 8) populations;  $\mu$  stands for the mean value of the trait  $P$ ;  $G$  stands for the genotype effect;  $S(G)$  stands for strains belonging to the same population;  $\alpha$  is the regression coefficient for ODmax;  $\beta$  the regression coefficient for Acetic acid; and  $\varepsilon$  for residuals of the model.

7) model 7:

$$P = \mu + \alpha ODmax + S_i + \varepsilon_i$$

$P$  stands for the value of the tested traits for the combination of  $i$  (1 to 8) populations;  $\mu$  stands for the mean value of the trait  $P$ ;  $S$  stands for the population effect;  $\alpha$  is the regression coefficient for ODmax; and  $\varepsilon$  for residuals of the model.

## Results and discussion

The purpose of this study was to evaluate the phenotypic variability of the *Brettanomyces bruxellensis* yeast species in five distinct environments (). To do so, a large-scale phenotyping approach was conducted, 31 parameters distributed in four classes were selected for their biological relevance: (1) Kinetics parameters included growth ability indicators (ODmax, OD.LP, OD.r, t90OD) and fermentation ability (CO2max, CO2.LP, CO2.r, t95CO2); (2) ten biochemical assays of metabolites of interests were performed (acetic acid, malic acid, glycerol, ethanol, SO2, Glucose\_Fructose, Maltose, Maltotriose, Sucrose, pH); (3) colorimetric impact was composed of six different measures (L, a, b, Int, Ton, delta); and (4) cytometry data included

seven parameters (alive\_pct\_1, Yeast\_Events\_2, alive\_pct\_2, alive\_FSC\_2, alive\_SSC\_2, dead\_FSC\_2, dead\_SSC\_2). These 31 traits were evaluated in five media, namely a commercial wine, a grape must, a commercial beer, a wort and a homemade kombucha must (Table 3). Overall, 151 strains were used, representative of the known genetic diversity and were clustered in eight populations (Joseph Schacherer's team, personal communications) (Figure 1). All kinetics and measurements were made in biological triplicates.

### a) Environment and genetic impacts

The different parameters measured in the five environments are presented on Table 3. On the PCA (principal component analysis) (Figure 2), the first axis, which represents more than 30% of the variance of the data allowed the separation of sugared rich media (must and wort) from beer, kombucha and wine media. The second axis, which accounts for 21.9% of the general variance, separated the must and wine from the wort and beer environments. The clustering according to the media reflected strong phenotypic differences depending on the environment. The phenotypic response of *B. bruxellensis* seemed thus to be highly environment dependent. A heatmap representation was constructed and kinetics, biochemicals, color and flow cytometry parameters were compared in the five media for the 151 strains (Figure 3). Several patterns of variations were observed at strains level, between environments, but also at population level, in the four groups of considered traits. For example, the *beer* and *kombucha-admixed* population appeared to be homogenous for kinetics and color parameters, which was not the case for the *wine 3-2* group. Flow cytometry parameters appeared to be more variable within populations compared to kinetics, biochemicals and color parameters.

In order to estimate the relative impact of three factors (namely, the environment (E), the genetic (G) at strains level and genetic by environment factors (GxE)), variance analysis (model 1) were performed on the 31 parameters.

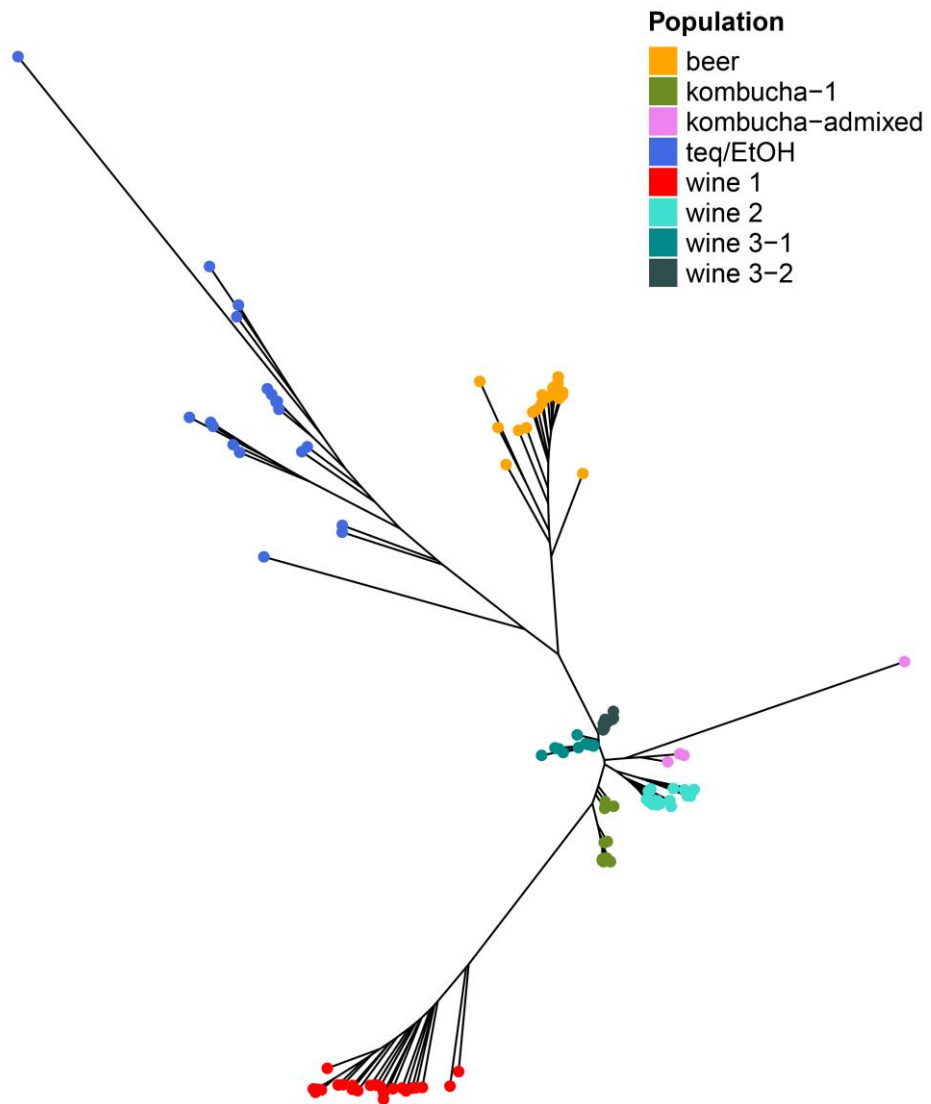


Figure 1: Phylogeny tree of the 151 strains used in this study. Colors represent the eight populations used in this study. Data shared by Schacherer's team. Whole genomes sequences were used to build this tree. The distance matrix was computed with "Merge branch 'devel'" (<https://github.com/szpiech/asd>) with SNP genotype data and clustered with fastStructure (Raj, Stephens, Pritchard 2014).

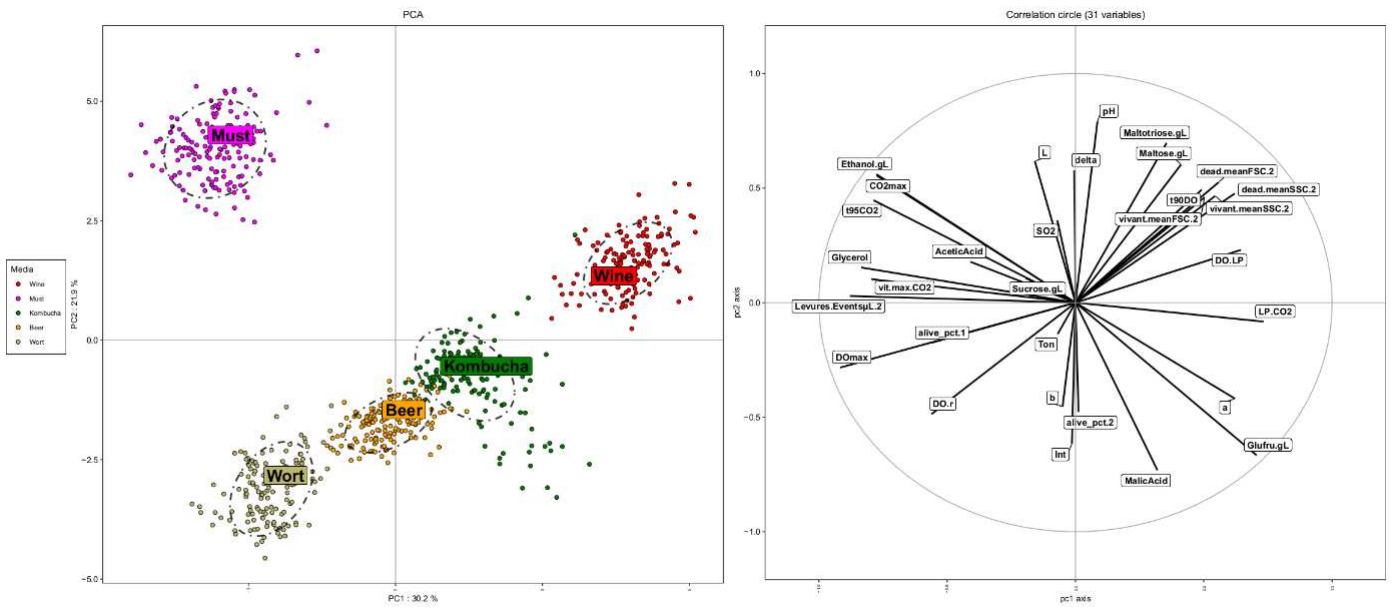


Figure 2: Principal component analysis, representing the 151 strains in the five environments, using 31 parameters. Left: Dots correspond to one strain (mean of the triplicates) in one environment. Dots are colored depending on the environment. Right: Correlation circle for the 31 parameters.

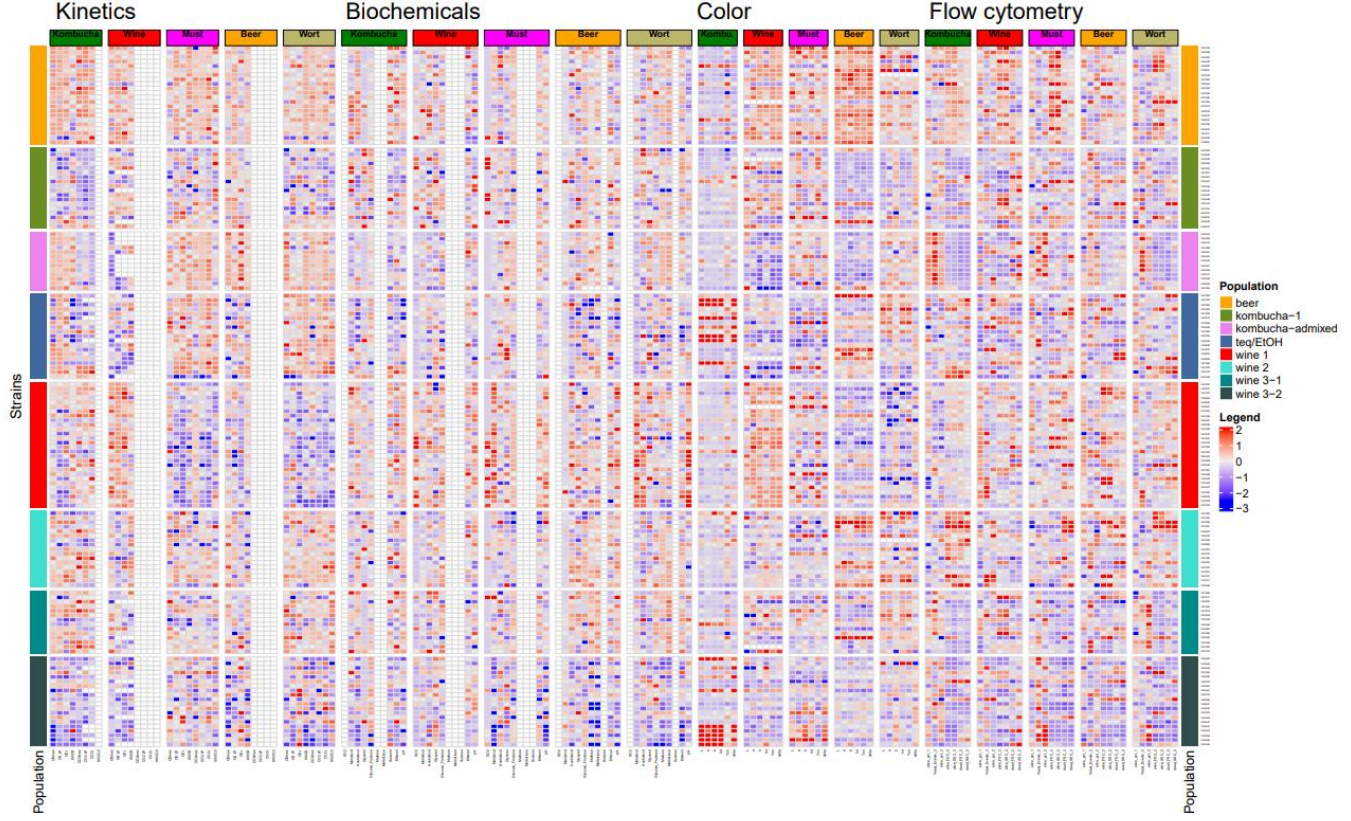


Figure 3: Heatmap representation of phenotypic diversity within *B. bruxellensis* strains. Values are normalized and missing values are in white. Strains are ordered and colored based on the genetic populations.

All factors significantly affected all 31 parameters (Figure 4A). Among parameters, the relative impact of each factor was variable; however, globally, environment played the major role, followed by GxE and genetic, which accounted for 58, 21.4 and 9.5%, respectively, of the total variance. The strong impact of the environment factor was expected, due to the diversity of the media used, in terms of sugar, ethanol content and color contrast. An additional variance analysis was conducted (model 2), which took into account the effect of the strains nested within populations, as well as its interaction with environment. The population factor explained 12.5% of the total variance, which was distributed into the genetic and GxE factors, 4.92% out of 9.5% and 7.61 out of 21.4% respectively (Figure 4B). In details, CO<sub>2</sub>max was strongly affected by environment, which may be due to the variation of fermentable sugars among the media, ranging from <2g/L to 240g/L. Glucose-fructose consumption, like maltose consumption, ethanol production and pH were strongly influenced by the environment. The high impact of the environment may indicate that these traits variations respond to environmental signals and are shared within the *B. bruxellensis* species (weak impact of GxE and G). On the contrary, SO<sub>2</sub> production was highly impacted by GxE and genetic factors, like acetic acid and glycerol production. This may suggest that SO<sub>2</sub>, acetic acid and glycerol production are mainly variable amongst isolates in response to environmental signal. Cytometry parameters were quite homogenous and mainly environment-dependent, indicating that cell morphology responds to environmental cues, with variations at the genotype level.

Phenotypic variations in *B. bruxellensis* have been regularly reported, with high strain specificity, however, never with such exhaustive population genetic comprehension (Agnolucci et al. 2009; Vigentini et al. 2008; Crauwels et al. 2017; 2015). Populations accounted for around half of the strain phenotypic variability. Strong phenotypic variations were observed in response to environmental and GxE factors which may highlight the plasticity of the species and its ability to respond to environmental changes.

## b) Signatures of adaptation

In order to test if the phenotypic differences within genetic populations could be the hallmark of adaptation to anthropized niches, *local adaptation* was tested by measuring the LF indice, with the `Local vs. Foreign` approach (Blanquart et al. 2013). Briefly, LF indices is defined as the mean value of the population in a given (local) environment, minus average value of all other populations in the same environment. Adapted populations are expected to have high LF for fitness traits in their preferred environment, while “maladapted” populations will show low LF indices. Measurement of the LF value was performed for each population in the five environments, and significance was assessed by 1000 bootstraps. Three kinds of responses were identified: (i) statistical hit for an increase of traits estimated as beneficial in the environment (ability to use sugar for example) were called “adaptation”; (ii) statistical hit for a decrease of traits estimated as being beneficial in the environment called “maladaptation”; (iii) statistical hit for traits whose relationships with fitness is unclear were called “undetermined” (Figure 5). For the beer group, no “maladaptation” was recorded for the five environments and adaptation to kombucha and beer environments were highlighted. The kombucha-1 was only “maladapted” in kombucha for fermentation parameters, without adaptation/maladaptation signal in other media, by contrast with the kombucha-admixed population, which presented numerous adaptation signatures for kombucha, must, beer and wort media and “maladaptation” signatures for wine (growth ability). The teq/EtOH and wine 2 groups did not show particular adaptation/maladaptation in these five environments. The wine 1 population showed strong adaptation signatures for wine environment (parameters related to growth), and “maladaptation” hits in must and wort. The wine 3-1 has statistical hits for adaptation in beer and wort environments (in maltose and maltotriose consumption and flow cytometry parameter), with no signal for “maladaptation”. Finally, the wine 3-2 group presented “maladaptation” hits for kombucha, wine, beer and wort. Other statistical signatures were found significant for color, biochemical or for flow cytometry parameters.



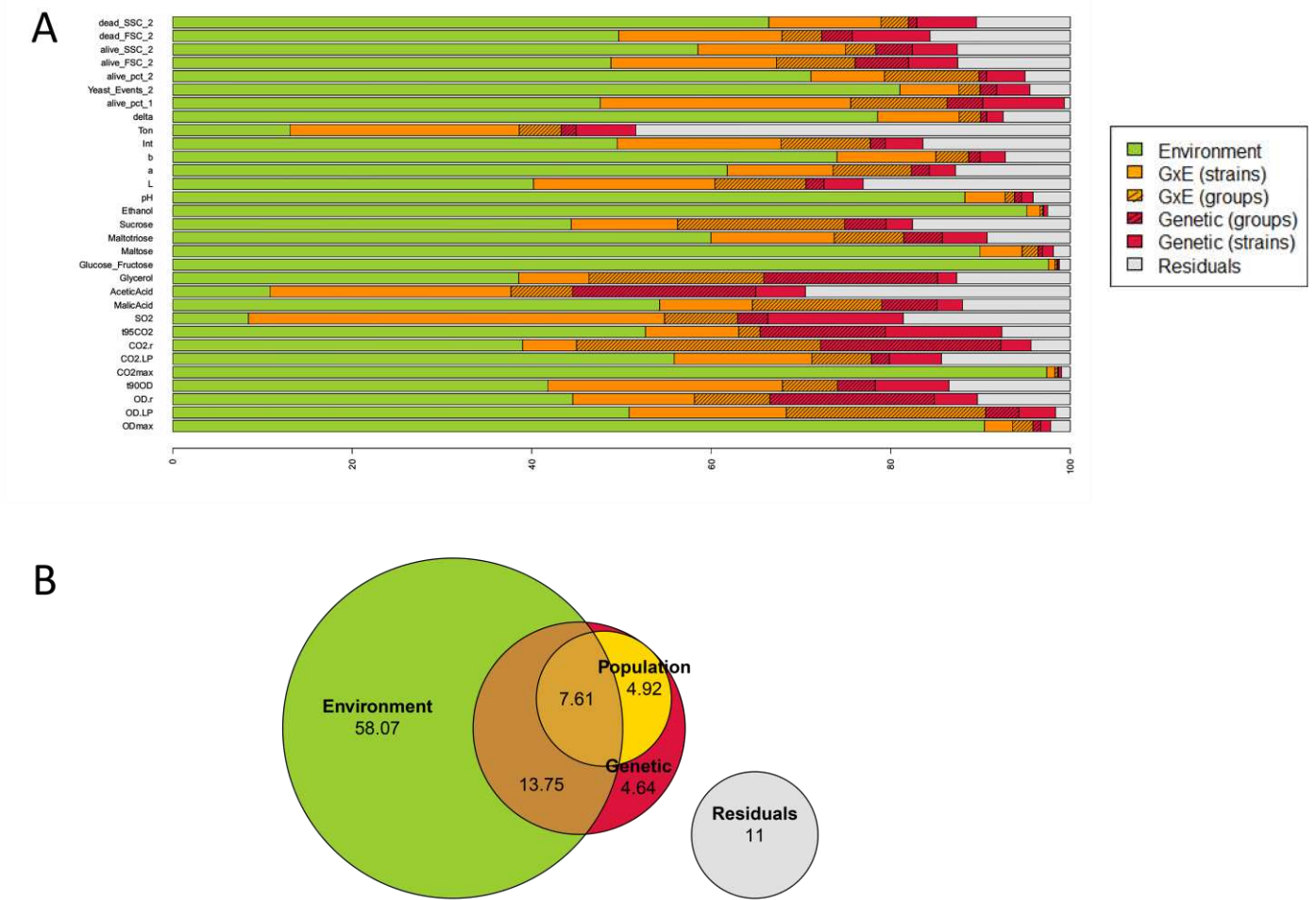


Figure 4: Variance analysis (ANOVA) of the 31 parameters, using the five environments, the strains and genetic by environment interactions. A: Percentage of variation associated with Environment, Genetic, Population factors and their interactions. The hatched part corresponds to the impact of the populations. B: Venn representation of the mean average of the different factors considered in the panel A.

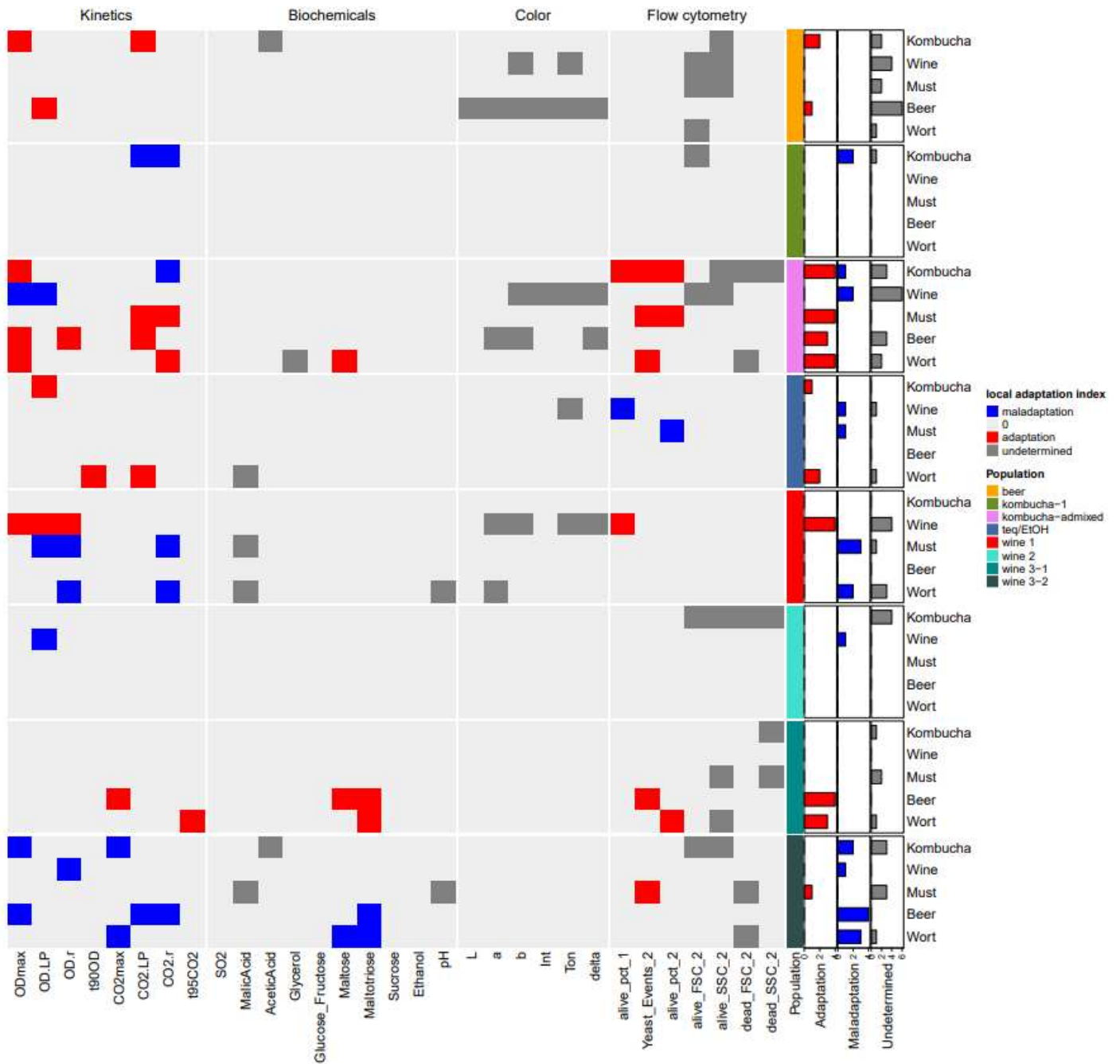


Figure 5: Heatmap representation of statistical hit for local adaptation. Each population is represented in each environment for the 31 parameters. Plots on the right summarize the number of hit (maladaptation, adaptation, of undetermined) for the population in the five environments

The beer, kombucha-admixed and wine 1 populations had several neutral hits for color parameters, in addition to adaptation or maladaptation signatures for kinetics or flow cytometry parameters. However, it appeared difficult to precise whether color modifications could hint at adaptation or maladaptation, or could simply be the consequence of neutral phenotypic divergence. The same observation was made for malic acid production, evidenced for wine 1 and wine 3-2 populations.

These results were the first investigations for large-scale phenotypic adaptation signatures in *B. bruxellensis* species to our knowledge. Several local adaptive signatures were highlighted for the different populations, such as *beer*, *kombucha-admixed*, *wine 1* and *wine 3-1* groups. Interestingly, while the first adaptive signature within *B. bruxellensis* stand for sulphite resistance of the *wine 1* population (most commonly used wine preservative); several other signatures were highlighted for this group in wine

environment for growth parameters. It indicated that beside sulphite resistance, this population showed strong growth ability in wine compared to other *B. bruxellensis* populations.

### c) Growth comparison assay

Special attention was carried out for the growth capacity of the 151 strains in all environments for its adaptive relevance. Five *S. cerevisiae* strains isolated from wine and beer environments were added as an out-group. To assess the growth ability of the strains, growth comparison were realized, each strain competed against each other 150 strains for the five environments (Figure 6, Supplemental figure 1

*Supplemental figure 1*). For each combination, the strain that reached the higher optical density was designated as “success” and the proportion of the strains was measured. Based on the comparison results, the proportion of “success” for each strain was calculated and compared at the population level (Figure 6, Supplemental figure 1). Variability was observed at two levels, within population and between population (using Kruskal-Wallis analysis). The *S.c* group (standing for the five *S. cerevisiae* strains) outcompeted all *B. bruxellensis* strains in kombucha, wort, beer and must, but was one of the less successful group in wine environment. The probability of success of the beer population was steady in all of the five environments being one of the best *B. bruxellensis* population. The kombucha 1 success was high in beer and wine environment, and low in the other media. The kombucha-admixed

population harboured a competitive growth in kombucha, beer and must environment, which was not the case in wine environment (being the weaker population). The *teq/EtOH* group had a competitive growth in wort and must (highly-sugared media) and a correct growth in the other environments. A previous study have shown that bioethanol isolates have a better growth rate in rich media than isolates from wine, however without assessing the genotype of the strains used (Da Silva et al. 2019). The wine 1 population had a poor percentage of success in all environment (particularly in environments with higher level of sugars) with the exception of the wine environment where it harboured the best growth capacity. The wine 2, wine 3-1 and wine 3-2 were similar, without no clear evidence of a particular growth capacity in the five environments.

To summarize, *B. bruxellensis* strains were able to grow in the five environments (with some exceptions in wine medium), though with a reduced growth rate compared to *S. cerevisiae* (with the exception of wine medium), which was congruent with previous studies (Ferremi Leali et al. 2022; Dias et al. 2003). Strong variability between the populations was highlighted. The fitness of *wine 1* population in wine environment was notable compared to the other environments, and the contrary was observed for the *kombucha-admixed* group, which was in accordance to local adaption signature observed. *Beer* population was competitive in the five environments, with reduced intra-variability. These results were in accordance with local adaptation signatures underlined above

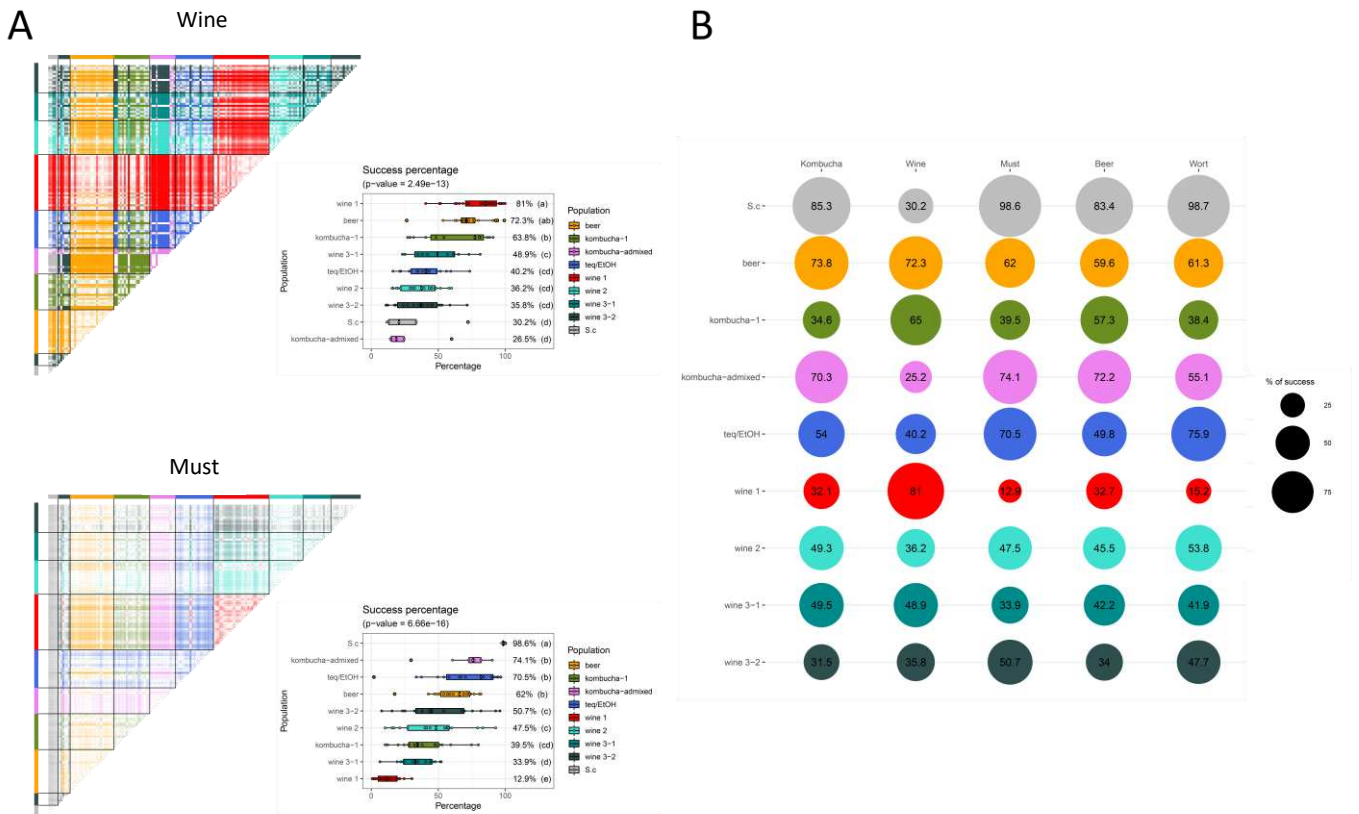
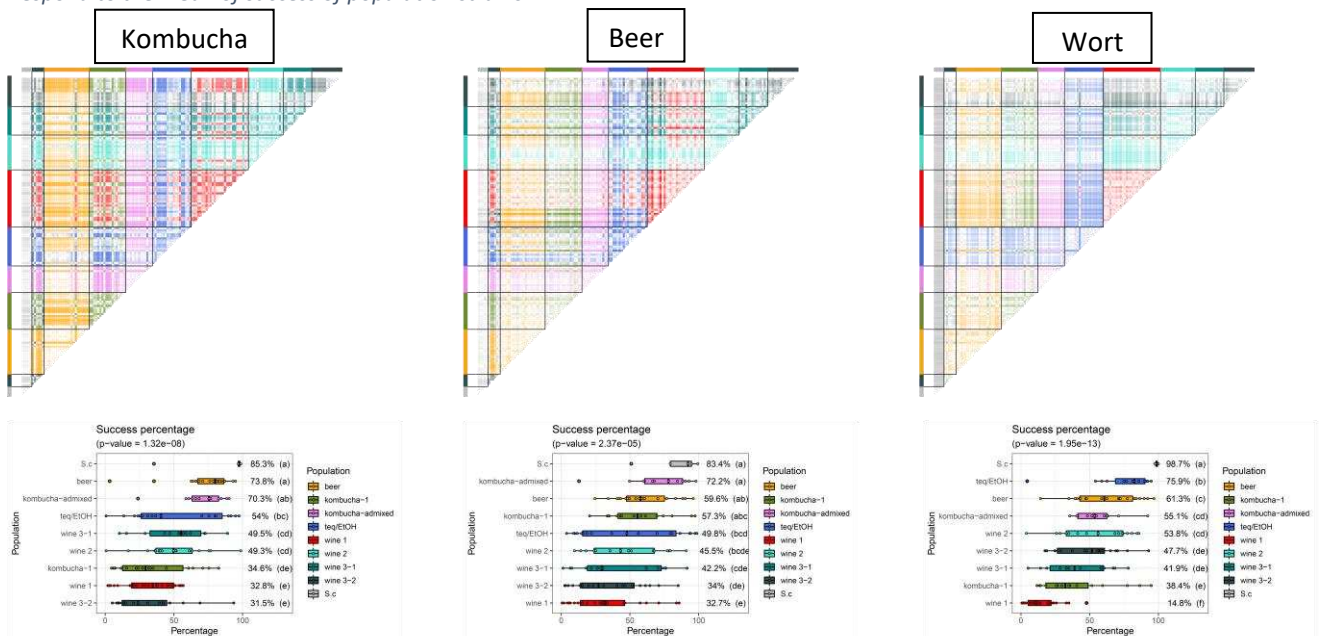


Figure 6: Comparison growth in wine and must environments. A: Comparison matrix in wine and must. Growths were performed in triplicates, and the average of the triplicates was used for each strain. The color intensity indicates the population of the fittest strain and the level of success. The percentage of success was calculated for each strain and represented on the plot. Means of the population are indicated and letters (between brackets) correspond to the statistical group (Kruskal-Wallis). B: Comparison result for the five environments. Points correspond to the mean of success of population strains.



Supplemental figure 1: Comparison growth experiments in kombucha, beer and wort environments. Growths were performed in triplicates, and the average of the triplicates was used for each strain. The color indicated the population of the fittest strain, which was the fittest. The color

intensity is correlated to the margin of success of a strain on the other. The percentage of success was calculated for each strain and represented on the plot. Strains were gathered following their population. Means of the population are indicated and letter (between brackets) correspond to the statistical group (Kruskal-Wallis).

#### d) Plasticity to environmental changes

To go deeper in the phenotypic variability of the strains, the environmental impacts on the 31 parameters for the 151 strains were investigated (Figure 7, model 3). Phenotypic plasticity can be defined as the ability of a genotype to change its phenotype in response to environmental factors (Grenier, Barre, Litrico 2016; David W. Pfennig 2021). Phenotypic plasticity has been proposed to facilitate adaptation in changing environments and to be the first step of adaptive divergence ('plasticity-first hypothesis') (David W Pfennig 2021; Levis, Pfennig 2016; Sun et al. 2020). All strains showed significant plasticity for all parameters across the five environments, but the extend of phenotypic plasticity varied greatly with some strains being more plastic than others.

Variation of the number of strains presenting strong environmental plasticity compared to the rest of the population were observed (from 0 to 32 strains) among parameters. Figure 7 shows that kinetics and flow cytometry parameters triggered more plastic strains than biochemical parameters. Interestingly, lower environmental effects were observed for sugar consumptions, as well as for SO<sub>2</sub> production and t95CO<sub>2</sub>. This indicates that sugar consumption was poorly plastic in *B. bruxellensis* species. The absence of plasticity of a trait (named "canalization") could arise from genetic selection (Grenier, Barre, Litrico 2016). The ability to efficiently consume sugars regardless of the environmental factors has already been observed (Smith, Divol 2018). Concerning individual plasticity, strains harboured 0 (16 strains) to 8 increased plastic traits (1 strain). Between populations, variations were observed, *wine 1*, *beer*, *wine 2* and *wine 3-1* having the less plastic strains and

*teq/EtOH*, *wine 3-2*, *kombucha-1* and *kombucha-admixed* groups encompassed the more plastic strains (Kruskal-Wallis analysis, *p*-value=0, data not show).

To go further, the plasticity of the life-history traits was explored. Flow cytometry was conducted both at the end of the acclimation step and the end of the experiment, allowing to estimate the variation of the FSC and SSC flow cytometry parameters. For example, Figure 8 shows SSC/FSC variations of viable cells between acclimation and final experiment, represented by an arrow (Figure 8). The plasticity of the parameters was investigated through the populations in the five media. Differences were highlighted, at environmental and population levels. In beer environment, a trend to reduce SSC and FSC parameter was observed for all the populations. In kombucha, the same trend was observed with the exception of the *beer* and *wine 2* groups. In must, FSC and SSC parameters tend toward an increase, in particular for *beer*, *kombucha-1* and *kombucha-admixed* groups. In wine and wort media, different responses were observed. The highest population plasticity was observed in must medium, with an increased FSC (size) and SSC (granularity) parameters at the end of the fermentation. In this environment, at the end of acclimation step, the sugar concentration was still high with low ethanol content, opposite as to the end of the fermentation. Nutrient depletion and ethanol increase could induce an increased cell size in *B. bruxellensis* in must but not in wort environments. Further analyses are needed to delve deeper in life-history traits response to environmental changes.

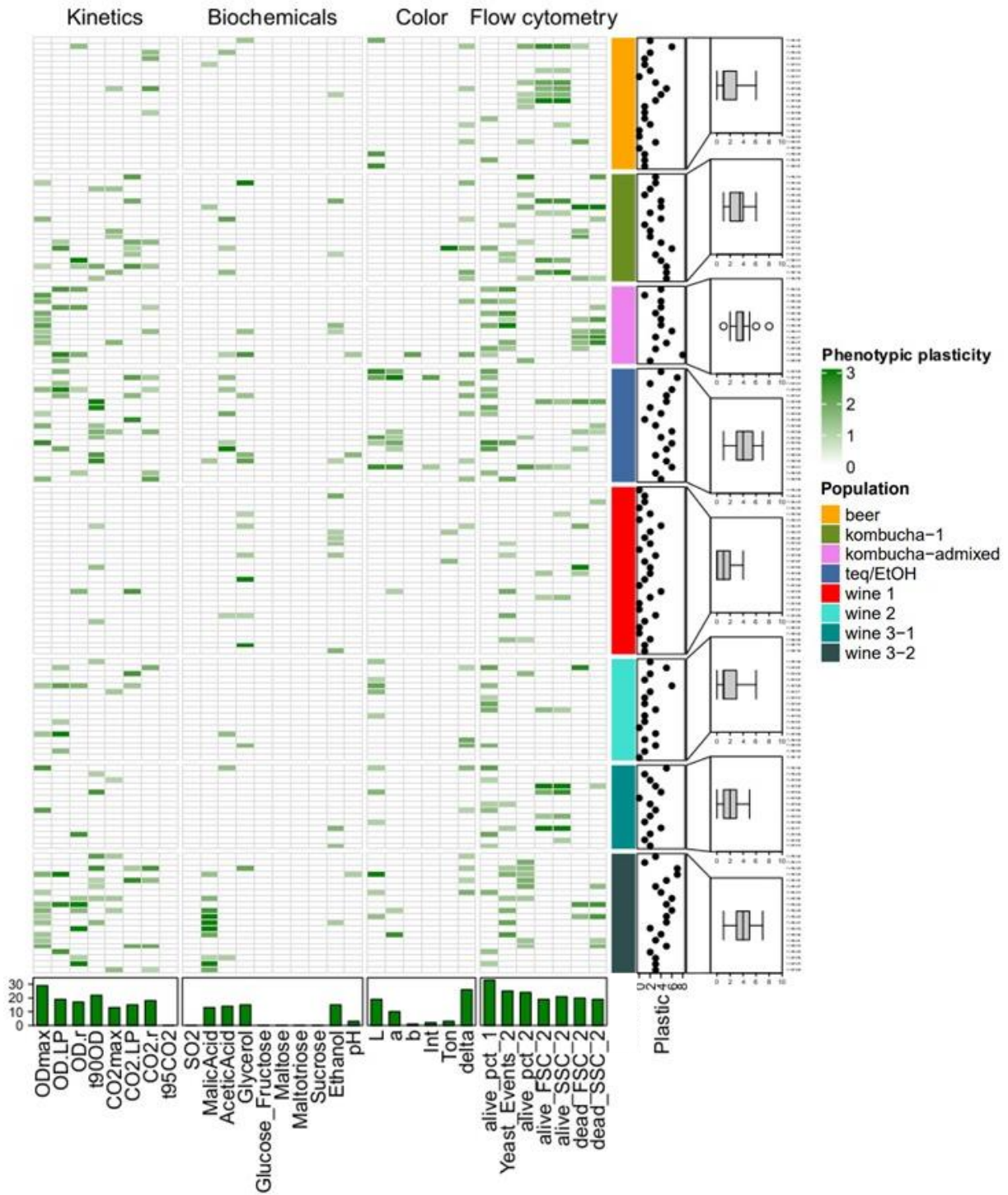


Figure 7 : Heatmap representation of the variance of the 31 parameter in the five environments for the 151 strains. Variances were normalized for each parameter with z-score. Z-scores showing more than one standard deviation above the mean were colored in green. Plots on the bottom indicate the number of plastic strains for each trait. On the right, points represent the number of plastic traits for each strain; boxplots represent the dispersion of the plastic traits within the genetic populations.

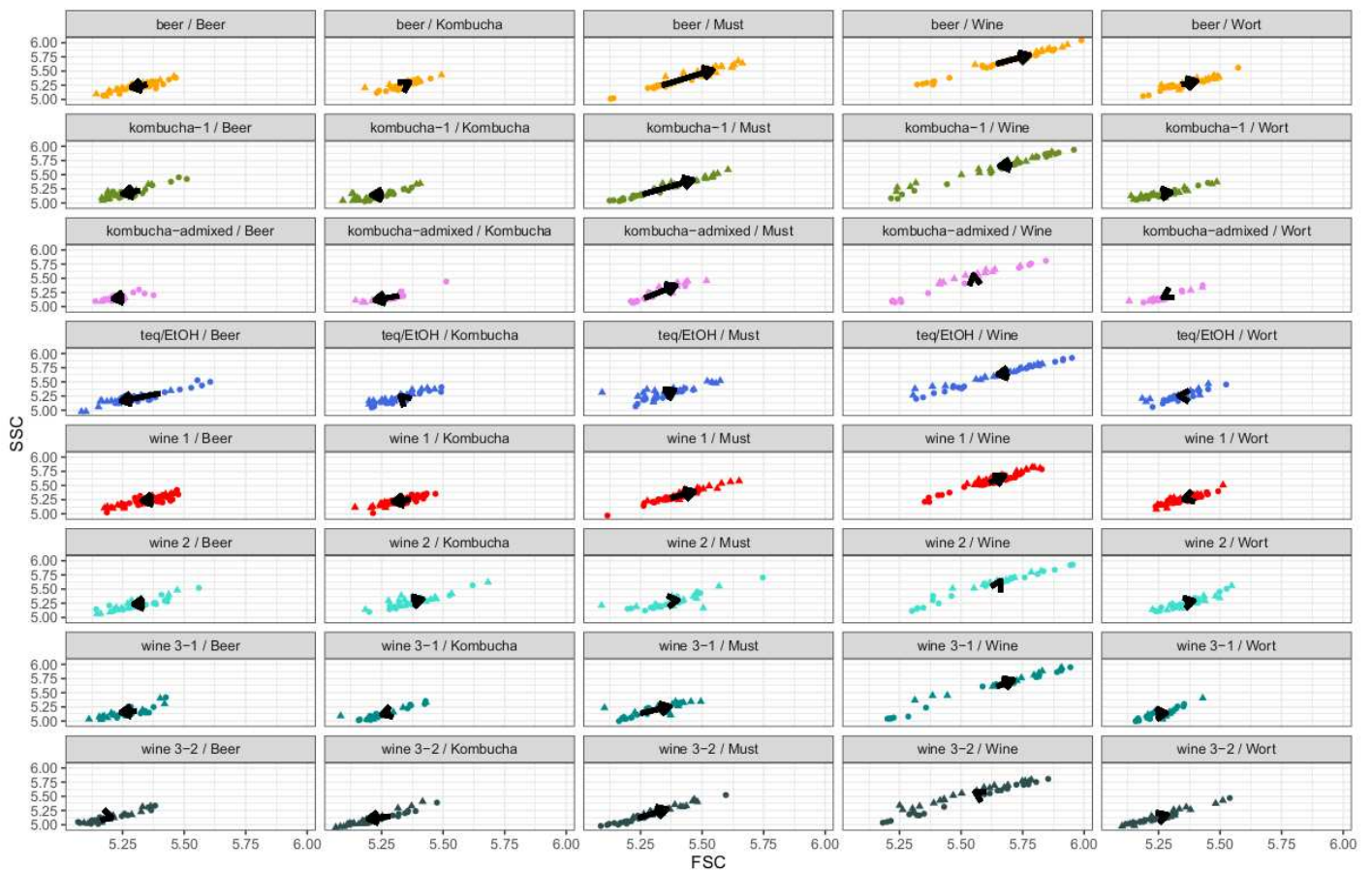


Figure 8: Visualisation of the eight populations FSC and SSC variations between acclimation step and experiment. SSC parameter (expressed in  $\log_{10}$ ) is expressed in function of the FSC parameter (expressed in  $\log_{10}$ ). Round dots represent the strains before the experiment and the triangle dots the strains after experiment. The black arrows are the vector sums of each population in the specific environment.

### e) Intra-population variability

The homogeneity (e.g. the ability of the strains of a same population to express the same phenotype in a given environment) was investigated within populations for the five environments. Variances between strains of the same population were measured for the five environments (model 4). The variances in the different media were estimated using Fligner-Killeen test (Figure 9). Strong difference of homogeneity was observed between populations. As observed in Figure 8, biochemical data were quite similar between populations. The *beer* group showed a good homogeneity (in blue) and low variability (in red) regardless of the environment considered. The *kombucha-admixed* population exhibited high degree of homogeneity in wort and kombucha, also in beer and must with less extend, and low variability in all environments. The *wine 3-1* showed low variability in

wort. The *wine 1* group showed no variability in wine, which was not the case in the other environments. The *teq/EtOH* and *wine 3-2* exhibited high degrees of strains variability. The homogeneity within the different populations were investigated. Results were variable between the populations, indicating specific strain environmental response for some populations, and that phenotypic response in specific environments were conserved for other populations. Adaptive evolution can reduce the phenotypical variance of a population, through directional or stabilising selection (Grenier, Barre, Litrico 2016). Most results were in accordance with local adaption signatures (e.g. reduced intra-variability for the media where populations showed local adaptive signature) which supports common selective pressures within specific populations.

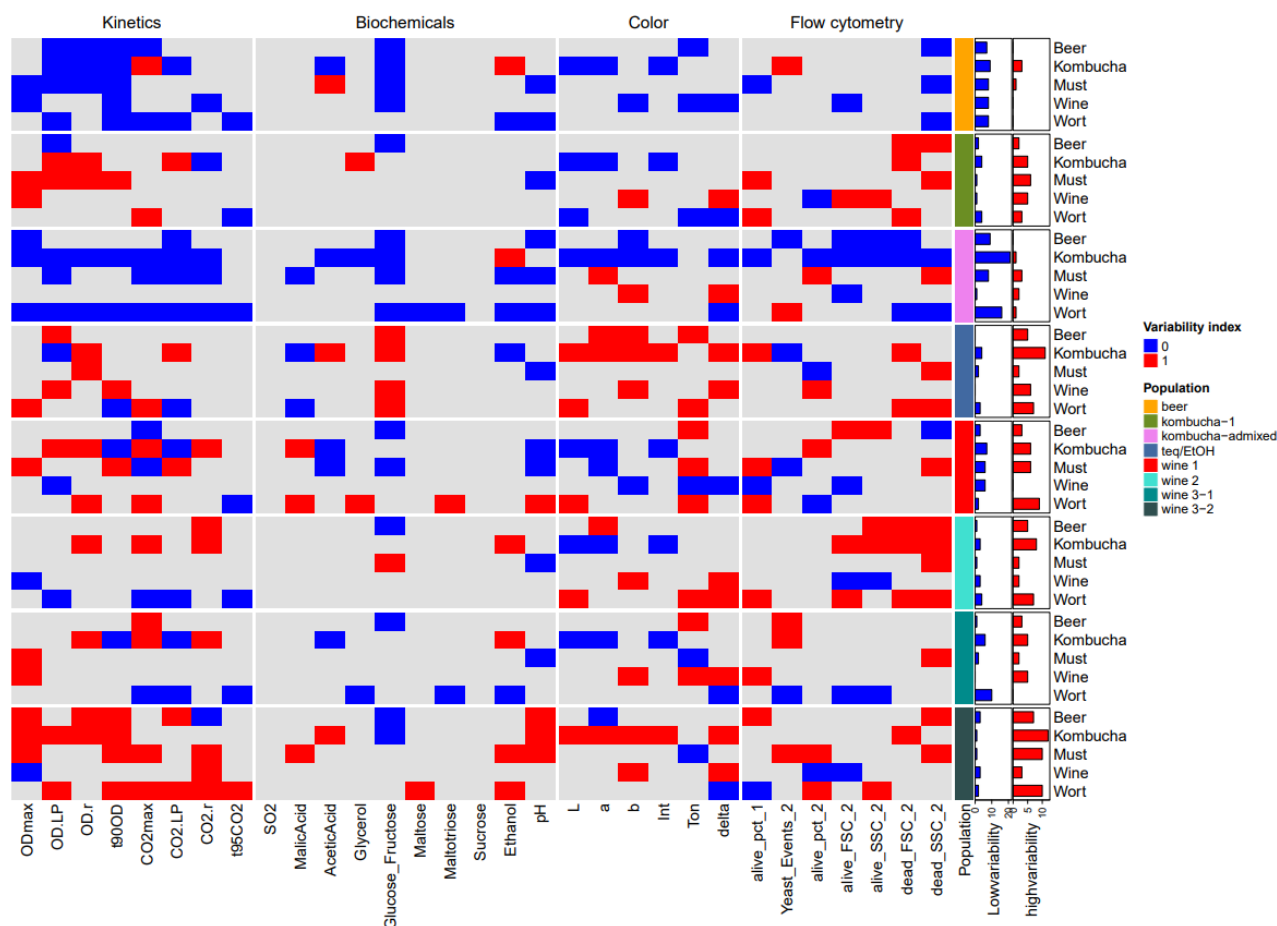


Figure 9 : Heatmap representation of the variability within the eight populations in the five environments. Variances of each population of the 31 parameters were compared (Fligner-Killeen test) independently in the five environments and only the less variable groups ("0") and the more variable groups ("1") were colored. Plots on the right summarize the number of variable or non-variable parameters for the population in the five environments.

## f) Fermentation kinetics

Fermentation kinetics were followed for all the strains, in all the environments. *S. cerevisiae* was used as an outgroup.

In must, almost all strains were able to ferment glucose and fructose, resulting in CO<sub>2</sub> release around 90 to 100 g/L (congruent with fermentable sugars measured in must 240g/L) (). Using K-means clustering approaches, FA kinetics were clustered in four main profiles (Figure 10). The first profile encompassed kinetics with low fermentation speed (122 kinetics); the second profile (208 kinetics) and the third profile (123 kinetics) encompassed intermediate kinetics speed. The last profile (15 kinetics) comprised kinetics with higher fermentation speed. The distributions of the four profiles in the eight populations were compared (Chi<sub>2</sub> test) and varied significantly. *S. cerevisiae* kinetics all belonged to the last profile, being able to complete the fermentation in 20 days. The *beer* group strains

mainly belong to the third profile, harbouring fast fermentation behaviour compared to other *B. bruxellensis* populations. The *kombucha-admixed*, *teq/EtOH* and *wine 2* showed kinetics belonging to the second profile, and thus had correct fermentation capability. The *wine 1* strains had the second and first profile and the *wine 3-2* strains had the first profile, showing slower fermentation ability in must compared to the rest of the strains. The *kombucha-1* and *wine 3-1* fermentation abilities were not statistically different from all strains.

In wort medium, some strains were not able to achieve the fermentation (around 40 g/L of CO<sub>2</sub>). Interestingly, for some strains, a clear pause in the fermentation kinetics was observed (around 18 g/L of CO<sub>2</sub> released). The kinetics were sorted into four profile types with K-means clustering (Figure 10). In the first profile (107 kinetics), kinetics harboured an extended lag-phase, slow fermentation rate between 0 to 18 g/L of CO<sub>2</sub> released, and some kinetics were able to reach 40 g/L. The second profile exhibit



kinetics (99) with a shorter lag-phase and improved fermentation rate between 0 to 18 g/L. However, kinetics showed an important delay around 18 g/L of CO<sub>2</sub>, with some kinetics not being able to reach 40 g/L of CO<sub>2</sub> released. The third profile (163 kinetics) encompassed kinetics with a short lag-phase, a little delay at 18 g/L of CO<sub>2</sub> and succeeded to reach 40 g/L of CO<sub>2</sub> released in around 30 days. Finally, the last profile (104 kinetics) had kinetics with a short lag-phase, without remarkable delay at 18 g/L, and kinetics reach 40 g/L of CO<sub>2</sub> within 30 days of fermentation. The distribution of the kinetics into the population was also analysed, within significant differences. *S. cerevisiae* strains belong to second profile, with strong fermentation rate but without being able to reach 40 g/L of CO<sub>2</sub> released. The *wine 3-1* population had a majority of profile four kinetics, as well as the *wine 2* population to a lower extent. The *beer* and *kombucha-admixed* group showed kinetics belonging mainly to the third profile with a good fermentation. The *teq/EtOH* group showed two types of profile, the second and the fourth, as well as the *wine 1* with first and third profile types. The *kombucha-1* and the *wine 3-2* exhibited mainly the first and second profiles.

Wort carbon sources are composed mainly of glucose and fructose (simple sugars), maltose and maltotriose (complex sugars). Yeast generally ferments simple sugar (easier to use) and then metabolize the most complex ones due to glucose catabolism repression system (GCR) (Kayikci, Nielsen 2015). GCR mechanism has been already observed in *B. bruxellensis* with individual variations (Da Silva et al. 2019). In our case, the fermentation of simple sugars corresponded to an average of 18 g/L CO<sub>2</sub> released (data not shown). The pause in fermentation kinetics at 18 g/L must thus corresponds to the end of glucose and fructose consumption and a delay in metabolism reorganisation to metabolize more complex sugars (maltose, maltotriose...). This delay is dependent of the populations. The *beer*, *wine 3-1*, *kombucha-admixed* and *wine 2* showed a reduced delay, and thus responded quicker from glucose/fructose depletion and to reorganize their metabolism for maltose consumption.

In wine, the CO<sub>2</sub> released was below our detection threshold, and considered as null, explained by the very low amount of fermentable sugars (2 g/L). In beer, low weight losses were recorded with little

variation at the population level (<6 g/L of CO<sub>2</sub>) (Supplemental figure 2). In kombucha media, the *beer* population and *S. cerevisiae* showed higher fermentation ability compared to others based on k-means clustering (Supplemental figure 2). However, the released CO<sub>2</sub> was low for all strains (0.6-8.6 g/L of CO<sub>2</sub>max in kombucha, median of 2.7 g/L) compared to the level of fermentable sugar (35 g/L of CO<sub>2</sub> release expected for 80 g/L sugars), although correct growth abilities were measured. More precisely, *S. cerevisiae* and the *beer* population showed the highest CO<sub>2</sub> release (mean values 5.6 g/L and 4.8 g/L respectively). The *kombucha-admixed* and *kombucha-1* groups were the less able to ferment sucrose (<2.5 g/L). Little sucrose consumption of a monoculture of *B. bruxellensis* in kombucha has been highlighted recently (Ferremi Leali et al. 2022; Tran et al. 2022; 2020). In kombucha, the microbial composition is complex, with association of yeasts (mainly *S. cerevisiae* and *B. bruxellensis*) and bacteria (*Acetobacter* and *Gluconobacter*) (Coton et al. 2017; Martínez Leal et al. 2018; Bishop et al. 2022). The low fermentation activity observed highlight the necessary microbial interaction between bacteria and yeast, to synergistically produce kombucha (Leali et al. 2022).

The fermentation capability of *B. bruxellensis* in terms of CO<sub>2</sub> release was congruent with the level of sugars present, except in kombucha. However, the rate of the fermentation was highly slower compared to that of *S. cerevisiae* in grape must and in wort environment. The two species have developed a "Make-Accumulate-Consume" life strategy independently, with parallel evolution such as promotor rewiring and the loss of specific regulatory element of respiratory genes (Guo et al. 2016; Rozpędowska et al. 2011). However, like for growth rate, fermentation rate of *B. bruxellensis* species was weaker than *S. cerevisiae* species. This could be explained by the fact that *S. cerevisiae* has been selected and domesticated for its alcoholic fermentation capability (Steensels et al. 2019). Moreover, this could also be due to the difficulty for *B. bruxellensis* to maintain the redox balance with acetic production in restrictive aerobic condition and the non-production of glycerol (Teles et al. 2018; Smith, Divol 2018), or by an adaptation to low nutrients environments (Smith, Divol 2018; 2016).

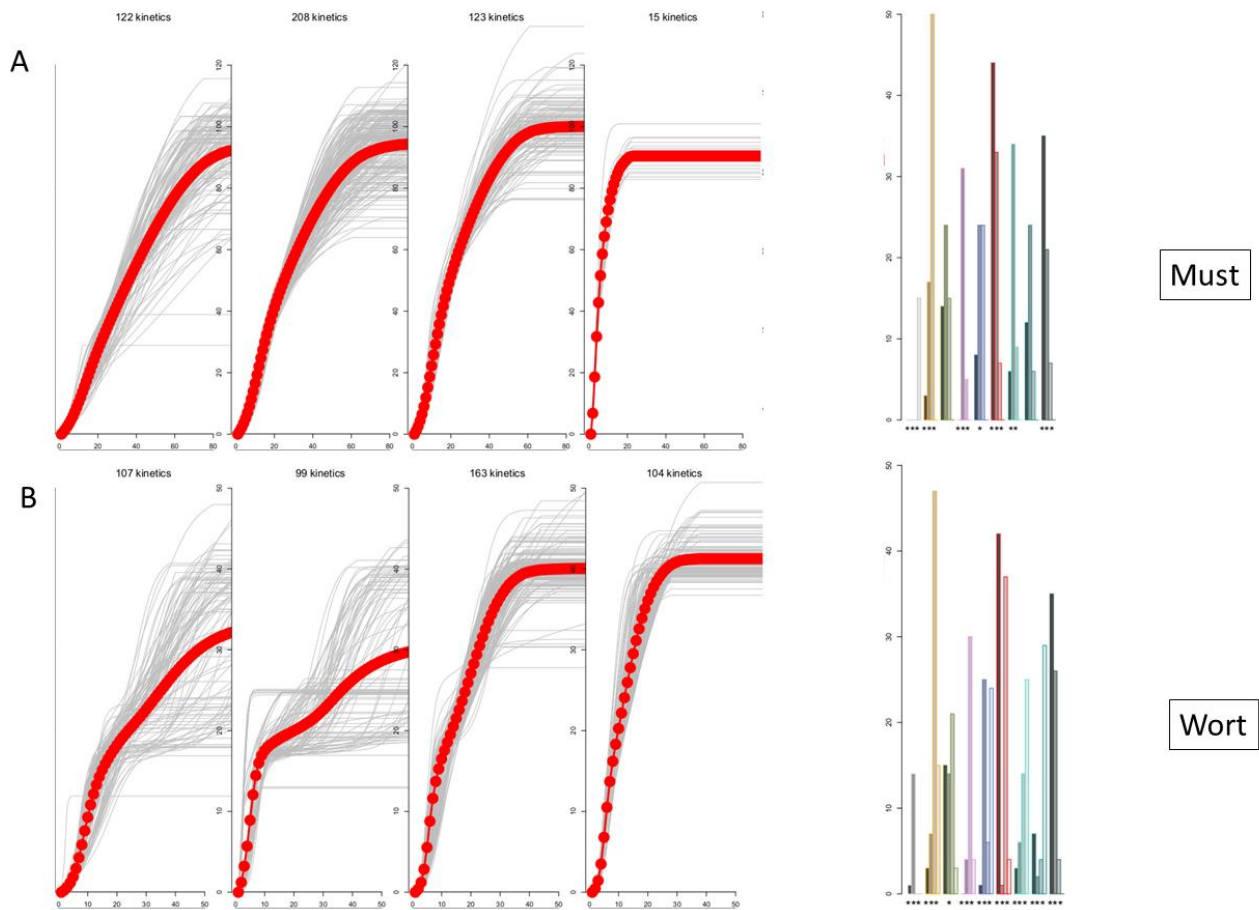
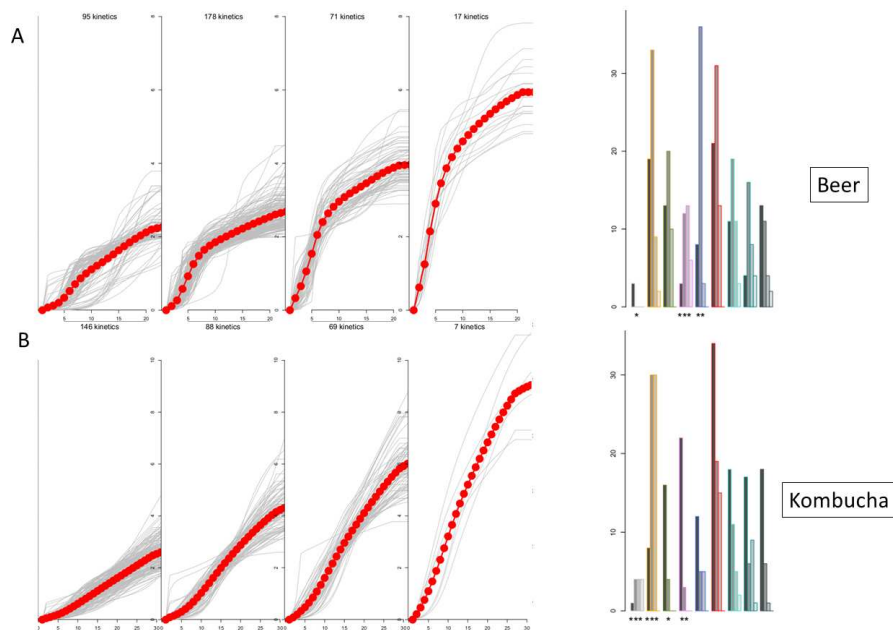


Figure 10: K-means clustering results for the must and wort fermentation (with four clusters) respectively. The  $CO_2$  released (g/L) is expressed in function of the time (day). Red lines and points represent the mean kinetic, grey line are the experimental kinetics measured. Profile are organised from the left to the right (profile 1, profile 2...). On the left, the distribution of the kinetics in the eight populations is showed. Stars on the bottom are depending of the significate differences of the distribution to one group to the distribution of the all population (conformity Chi2 test). One star stands for  $p$ -value $<0.05$ , two stars stand for  $p$ -value $<0.01$ , and three stars stand for  $p$ -value $<0.001$ .



Supplemental figure 2 : K-means clustering results for the kombucha and beer fermentation (with four clusters) respectively. The  $CO_2$  released (g/L) is expressed in function of the time (day). Red lines and points represent the mean kinetic, grey lines are the experimental kinetics measured. On the left, the distribution of the kinetics in the eight population is showed. Stars on the bottom are depending of the significate differences of the distribution to one group to the distribution of the all population (conformity Chi2 test). One star stands for  $p$ -value $<0.05$ , two stars stand for  $p$ -value $<0.01$ , and three stars stand for  $p$ -value $<0.001$ .

However, most of the strains were able to release more CO<sub>2</sub> in wort and beer compared to *S. cerevisiae* (around 5 g/L more in wort), highlighting the  $\alpha$ -glucosidase activity of this species (Serra Colomer et al. 2020).

#### **g) Variability of the life history traits**

Several flow cytometry measures were made during the experiment. It allowed the measurements of various life-history traits, such as cell size (using the FSC measurement as proxy), the granularity (SSC proxy), cells concentration, percentage of viable cells and the size and granularity of the viable and dead cells. Cell size is known to be impacted by the ploidy level (Sugimoto-Shirasu, Roberts 2003). In our case, different ploidy levels were evidenced in *B. bruxellensis* species in relation to the population groups, ranging from 2n to 3n (Eberlein et al. 2021). We investigated if cell size of viable cells in the five environments is related to ploidy, and could help to identify polyploids strains of *B. bruxellensis* for further analysis (Figure 11A). The distribution of the cell size of the diploid and triploid individuals were different, but highly over-lapping in the five media, and particularly in wine environment. A variance analysis was performed (ANOVA 5); the environment factor explained 54% of the total variance and the ploidy level less than 3% of the total variance (similar results were obtained for cell granularity- SSC parameter, data not show). The distribution of the size was investigated in the different populations (Figure 11B). Differences were evidenced again, but

with strong over-lapping between populations. Once again, strong variations were observed between environments (54% of the variance) and between population to a lesser extend (5% of the variance). Using linear model, we tested whether the ploidy level and/or the different populations could be predicted using the different flow cytometry parameters. The correct assignation of the ploidy level and populations was computed (Supplemental Table 1). The percentage of correct assignations varied between parameters, with higher assignation scores observed for FSC and SSC of alive cells (ranging from 64% to 77% of correct assignations). Variations were also found depending on the media, kombucha medium had the higher assignation score (77%). However, the levels of correct assignations were globally too low to envision a pertinent prediction of the ploidy and populations based on flow cytometry parameters. In this work, several flow cytometry data were collected for the 151 strains over five environments. Flow cytometry parameters (such as FSC and SSC of the total yeast population, viable cells and dead cells) were highly dependent of the environment and strongly over-lapped from one population to another. This over-lapping of morphological parameters was already observed on previous study (Lebleux et al. 2021). With our results, we show that without any specific flow cytometry dyes, the determination of the populations, as well as the ploidy level, with only flow cytometry is highly uncertain and environment dependent as also shown by Longin et al. (2017).

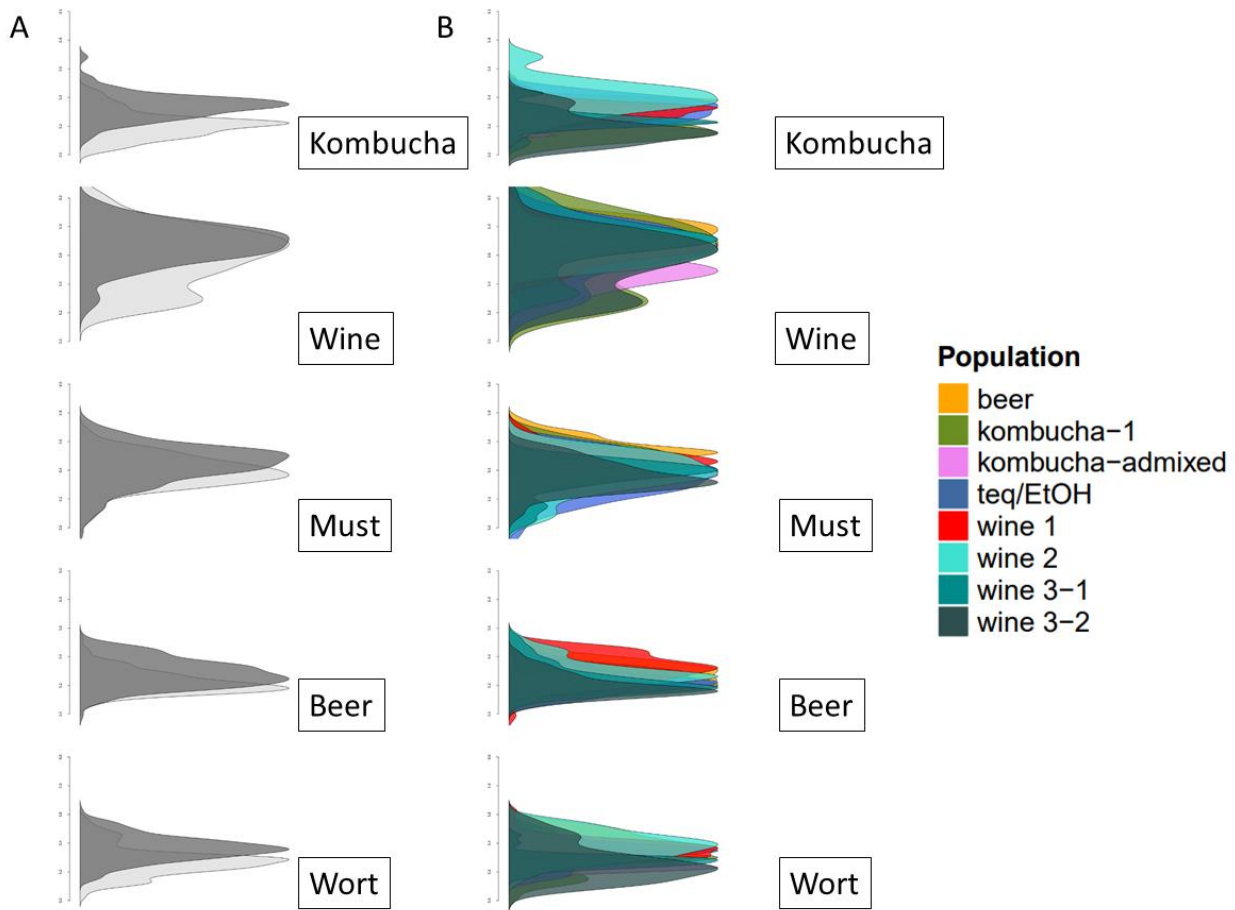


Figure 11 : Distribution of the FSC parameter (expressed in log10, cell size proxy) in kombucha, wine, must, beer and wort for viable cells at the end of the experiment. A: The two groups represent the diploid (in light grey) and triploid (in dark grey) groups. B: The distribution of the different populations is shown (same color as before).

Supplemental Table 1 : Percentage of correct assignments using linear model for ploidy and populations in the five environments on the seven flow cytometry parameters.

Ploidy determination	Environments				
	Kombucha	Wine	Must	Beer	Wort
alive_FSC_2	75,60%	63,01%	59,04%	61,29%	73,11%
alive_SSC_2	76,91%	61,72%	60,57%	59,35%	72,22%
dead_FSC_2	69,72%	51,18%	67,76%	53,98%	59,11%
dead_SSC_2	64,27%	60,22%	68,19%	63,01%	60,89%

Population determination	Environments				
	Kombucha	Wine	Must	Beer	Wort
alive_FSC_2	33,12%	22,80%	23,53%	22,80%	26,44%
alive_SSC_2	36,82%	19,14%	23,53%	20,65%	22,67%
dead_FSC_2	29,63%	15,91%	24,40%	15,27%	20,00%
dead_SSC_2	34,42%	17,63%	23,97%	22,80%	24,89%

## h) *B. bruxellensis* metabolism variation

Specific parameters were investigated in more details in some environments through the different populations. In all populations, some strains were able to consume maltose and maltotriose in wort and beer media (Figure 12A, Supplemental figure 3, Kruskal-Wallis test). However, some differences were evidenced within populations, the *wine 3-2* was the less able to fully consume them. The *kombucha-admixed* and the *wine 3-1* and *beer* groups showed the higher affinity for these two sugars types. Moreover, the *kombucha-admixed* and the *wine 3-1* were highly homogenous compared to other groups. These results were correlated to the fermentation behaviours observed (quantities of released CO<sub>2</sub>). Variability in maltose assimilation have been already observed, and beer strains showed a better maltose utilization compared to wine isolates (Serra Colomer et al. 2020). Besides some variabilities observed, maltose and maltotriose consumption appeared to be a common trait within *B. bruxellensis* strains, which seemed to be under different selective pressures. Adaptation to anthropic environments can result in relaxed selection on traits that are not necessary to the new environment, a phenomenon called “degeneration”. For example, relaxed selection has played a major role in animal domestication (Larson, Fuller 2014). For example, domesticated pigs have accumulated several nonsynonymous mutation in the *MRC1* gene responsible of the coat color, which are not present in wild boar (Fang et al. 2009). For some populations (*wine 3-2*, *wine 1*, *kombucha-1*), consumption of  $\alpha$ -glucoside compounds seemingly under relaxation, in contrary to other groups (*kombucha-admixed*, *wine 3-1*), suggesting different patterns of selection.

In must environment, the malic acid concentration was lower compared to the control ones, indicating a probable consumption of this carbon source by *B. bruxellensis* strains (Figure 12B). However, variations were also observed within the populations. Most of the populations consumed around 0.5 to 0.8 g/L of malic acid in must, with the exception of the

*wine 1* and *wine 3-2*. The *wine 3-2* was able to consume between 0.5 to 2 g/L of malic acid, and the *wine 1* strains consumed less than 0.5 or even produced 0.5 g/L of malic acid in must. In wort, weak production of malic acid was recorded, ranging from 0 to 0.2 g/L for the population with the exception of the *wine 1* which produced between 0.1 to 0.6 g/L in wort. This peculiar metabolic trait influenced the pH value, which was significantly lower for the *wine 1* strains compared to the other groups (Figure 12C). Utilization of malic acid has been already highlighted in *B. bruxellensis* species, however without no such difference between isolates and populations (Smith, Divol 2018; Vigentini et al. 2008). Malic acid production has only been reported once through the literature for one strain to our knowledge (Valdetara et al. 2020). This production was observed during the fermentation in semi-anaerobic condition of a synthetic matrix with low amount of malic acid (0.5 g/L). This malic acid production/consumption could be influenced by the malic acid medium concentration and seems also to be link to the oxygenation (Smith, Divol 2018), and the production could arise from a leak due to imbalance between inner cell and the medium during fermentation or due to a specific efflux pump. This ability to consume malic acid could help the establishment of *B. bruxellensis* in red wine between the alcoholic and malolactic fermentation, when sugar quantity is low and malic acid is not yet consumed by *Oenococcus oeni* bacteria (Renouf, Lonvaud-Funel, Coulon 2007; Berbegal et al. 2018; Dubois et al. 2020).

In wine environment, variation of SO<sub>2</sub> content was measured (Figure 12D). While most of the genetic groups had no or few SO<sub>2</sub> content at the end of the experiment, strains (mostly belonging to the *wine 1* group) showed little amount of SO<sub>2</sub> (from 0 to 20 mg/L). The *wine 1* population was shown to be tolerant to SO<sub>2</sub> addition (Avramova, Vallet-Courbin, et al. 2018). This intriguing feature could be due to SO<sub>2</sub> capture and release or a production during growth, and could be a track to follow in future studies to fully understand the sulphite tolerance mechanisms within the *B. bruxellensis* species.

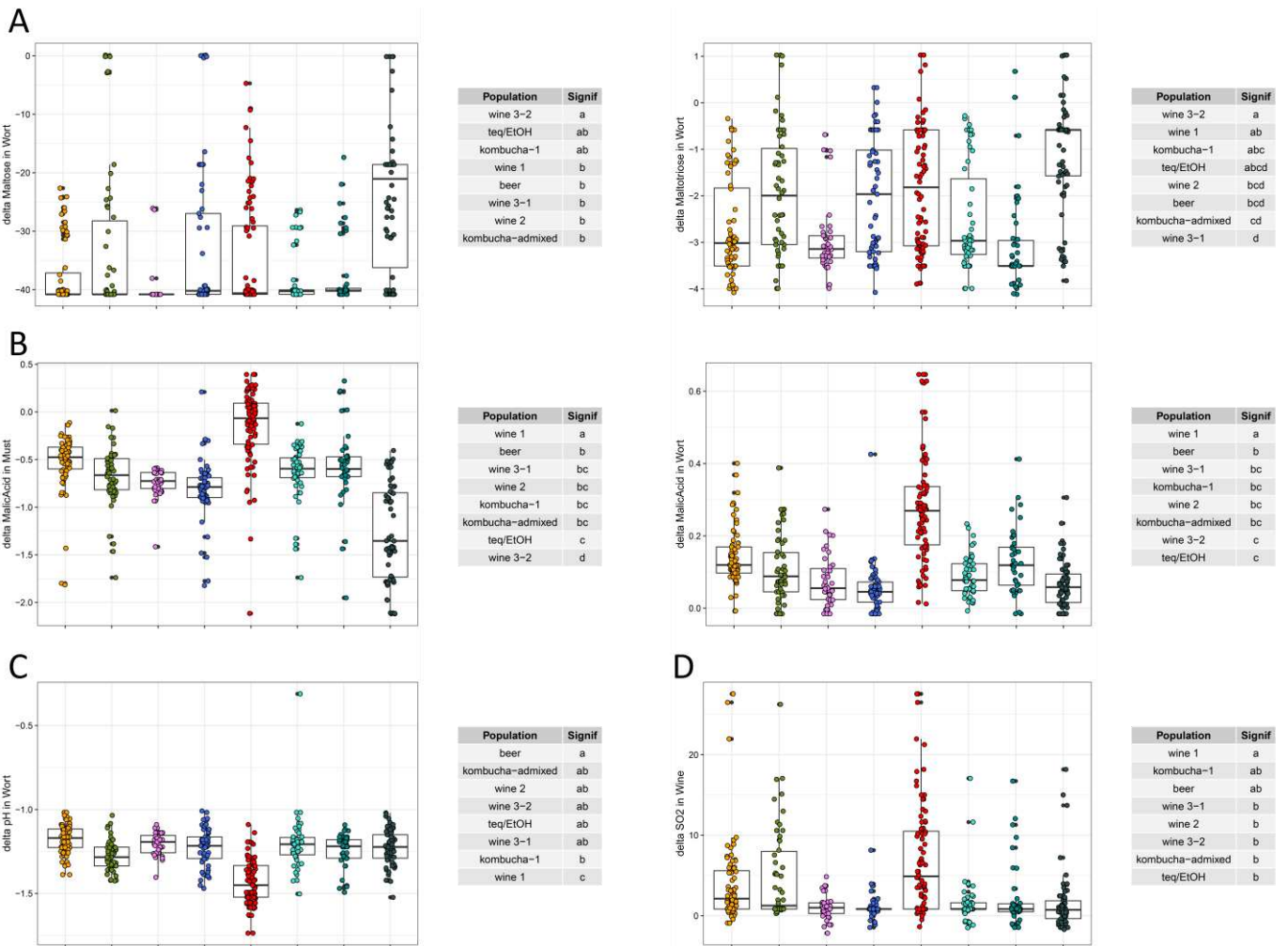
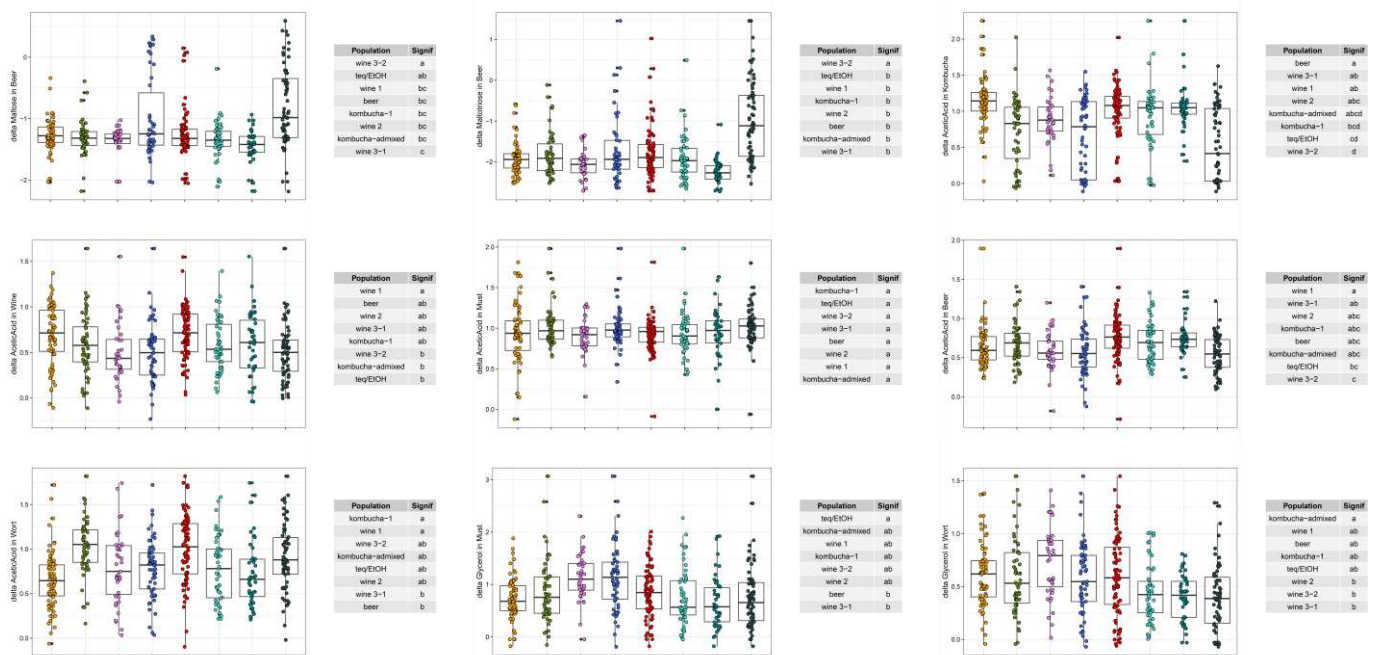


Figure 12 : Focus on specific traits in defined environments. Tables summarise Kruskal-Wallis test, letters indicate significant differences between populations ( $p$ -value $<0.05$ ). A: Maltose and maltotriose concentration differences between samples and control samples in wort medium, depending on the populations, expressed in g/L. A dot corresponds to one biological experiment. B: Malic acid concentration differences between samples and control samples in must and wort media, depending on the populations, expressed in g/L. A dot corresponds to one biological experiment. C: pH differences between samples and control samples in wort medium, depending on the populations. A dot corresponds to one biological experiment. D:  $SO_2$  concentration differences between samples and control samples in wine medium, depending on the populations, expressed in mg/L. A dot corresponds to one biological experiment.

Others phenotypic traits have been showed to be shared by the species, with low variation at the population and strain levels. *B. bruxellensis* strains produced acetic acid (between 0.5 to 1.5 g/L) (Supplemental figure 3) in all media. Acetic acid production was correlated to the growth, which was congruent with the literature (Reis et al. 2014).

Glycerol production was also underlined in wort and must (ranging from 0.5;g to 1.2;g/L) (Supplemental figure 3). This production during fermentation was lower than *S. cerevisiae* production in accordance with previous studies and could explain the lower fermentation ability of this species (Reis et al. 2014; Leite et al. 2012).



Supplemental figure 3 : Focus on specific traits in defined environments. Tables summarise Kruskal-Wallis test, letters indicate significant differences between populations ( $p$ -value<0.05).

### i) *B. bruxellensis* affects wine color

A novel characteristic was assessed in this study, the impact of *B. bruxellensis* on the final beverage color, using CIELAB analysis. Impacts of *B. bruxellensis* were measured in all five media and variations between the different populations were evidenced (data not shown). We decided to focus on these variations of color intensity in wine medium (Figure 13). Positive values of  $a^*$  were recorded (indicating a variation in the direction of redness), as well as negative values of  $b^*$  (indicating a variation in the direction of blueness) (Wrolstad, Durst, Lee 2005). This might indicate that *B. bruxellensis* affected the pigments present in wine (flavonoids, mainly anthocyanins) (Chris Somers, Evans 1979; Zoecklein et al. 1990). Multi-regression analysis was conducted to explore the impact of OD.max, Acetic acid and the strain and population level (model 6) (pH was also tested without significant impact). Besides the strong impact of the strains, the growth ability, the population and the acetic acid production were found significant (Supplemental Table 2). The  $a^*$ ,  $b^*$ , Ton and delta parameters were the most affected by the population factor. This reveals that the wine color was significantly impacted by the populations. In wine, anthocyanins are glycosides arising from the skin of the berries and are the main compounds responsible for the color of red wines (reaching 500mg/L) (Burns

et al. 2002; Ruta, Farcasanu 2019). Anthocyanins can interact with hydroxycinnamic acids (e.g., caffeic, coumaric, ferulic) or vinyl-phenols, resulting in an increased bluish red color (De Freitas, Mateus 2011; Ruta, Farcasanu 2019), which is in accordance with our observations (increased  $a^*$  and decreased  $b^*$  values). This could imply that hydroxycinnamic acids and vinyl-phenols released content and/or ratio were different between the populations and affected by the growth ability. Strong variations of volatile phenols production have been widely evidenced in the literature (Agnolucci et al. 2009; Conterno et al. 2006; Romano et al. 2017; Zepeda-Mendoza et al. 2018); however, never in view of the recent genetic diversity. Moreover, anthocyanins are phenolic molecules composed of a flavylum ion glycosylated with a  $\beta$ -glycosidic bond. Breakage of this glycosidic bond liberates the corresponding anthocyanidin, which is spontaneously converted to colorless molecules. Strong microbial  $\beta$ -glucosidase activity has been shown to decrease the color of wine (Kähkönen, Heinonen 2003; Fleischhut et al. 2006; Suárez et al. 2007; Sánchez-Torres, González-Candelas, Ramón 1998). Once again,  $\beta$ -glucosidase activity has been shown to be strain dependent (Mansfield, Zoecklein, Whiton 2002; Crauwels et al. 2017; Daenen et al. 2007; Vervoort et al. 2016), but has never been investigated in the light of the recent genomic data. Another hypothesis concerning this color

modification can be a precipitation or an absorbance by the cell wall (Ruta, Farcasanu 2019; Morata et al. 2003). This is congruent with the interaction with the growth ability, and the differences between populations could come from the difference of the cell wall composition, which was shown to be

different between populations in preliminary study (Dimopoulou et al. 2019). The fact that the populations affect differently the color, must indicate that the different populations interact specifically with the wine pigmented molecules.

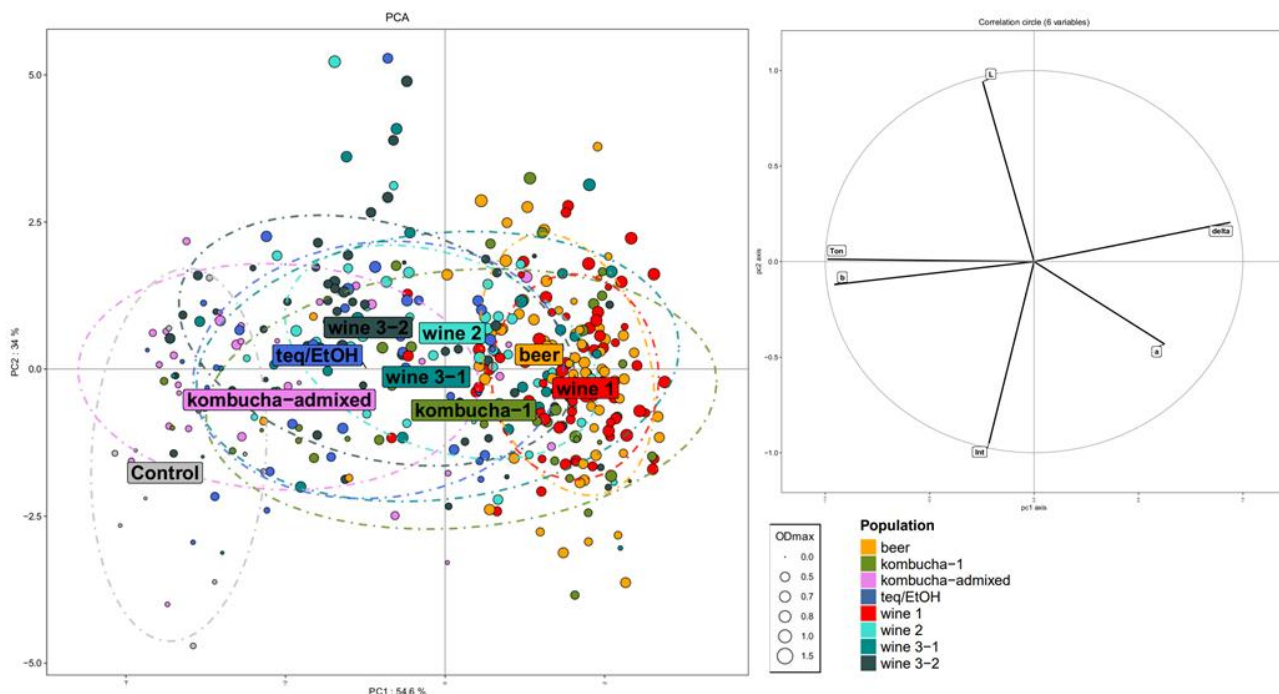


Figure 13 : Principal component analysis (PCA) on the wine color parameters and the correlation circle. Point are colored following the population and the size of the point is correlated with the ODmax measured.

Supplemental Table 2: ANOVA analysis on regression on the color parameters in wine (model 6). Numbers are the proportion of explained variances. Stars stand for the level of significativity (\*=p-value<0.05, \*\*=p-value<0.01, \*\*\*=p-value<0.001).

Factors and variables	Color parameters					
	L	a	b	Int	Ton	delta
<b>ODmax</b>	3,3% **	2,5% ***	25,5% ***	18,3% ***	24,9% ***	25,3%***
<b>Pop.</b>	9,4% *	23,1% ***	21,1% ***	3,90%	25,5% ***	17,9% ***
<b>Acetic acid</b>	0,20%	0,30%	4,9% ***	0,40%	3,9% ***	4,3% ***
<b>Strains</b>	39%	38,8% *	30,5% **	33,50%	39,9% ***	33,1% ***
<b>ODmax\$Pop.</b>	1,40%	2,2% *	1,4% **	1,30%	2,5% ***	1,3% *
<b>ODmax*Acetic acid</b>	2,4% *	0,8% **	0,10%	2,10%	0,3% **	0
<b>Acetic acid*Pop.</b>	1,90%	3,00% **	0,9% *	1,40%	0,6% *	0,80%
<b>ODmax*Strains</b>	23%	14,60%	0,97%*	22,20%	7,1% *	10,70%
<b>Acetic acid*Strains</b>	12,90%	13,10%	5,10%	13%	4,70%	5,70%
<b>ODmax*Acetic acid*Pop.</b>	1,10%	0,40%	0	1%	0	0,10%
<b>Residuals</b>	3,90%	1%	0,40%	2,80%	0,30%	0,50%



## j) Volatolomic data

On a small subset of strains (16), the volatile fingerprint of *B. bruxellensis* in wine, beer, must and wort was assessed using a comprehensive two-dimensional gas chromatography (GCxGC) coupled with time-of-flight mass spectrometry (TOF-MS). We were able to quantify more than 10,000 volatiles compounds over the four media (volatolome), which allowed a good separation of the four media in a PCA (Figure 14A). Strong volatile compounds differences from the control samples were observed in must and wort media, indicating that fermentations had strongly affected the volatile molecule content, compared to wine and beer environments. Out of the 10,616 molecules quantified, 4,988 were identified and 411 compounds were found to be significantly more or less abundant in most *B. bruxellensis* strains. 4-Ethyl phenol and 4-ethyl guaiacol were identified and quantified in the four environments (Supplemental figure 4). All the tested strains were able to produce these two compounds in the four environments. Globally, in brewery environments (wort and beer), the abundance of 4-ethyl guaiacol was higher than the abundance of 4-ethyl phenol, which is in accordance with the literature (Vanbeneden et al. 2008; Lentz 2018). Little inter population variations was observed except in wine, where the production was dependent on the growth ability (ANOVA analysis, result not shown). These observations highlighted the ability of *B. bruxellensis* to produce volatile phenols, in oenological conditions as well as in brewery environment.

Then, the 411 differential compounds were clustered in chemical families and their abundance fold change was compared (Figure 14B). Several chemical families were significantly impacted compared to the control samples, such as acid, acetate ester, ester and phenol classes, which were more abundant. The increased abundance of the acid family could be attributed, at least in part, to the strong production of acetic acid of the species (Freer 2002). Ester (and acetate ester) family is known to be produced by yeast species during grow and fermentation and is an important group of impact aroma compounds in fermented beverages (Saerens et al. 2010; Mason, Dufour 2000; Peddie 1990). It has also been hypothesized that ester synthesis tunes intracellular redox balance and maintains membrane fluidity (Mason, Dufour 2000).

Phenols compounds were also significantly more abundant. The strong production of 4-ethyl guaiacol and phenol can explain a part of this enrichment. Phenolic compounds can have microbiological activities in sufficient quantities (Dzialo et al. 2017). Carbaldehyde family was found to be less abundant. Carbaldehydes can be formed by oxidation of phenol molecules (Alnaizy, Akgerman 2000). These variations of phenols and carbaldehydes with or without *B. bruxellensis* presence can be attributed at least in part to the oxygen consumption of the strains, limiting the reduction of phenolic compounds into carbaldehydes.

The enrichment of metabolic pathways was tested (with the KEGG metabolic database), and several pathways were identified as overrepresented, such as monoterpenoid biosynthesis or sulphur metabolism (Figure 14C). As an example, the concentration of geraniol (an odorant monoterpene) in wine was investigated (Supplemental figure 4). Its concentration was correlated with the maximal optical density (OD<sub>max</sub>, Spearman correlation ~0.75, model 7 and ANOVA analysis 59.9% of the variance), also impacted by the populations (17.4% of the variance) and the interaction between the two (13.0% of the variance). While not directly synthesized by yeasts, monoterpenes are sometimes released from odourless precursors by yeast-derived enzymes and can present antimicrobial activity (Marmulla, Harder 2014). This production of geraniol was also observed in must medium and may indicate that precursors originate from the grape berries. More than four hundred identified compounds have been affected by *B. bruxellensis*. The production of aromatic volatile molecules, such as ester, acids by yeast, plays a central role in their lifestyle, attracting animal vectors (particularly the vinegar flies *Drosophila melanogaster*), inhibiting microbial growth or contributing to cell-cell communication (Becher et al. 2018, for review: Dzialo et al. 2017). *S. cerevisiae* domestication has influenced the volatile fingerprint of the industrial strains, increasing their ability to produce desired aromas and reducing the unwanted ones (Gallone et al. 2016; Gonçalves et al. 2016; Legras et al. 2018). It could be interesting to compare the volatile fingerprint of the different populations and investigated “if”, “how” and “which” anthropized adaptation has reshaped volatile productions of *B. bruxellensis*.

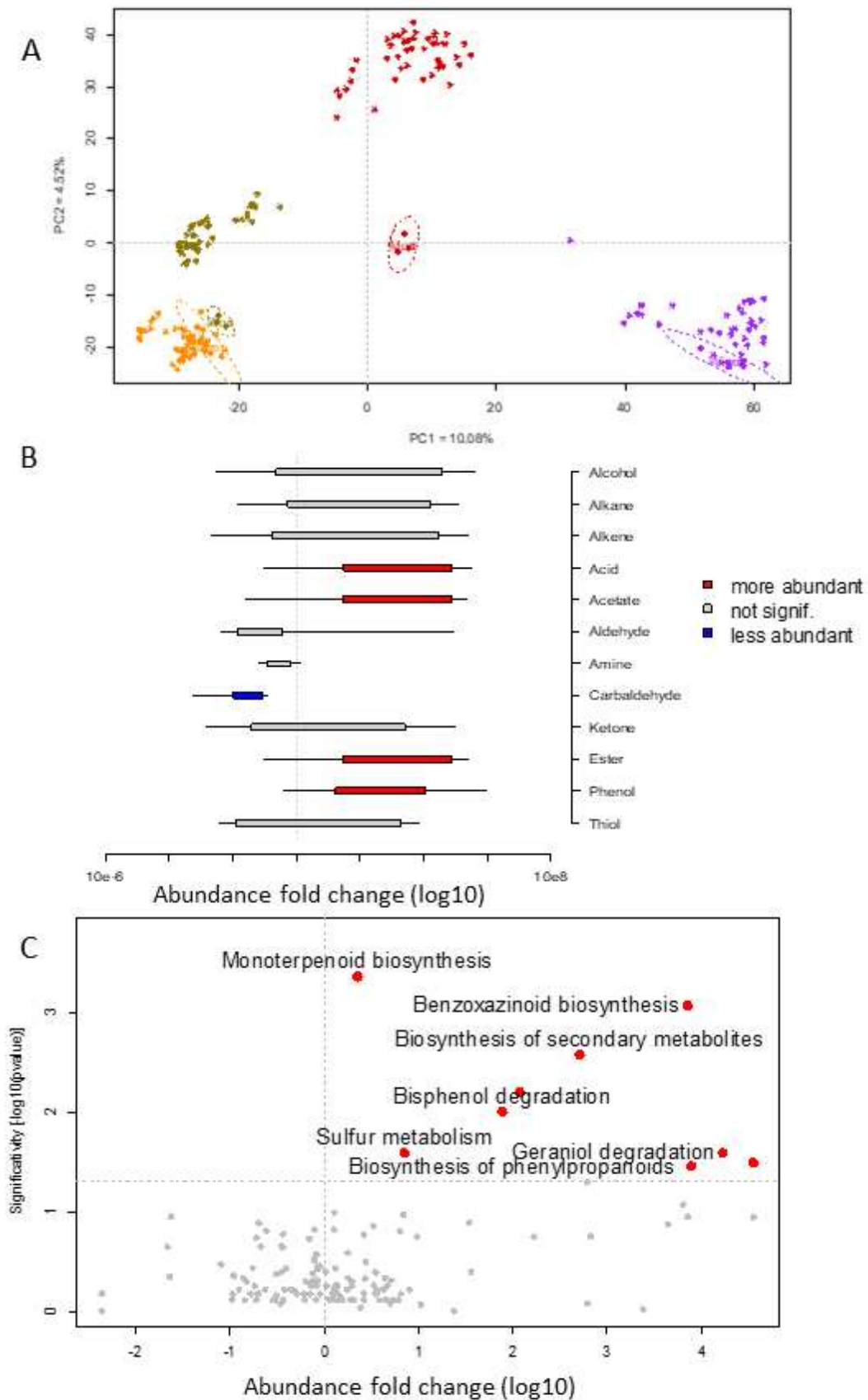
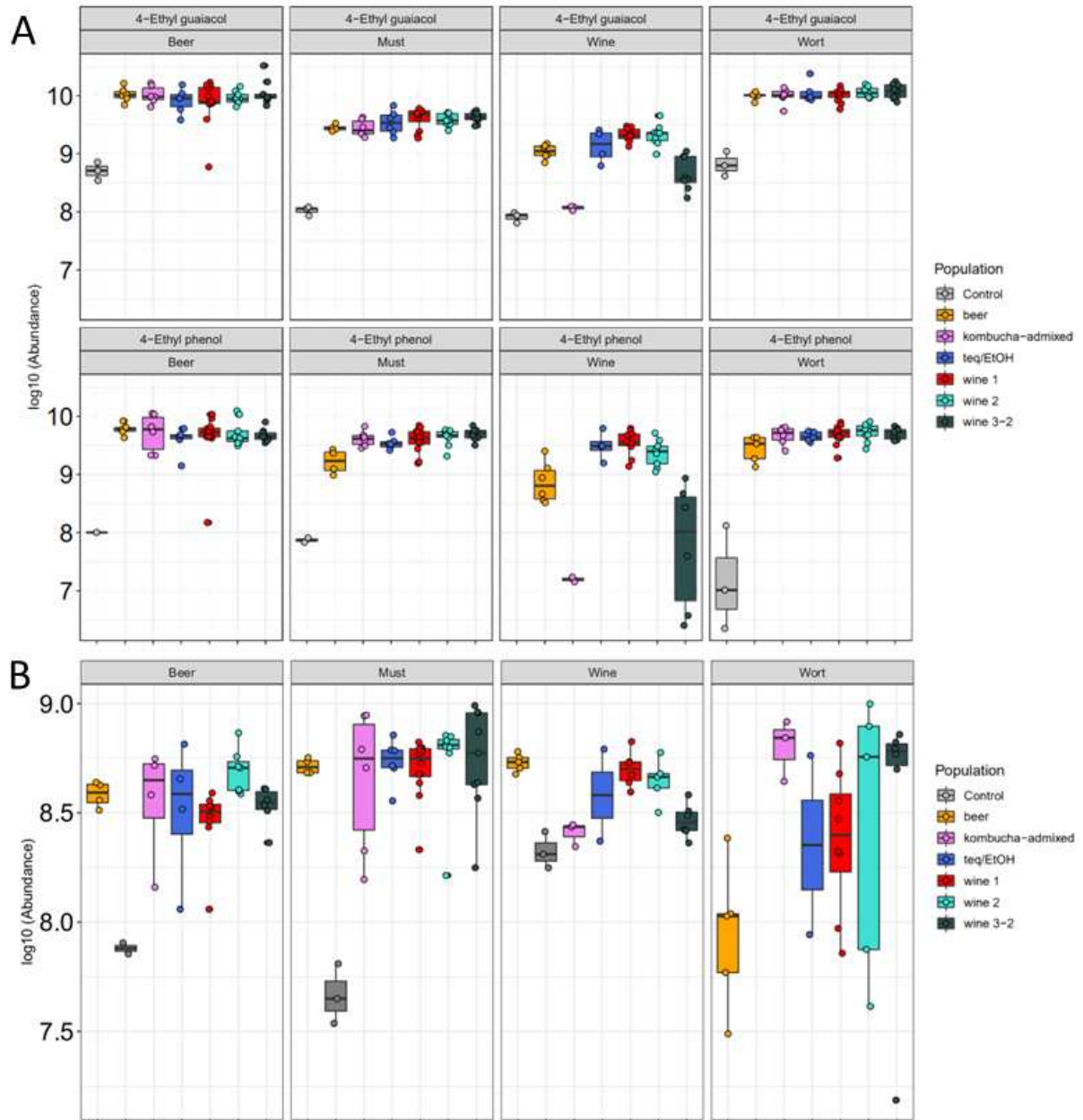


Figure 14 : A: Principal Component Analysis (PCA) on the 16 strains in wine, beer, must and wort media (stars). The corresponding controls (media without *B. bruxellensis* inoculation) were added (full dots) for the four environments. B: The fold abundance change compared to the control of the molecules identified as impacted by *B. bruxellensis* in the four media. Molecules are clustered by their family belonging. C: Enrichment of KEGG metabolic pathways.



Supplemental figure 4 : A Ethyl phenol and ethyl guaiacol abundance ( $\log_{10}$ ) in the four environments (beer, must, wine, wort) in the different populations with control samples (grey). B: Geraniol abundance ( $\log_{10}$ ) in wine and must environments for the different populations with control samples (grey).

These results are valuable data to study the volatile fingerprint of *B. bruxellensis* in distinct environments, but also to investigate volatile molecule production linked to the populations and require in-depth study.

## Conclusions

This study aimed to investigate the phenotypic variability of 151 strains representative of the genetic diversity recently highlighted (Eberlein et al. 2021), in five actual matrices. Numerous parameters have been measured, from growth and fermentation ability, to metabolic traits, colorimetric analysis and life-history traits with flow cytometry technics. Phenotyping is time consuming and high throughput analyses are particularly difficult to set-up using this type of media. Our results show that the phenotypic diversity of the yeast *B. bruxellensis* is highly environment- and strain-dependent but also associated to the genetic structure of the population. Indeed, the phenotypic variance of 31 parameters measured for five environments showed that the individual genotype and the population level account equally for 50% of the genetic (G) factor (4.64 and 4.92%, respectively) (Figure 4). Population factor affects also the genetic environment interaction (GxE), influencing 35% of the GxE variance. To our knowledge, it is the first time that such a large number of phenotypic traits are looked through the genetic diversity and that the population phenotypic impact is clearly assessed. Strong divergences were evidenced between populations, in terms of variability within the population, local adaptation signatures, growth and fermentation ability, and in some specific metabolic traits (Table 4).

### **Some *B. bruxellensis*' populations show signature of adaptation to anthropized environment**

Recent genomic and microsatellite studies were able to separate the *B. bruxellensis* diversity in 5 to 6 groups (Avramova, Cibrario, et al. 2018; Serra Colomer et al. 2020; Gounot et al. 2020; Eberlein et al. 2021). This phenotypic study went deeper into this genetic diversity, and eight populations with different phenotypic patterns were highlighted. The allotriploid *beer* population encompassed strains harbouring low phenotypic plasticity, robustness for the five environments, and local positive adaptation

signs for kombucha and beer environments. In addition, this group was competitive in all tested media and possessed good fermentation abilities. The allotriploid *wine 1* population exhibited traits which are highly marked in wine environment. Indeed, this group was the slowest growing group in all the environments, except in wine where it was the most competitive. Numerous local adaptation signs and reduced intra-variability were evidenced for this group, but only in wine. Interestingly, this population encompassed strains with sulphite resistance (Avramova, Vallet-Courbin, et al. 2018; Varela et al. 2019). This group was not isolated before 1960, but became the main group of wine isolates since 2000 (Cibrario et al. 2019; Curtin, Kennedy, Henschke 2012). Intriguingly, in wine environment, the amount of final sulphites was increased with the *wine 1* group compared to the other groups (Figure 12). This could be due to SO<sub>2</sub> production in wine environment, or a slow release of sulphite after capture and could cover additional sulphite resistance mechanisms than those associated with *SSU1* gene (Varela et al. 2019; Valdetara et al. 2020). *Wine 1* strains produced malic acid in wort and must, which resulted in lower final pH in wort. This malic production was only recorded in media in which the fermentation was clearly observed, and may be linked with fermentation process and can explain the slow fermentation rate of this group. This characteristic could be interesting for brewers, as *B. bruxellensis* is used to enhance acid notes (Serra Colomer, Funch, Forster 2019). Another particular population is the *kombucha-admixed*. This group showed strong adaptation signatures in kombucha, wort, must and beer environment with reduced intra-variability, maltose and maltotriose consumption capability, and a strong growth competitiveness in these environments. However, this group showed limited growth in wine environment. The *wine 3-1* population showed also an important capability to consume maltose and maltotriose in brewery environments associated with strong fermentation capability in wort.

### **Degeneration of maltose and maltotriose capability and wine adaptation**

In our results, all populations of *B. bruxellensis* were more or less able to consume complex wort sugars (maltose and maltotriose), and inability was evidenced at the strain level. Moreover, all strains

were able to grow in beer, which was not the case in wine medium. Maltose and maltotriose utilization were more specialized to a specific population in large phenotypic studies on *S. cerevisiae* (Gallone et al. 2016; Gonçalves et al. 2016). These observations suggest that the *B. bruxellensis* species as a whole is adapted to cereal environments, and that maltose and maltotriose assimilation is a species trait rather than a population specificity. Maltose and maltotriose assimilation ability is probably under relaxed selection in some anthropic environments such as grape must, wine or kombucha. Genetic investigations are required to uncover the maltose and maltotriose metabolism in *B. bruxellensis* (Serra Colomer et al. 2020). This hypothesis gives new insights to understand the ecology of *B. bruxellensis*. Indeed, this species has always been identified in human associated niches, and no “natural” or “wild” isolate has been described so far. Concerning wine environment, most of the strains were able to grow, with strong variations between populations (the *wine 1* and *beer* populations showing the best wine contamination ability). This ability to grow of all the populations compared to *S. cerevisiae* confirms that *B. bruxellensis* species is tailored for harsh environments with high ethanol content (Smith, Divol 2018; 2016). Therefore, these variations limited for some populations level could reflect divergent adaptive signatures to the oenology process such as SO<sub>2</sub> tolerance.

### **Phenotypic plasticity as a mechanism of anthropic adaptation in *B. bruxellensis*?**

Phenotypic plasticity was evidenced in the five media for *B. bruxellensis*. This ability to modulate the phenotype response to environmental changes is called “phenotypic plasticity” (David W. Pfennig 2021). Adaptation to environmental changes has been hypothesised to be promoted by phenotypic plasticity (“Plasticity-first”), because it allows the development of individuals upon which selection can act (Levis, Pfennig 2016; Sun et al. 2020; West-Eberhard 2005). *B. bruxellensis* species is regarded in the different environments principally as spoiler and its management aims to suppress/eradicate it. Plasticity may be a key source of evolutionary innovation, allowing organisms to cope with the large magnitude and rapid rate of human-induced environmental changes (Diamond, Martin 2016). Phenotypic plasticity could be advantageous in very stressing environments and may explain its persistence ability. However, these questions have not yet been assessed to microbial species, and species comparison are needed to evaluate the phenotypic plasticity of this species.

Thus, *B. bruxellensis* could be an interesting species to dissect genetic adaptation and phenotypic plasticity in response to anthropic environment. All these clues allow making the hypothesis that the genetic drift is not the only responsible of the phenotypic divergence of the different populations, and that anthropized environments have shaped the *B. bruxellensis* diversity.

Table 4: Main results highlighted in this study.

Population	Ploidy	Local adaptation signs	local "maladaptation" signs	Strains phenotypic plasticity	Intra-variability	Comparison results (environment of success)	Fermentation behaviours	Metabolic traits
<b>beer</b>	3n (allotriploid)	kombucha, beer	none	low	reduced in all environments	kombucha, wine, must, beer, wort	high	correct assimilation of maltose and maltotriose
<b>kombucha-1</b>	2n	none	kombucha	high		wine		
<b>kombucha-admixed</b>	2n	kombucha, must, beer, wort	kombucha, wine	high	reduced in beer, kombucha and wort	kombucha, must, beer, wort		good assimilation of maltose and maltotriose
<b>teq/EtOH</b>	3n (allotriploid)	kombucha, wort	wine, must	high	Strong in all the environments	must, wort	high in must, contrasted in wort	
<b>wine 1</b>	3n (allotriploid)	wine	must and wort	low	reduced in wine	wine	low	malic acid production in must and wort, SO <sub>2</sub> presence in wine
<b>wine 2</b>	3n (autotriploid)	none	wine	low	Strong in all the environments	none	correct in must, high in wort	correct assimilation of maltose and maltotriose
<b>wine 3-1</b>	2n	beer, wort	none	low	reduced in wort	none	high in must, very high in wort	good assimilation of maltose and maltotriose
<b>wine 3-2</b>	2n	must	kombucha, wine, beer, wort	high	Strong in must, wort, beer and kombucha	none	low	difficulties for maltose and maltotriose consumption, strong malic acid consumption

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## Perspectives et poursuite des travaux

Ces travaux ont permis la collecte importante de données phénotypiques sur l'espèce *B. bruxellensis* au travers de cinq environnements. Des différences phénotypiques importantes ont été mises en évidence entre les populations génétiques de *B. bruxellensis* associées à des changements de valeur adaptative dans des environnements anthropisés. Cependant, l'analyse de ces données n'est pas encore achevée, et de plus amples investigations sont nécessaires (corrélations entre les variables par exemple). Plusieurs pistes seront plus précisément étudiées, comme la variabilité intra-groupe dans les différents milieux (méthodes de mesure, représentations graphiques). La comparaison entre milieux primaires (moût de raisin, wort) et milieux secondaires (vin, bière) pourrait aussi apporter des observations intéressantes concernant le mode de vie de *B. bruxellensis* en tant que premier ou second colonisateur d'un milieu. Enfin, l'analyse approfondie des données des empreintes volatiles des 16 souches dans les quatre milieux (vin, moût, bière, wort) sont nécessaires. Il s'agit d'un jeu de données important (de par sa taille) mais aussi de sa complexité (composés identifiés et non identifiés, nom des composés) et l'aide de collègues chimistes sera nécessaire. Ce jeu de données pourra aussi être utile pour identifier des changements dans la composition (qualitatif ou quantitatif) des molécules volatiles spécifiques au regard des différentes populations de *B. bruxellensis*.

Avec le séquençage des souches utilisées dans cette étude, il est aussi pertinent d'étudier le lien phénotype-génotype. Une première tentative a été réalisée par l'équipe GMGM de Strasbourg (Victor Loegler en thèse et Anne Friedrich) en utilisant une approche GWAS (Genome-Wide Association Studies) sur les différents phénotypes des 151 souches avec l'utilisation du nombre de copies de gènes (CNVs) et des variations nucléotidiques uniques (SNPs). De nombreux *locis* ont été identifiés ; cependant, le manque d'annotations fonctionnelles du génome de *B. bruxellensis*, la complexité des phénotypes étudiés, le faible nombre d'individus caractérisés et la population très structurée de l'espèce sont des freins importants dans l'association phénotype-génotype avec cette approche.

En complément de ces analyses, des expériences supplémentaires ont été commencées pour mieux comprendre la croissance de *B. bruxellensis* dans le vin rouge. Pour cela, des cinétiques de croissances de 30 souches avec des prélèvements en fonction du temps et/ou de la croissance (5 prélèvements en duplicatas biologiques répartis sur 45 jours) ont été réalisés dans un vin rouge. Les dosages de métabolites (acide acétique, éthanol, SO<sub>2</sub>, glucose, fructose, glycérol, malique), des analyses par cytométrie de flux, ainsi que des mesures quotidiennes d'oxygènes dans le milieu ont été réalisées. Les échantillons collectés ont été centrifugés et les cellules récupérées ont été préparées pour réaliser des analyses des protéomes (en association avec la plateforme de protéomique PAPPSO, UMR Génétique Quantitative et Évolution du Moulon) mais aussi des transcriptomes (laboratoire GMGM Strasbourg). En complément, le surnageant a été analysé en LC-MS (chromatographie en phase liquide couplée à une spectrométrie de masse) pour analyser les composés non volatiles de manière non ciblée. L'analyse des résultats obtenus n'est encore que très préliminaire, mais l'étude des cinétiques permet de mieux comprendre les dynamiques de production/consommation de certains composés. L'étude conjointe des transcriptome/protéome/métabolome devrait permettre de mettre en lumière les voies métaboliques impliquées dans la croissance dans le vin. L'analyse des composés non volatiles permettra d'étudier des composés du vins (tanins, anthocyanes, etc) qui pourraient être liés avec les variations de couleurs mises en évidence dans l'article précédent et mieux comprendre l'empreinte au niveau moléculaire de *B. bruxellensis* et de ses différentes populations.

## Chapitre 3 : Variabilité de l'assimilation des nitrates chez *Brettanomyces bruxellensis*

Remarque :

Les résultats obtenus sont présentés sous forme d'un article scientifique. L'analyse des génomes n'est pas encore totalement terminée. La publication de ces résultats est dépendante de la publication des génomes par nos collaborateurs de Strasbourg (GMGM).

Contexte de l'étude :

*B. bruxellensis* est une levure qui se développe majoritairement après les principales étapes de la fermentation alcoolique réalisée généralement par *Saccharomyces* spp. Cette capacité est permise par une tolérance à de forte quantité d'éthanol dans l'environnement mais aussi à la capacité d'assimilation d'une vaste gamme de nutriments souvent délaissés par les autres levures. Parmi ces nutriments, les nitrates ont été décrits comme étant assimilés par *B. bruxellensis* (de Barros Pita et al., 2011). La quantité de nitrates dans les différentes matrices où est isolée *B. bruxellensis*, est très variable (de <2mg/L à 100mg/L, (Colla et al., 2018)). Certaines études ont montré une hétérogénéité sur cette capacité d'assimilation avec 25% des souches incapable d'assimiler cette source d'azote (Conterno et al., 2006). Les questions principales de ce chapitre sont de savoir si l'assimilation des nitrates est un caractère commun à l'espèce *B. bruxellensis* et/ou s'il existe des évolutions divergentes entre les différentes populations, soulignant de potentiels signaux d'adaptations aux niches colonisées. Les 151 souches (réparties en 8 populations) ont été utilisées dans cette études.

# LOSS OF NITRATE ASSIMILATION IN THE YEAST *BRETTANOMYCES BRUXELLENSIS* IS SHAPED BY POPULATION STRUCTURE AND COPY NUMBER VARIATION

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## Abstract

The budding yeast *Brettanomyces bruxellensis* is a major contaminant in various anthropized environments, such as winery, brewery and bioethanol industry. In these processes, *B. bruxellensis* development occurs when nutrients are scarce. Its spoilage ability is mainly attributed to its capacity to metabolize complex carbon and nitrogen sources that other microorganisms cannot assimilate. Thus, *B. bruxellensis* is described as a “scavenger”. The utilization of nitrate as a nitrogen source has been reported, however, with variability within the species. Recent genetic and genomic data have highlighted a complex diploid-triploid species structured by the isolation niche. In order to study the distribution of nitrate assimilation within *B. bruxellensis* species, a screening of 151 strains representative of the genetic diversity was performed on rich and minimum media with ammonium or nitrate as nitrogen sources. Three main behaviours were observed: 56% (84/151) of the tested strains were able to consume nitrate, 25% (36/151) were not able to consume it, and 20% (31/151) were not able to grow on nitrate and ammonium minimal media. Behaviours were structured by the genetic structure of the species with some population enriched in strains unable to assimilate nitrate. Investigation at the genomic level revealed that the ability to assimilate nitrate was strongly shaped by copy number variation of the NIT genes cluster, a genomic trigon encompassing the three genes of the nitrate assimilation pathway. Loss of heterozygosity events for the NIT genes was highlighted between populations. Overall, these results show that assimilation of nitrate is under divergent selective pressures (positive and relaxed) among *B. bruxellensis* populations. Nitrate assimilation was probably an adaptive trait in the ancestral *B. bruxellensis* population that have since become useless for some populations. Loss of heterozygosity and copy number variation play a major role in the evolutionary degeneration of this function.

## Introduction

*Brettanomyces bruxellensis* is considered as a major contaminant in many fermentation processes. In wine production, *B. bruxellensis* causes organoleptic deviations, known as “*Brett taint*”, due to the production of volatile phenols (Chatonnet et al., 1992). In beer process, *B. bruxellensis* is commonly isolated as a spoilage yeast, producing high amount of acetic acid and volatile phenols (Suiker and

Wösten, 2022). In bioethanol industry, this yeast is considered as the major yeast contaminant in continuous fermentation (de Souza Liberal et al., 2007). *B. bruxellensis* growth induces an ethanol yield decrease, and can overcome *S. cerevisiae* during industrial fermentation (de Barros Pita et al., 2011). In these industrial processes, *B. bruxellensis* contaminations generally occur after the fermentation stage, when nutrients are scarce (Smith

and Divol, 2018). *B. bruxellensis* is responsible for strong economic losses, making its management essential.

*B. bruxellensis* has attracted attention for its industrial entanglement as well as for its genomic composition: recent genomic and genetic population studies have revealed a diploid-triploid complex structured according to substrate isolation and geographic origin (Avramova et al., 2018a; Eberlein et al., 2021; Gounot et al., 2020; Serra Colomer et al., 2020a). More than half of the isolates are triploids that arose from interspecific or intraspecific hybridizations (Borneman et al., 2014; Curtin et al., 2012). At least three independent interspecific hybridization events involving three unknown species have been described to date, which result in the merging of a diploid core-genome with a supplemental divergent haploid genome (2-3% of genetic divergence). One intraspecific hybridization has been recorded, which involved a less divergent haploid genome (<1% of genetic divergence) (Eberlein et al., 2021). Genetic population studies also demonstrated a strong relationship between process isolation and genetic clusters suggesting adaptation to anthropized environments, the extent of which remains to be investigated (Harrouard et al., 2022).

This yeast species is described in the literature as “prepared for the apocalypse”, meaning that *B. bruxellensis* is adapted to high ethanol content, low pH and scarce nutrients availability (for review Smith and Divol, 2016). Indeed, *B. bruxellensis* is able to consume a large range of nutrients including diverse carbon and nitrogen sources such as nitrate (Cibrario et al., 2020; Conterno et al., 2006; Crauwels et al., 2015; Smith and Divol, 2018). However, the range of assimilable nutrients differs at strains level (Crauwels et al., 2017). The genetic diversity seems to be mirrored at the phenotypic level, but large-scale phenotyping studies are lacking (Harrouard et al., 2022). In particular, *B. bruxellensis* has been described to consume nitrate with strain variations (Conterno et al., 2006). In bioethanol production, nitrate assimilation strongly affects *B. bruxellensis* metabolism: nitrate addition in aerobic condition is associated with high acetic acid production and a slow growth rate due to oxidative stress (de Barros Pita and Tiukova, 2013; Galafassi et al., 2013; Peña-Moreno et al., 2021), while in anaerobic condition,

nitrate assimilation is associated with increased ethanol production and yield (Galafassi et al., 2013; Neto et al., 2014; Peña-Moreno et al., 2019). Interestingly, when ammonium and nitrate are both present, *B. bruxellensis* is able to co-consume them (Peña-Moreno et al., 2019). However, all these studies only focus on one bioethanol strain GDB 248.

In the methylotrophic yeast *Hansenula polymorpha*, a closely-related species of *B. bruxellensis* (both belonging to the *Pichiaceae* phylum) (Hittinger et al., 2015), three genes are involved in nitrate assimilation. *YNT1*, *YNR1* and *YNI1* encode a nitrate transporter, a nitrate reductase and nitrite reductase respectively and are organised into the NIT gene cluster (Siverio, 2002). Two supplemental transcriptional co-factors, *YNA1* and *YNA2*, have also been identified in the same species (Silvestrini et al., 2015). Nitrate gene orthologs have been identified in *B. bruxellensis*, also forming a NIT cluster in the subtelomeric region of chromosome VI (Borneman et al., 2014; Serra Colomer et al., 2020b). However, comparative genomics on four strains noted different genomics outcomes with CNV linked to the NIT gene cluster (Borneman et al., 2014; Serra Colomer et al., 2020a). This variation may indicate different selective pressure.

In this study, we investigated the ability to use nitrate as sole nitrogen source in aerobic condition across the spectrum of the different *B. bruxellensis* populations recently highlighted (Avramova et al., 2018a; Eberlein et al., 2021). 151 strains were screened and the NIT cluster genes content was compared to decipher the genotype-phenotype relationship. The different populations showed phenotypic variability for nitrate assimilation, associated with different genetic architecture of the NIT cluster.

## Material and Method

### 1. *B. bruxellensis* strains

The various *B. bruxellensis* strains with their genetic groups are indicated in table 1.



table 1: *B. bruxellensis* strains used in this study. All strains are from Strasbourg collection.

<i>B. bruxellensis</i> population	Number of strains	Strain
<i>beer</i>	22	YJS7816; YJS7817; YJS7818; YJS8081; YJS8657; YJS8229; YJS8010; YJS7966; YJS7895; YJS8048; YJS7868; YJS7810; YJS7926; YJS8251; YJS7944; YJS7813; YJS8089; YJS8072; YJS7995; YJS5397; YJS5400; YJS5454
<i>kombucha-1</i>	18	YJS8285; YJS8013; YJS8070; YJS7909; YJS7910; YJS7921; YJS7923; YJS7947; YJS7831; YJS8104; YJS7879; YJS5310; YJS5340; YJS5344; YJS5363; YJS5368; YJS5407; YJS5420
<i>kombucha-admixed</i>	13	YJS8090; YJS7958; YJS7889; YJS5301; YJS533; YJS5349; YJS5384; YJS5398; YJS5402; YJS5406; YJS5413; YJS5417; YJS5431
<i>teq/EtOH</i>	19	YJS8003; YJS8004; YJS7829; YJS7828; YJS7800; YJS7805; YJS7839; YJS7860; YJS7885; YJS8068; YJS7821; YJS7819; YJS7820; YJS7890; YJS8028; YJS7952; YJS8014; YJS7953; YJS7954
<i>wine 1</i>	28	YJS7803; YJS8082; YJS8091; YJS8092; YJS8757; YJS7948; YJS8758; YJS7886; YJS7900; YJS8095; YJS7903; YJS7905; YJS7902; YJS7980; YJS7827; YJS7838; YJS7972; YJS7881; YJS7884; YJS5408; YJS5434; YJS5445; YJS5459; YJS5469; YJS5473; YJS5476; YJS5478; YJS5487
<i>wine 2</i>	17	YJS7804; YJS7801; YJS8045; YJS8063; YJS7807; YJS7989; YJS8187; YJS7894; YJS7809; YJS7891; YJS7853; YJS7824; YJS782; YJS7811; YJS7812; YJS8000; YJS5382
<i>wine 3-1</i>	14	YJS7802; YJS7967; YJS7955; YJS7874; YJS7844; YJS7973; YJS7848; YJS7849; YJS7862; YJS7911; YJS7888; YJS7806; YJS5392; YJS5449
<i>wine 3-2</i>	20	YJS7875; YJS7856; YJS7990; YJS5302; YJS5319; YJS5320; YJS5345; YJS5347; YJS5357; YJS5373; YJS5385; YJS5422; YJS5426; YJS5440; YJS5447; YJS5453; YJS5458; YJS5461; YJS5479; YJS5485

## 2. Media and growth condition

Yeasts were first grown in YPD liquid media (Yeast extract 10 g.L<sup>-1</sup>, Peptone 20 g.L<sup>-1</sup>, Dextrose 20 g.L<sup>-1</sup>) for 48h in 24°C without shaking. The cells were washed in sterile water and viable cell concentration was assessed using flow cytometry approach (Cytotflex, Beckman Coulter, Brea CA, USA). Fifty µL of the homogenised culture with 3 µL of propidium iodide (PI) were diluted in 1 mL final in McIlvaine buffer (sodium phosphate dibasique 0.2 M, citric acid 0.1 M) and was analysed by flow cytometry (Zimmer et al., 2014). Fifty thousand events were recorded per experiment. The viability of the cells was estimated using 488 nm excitation (40 mW argon laser), with a 610/20 nm long bandpass filter to detect PI fluorescence (dead cells). Growth assay was performed in liquid synthetic medium containing 1.7 g.L<sup>-1</sup> of YNB wo/wo (Yeast Nitrogen Base without ammonium and amino-acids) (DIFCO, Thermofisher, MA, USA), 20 g.L<sup>-1</sup> of D-glucose and supplemented with (i) 0.66 g.L<sup>-1</sup> of ammonium sulphate, to obtain 5 mM (NH<sub>4</sub><sup>+</sup>), (ii) or with 0,4 g.L<sup>-1</sup> of sodium nitrate, to obtain 5 mM (NO<sub>3</sub><sup>-</sup>). Growth in YPD liquid media was

used as control. Growth was monitored on microplates 96 wells at 24°C, 200 µl of medium per well under agitation (CLEARLine, Thermofisher, MA, USA) and measured every two hours for 96 hours by spectrophotometer at 600nm (FLUOstar Omega, BMG LABTECH). Each strain was inoculated at 2.5x10<sup>5</sup> viable cells/mL in triplicate for each medium.

## 3. Determination of growth parameters

Growth parameters were determined using the package “growthcurver” on R software (<https://www.rdocumentation.org/packages/growthcurver/versions/0.3.1>) for all individual wells (>1500). Three parameters were kept, *K* standing for the maximal optical density, *r* for the maximal growth rate and *lag.phase* as the time to reach 0.3 optical density unit.

#### 4. Phylogenetic tree of the 151 *B. bruxellensis* strains

Whole genomes sequences were used to build the phylogenetic tree (diploid core genome and supplemental haploid one for triploid strains). The distance matrix was computed with “Merge branch ‘devel’” (<https://github.com/szpiech/asd>) with SNP genotype data and clustered with fastStructure (Raj et al., 2014).

#### 5. Copy number estimation and allelic frequency

The determination of the Copy number of the NIT genes was conducted as described in Peter et al., (2018). Briefly, the determination of the number of copies of the genes was conducted with Control-FREEC v11.6 (Boeva et al., 2012): it was run on BAM files, over 1kb windows along the genome, taking strain ploidy into account and using parameters: breakPointThreshold = 0.6, window = 1,000, telocentromeric = 6,000, step = 200, minExpected GC = 0.3, and maxExpectedGC = 0.5. Genes with 100% of its length located in a duplicated region were considered as gained. Genes with 90% of its length located in a deleted region were considered as lost.

The determination of the allelic frequency was conducted as described in (Peter et al., 2018). Illumina short paired-end reads were mapped against the *B. bruxellensis* UMY321 genome (Gounot et al., 2020) using bwa 0.7.17-r1188 mem. Samtools 1.13 was used to sort the alignments, remove reads duplicates and filter the alignment for mapping quality  $\geq 30$ . Variants were called as diploid irrespective of real ploidy using bcftools 1.13, with a minimum base quality of 30 for the input. Variants were filtered to pass a genotype quality of  $\geq 20$ , a read depth of  $\geq 5$ , with at most 10% samples not being genotyped. Sites were included if they were within the 5<sup>th</sup> to 95<sup>th</sup> percentile of mean coverage across samples.

#### 6. Statistical analysis

The 687 SNPs present in the NIT genes were retrieved from the variant calling analysis. To consolidate the analysis, strains with more than 200/687 missing genotypes and SNPs with more than 10 % of missing strains were removed. This filtering step led to a dataset of 923 sequenced strains (unpublished data)

and 668 SNPs. Genotype information was converted to allelic frequencies. For each SNP allelic frequencies were centred and missing data replaced with population mean value. This dataset was then used to perform Principal component analysis (PCA) using R package ‘adeqnet’ on SNPs frequencies.

Analyses of variance (ANOVA) were performed followed by post-hoc Tukey tests (HSD.test function from agricolae package, R software)

## RESULTS

### 1. Growth ability

In order to explore the phenotypic diversity of nitrate assimilation within *B. bruxellensis*, we selected 151 strains representative of the genetic diversity of the species and distributed into eight populations (Avramova et al., 2018a; Eberlein et al., 2021). The ability to assimilate nitrate as the only nitrogen source was evaluated. The growth in synthetic media with nitrate ( $\text{NO}_3^-$ ) was compared to two control media, the growth in synthetic media with ammonium ( $\text{NH}_4^+$ ) and in rich medium (YPD). Growths were monitored in 96 well microplates for 96hours in aerobic condition in triplicates for each strain and each condition, resulting in more than 1500 growth curves. Three main behaviours were recorded: strains able to grow on each condition (YPD,  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ ), some able to grow on YPD and  $\text{NH}_4^+$  and a last group of strains only able to grow on YPD. These three behaviours are shown for three representative strains in figure 1A. These strong differences confirmed that the phenotypic variation in nitrate assimilation in *B. bruxellensis* was not rare (Conterno et al., 2006).

In order to compare growth curves, kinetics parameters were extracted, the maximal growth rate ( $r$ ), maximal optical density ( $k$ ) and lag-phase (lag.phase). The heritability was tested for the three parameters and was high ( $>0.99$ , ANOVA analysis). The strains that failed to reach 1 unit of optical density after 96 hours were considered as not being able to grow. Growth parameters were first compared according to media. For this analysis only the strains that succeeded to grow in all the media ( $n=84$ ) were kept and compared (figure 1B). The maximal optical density after 96 hours of growth was quite similar: a mean of 3.79, 3.66 and 3.57 optical

density for YPD,  $\text{NH}_4^+$  and  $\text{NO}_3^-$ , respectively. There was only a significant difference between YPD that is 5.8% higher than  $\text{NO}_3^-$ . Higher effect was found for growth rate between YPD (mean of 0.17 OD/h) and  $\text{NH}_4^+$  and  $\text{NO}_3^-$  (mean of 0.13 OD/h for both). The same was observed for the lag-phase parameter.

Thus, the growth was higher on YPD than on  $\text{NH}_4^+$  and  $\text{NO}_3^-$ , but no significant difference between  $\text{NH}_4^+$  and  $\text{NO}_3^-$  was observed. This result showed that *B. bruxellensis* is able to metabolize  $\text{NO}_3^-$  or  $\text{NH}_4^+$  as nitrogen sources without significant difference on growth capacity in aerobic conditions.

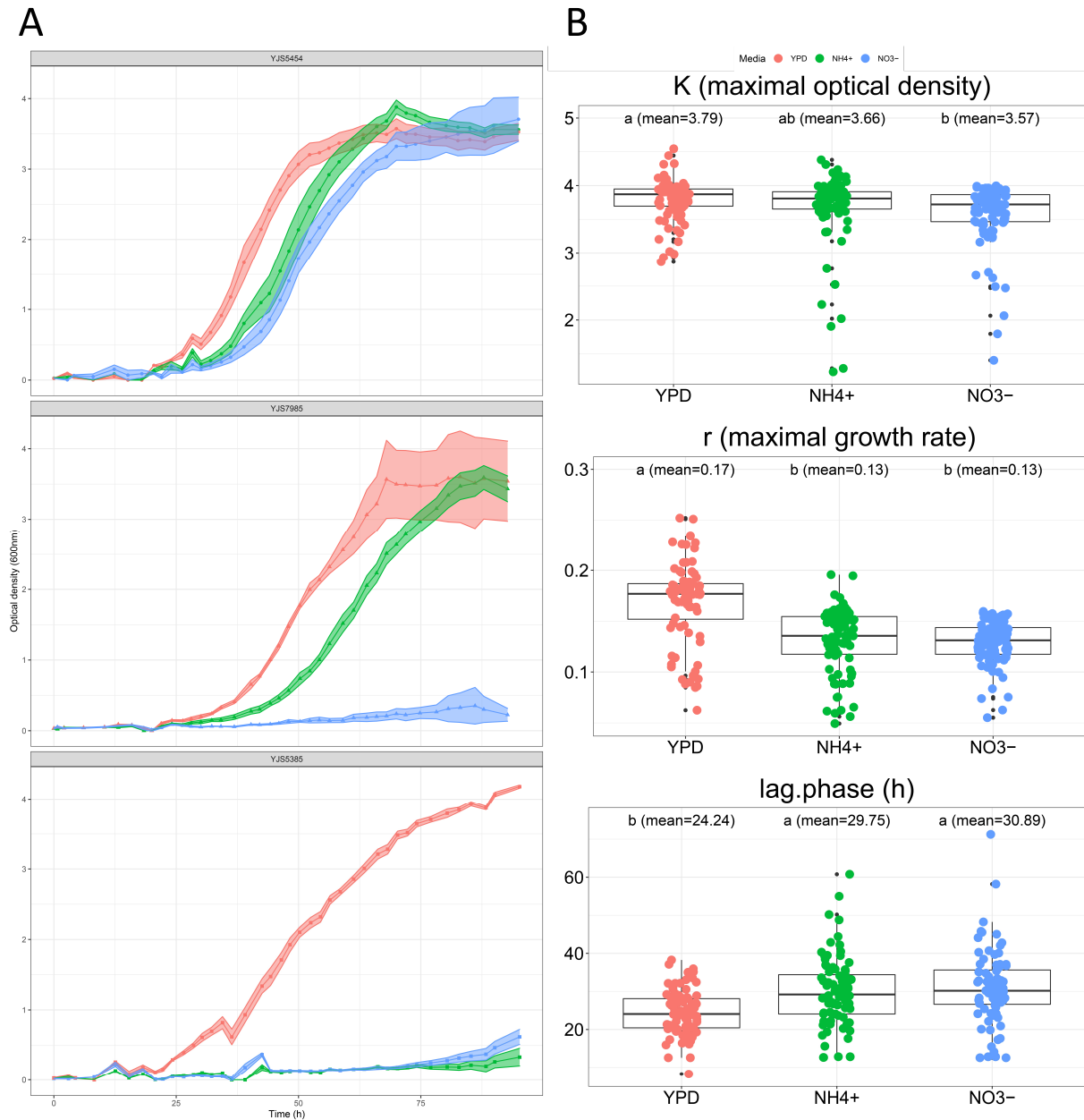


figure 1 : A. Growth in the three different conditions for three strains representative of the phenotypic behaviours observed. Shaded curves represents standard deviations between triplicates.

B. The panels represent three measured parameters (the maximal optical density, the maximal growth rate and the lag.phase) for the strains able to grow on  $\text{NO}_3^-$  (n=84). Upper letters represent significance groups (ANOVA followed by Tukey's post-hoc test,  $\alpha = 5\%$ )

## 2. Variation of nitrate assimilation amongst *B. bruxellensis*' population

We next aimed to compare strains growth profile with the maximal optical density for its relevance regarding the ability to use nitrate as sole nitrogen source. We performed hierarchical clustering in order to group them according to their phenotypic behavior (figure 2A). Three clusters were identified: (i) the first cluster contained 84 strains growing in all three conditions (so-called NIT+), (ii) a second cluster contained 36 strains growing in YPD and NH<sub>4</sub><sup>+</sup> but not in NO<sub>3</sub><sup>-</sup> (called NIT-), (iii) and the last cluster contained 31 strains growing only in YPD (AMMO-). The distribution of the isolates

in the three clusters was structured in part according to genetic group (figure 2B). Briefly, the *wine 1* and *wine 3-2* populations were enriched in isolates NIT+ or AMMO-. The *wine 2* and *wine 3-1* groups were represented by strains with NIT- phenotype. The *teq/EtOH* and *beer* strains mostly NIT+. The *kombucha-admixed* was only represented by strains NIT+. Finally, the *kombucha-1* presented strains distributed in each cluster. The distribution of the three phenotypic clusters were significantly different in the population compared to the global population (K<sub>hi</sub><sup>2</sup> test, *p*-value < 2.2 × 10<sup>-16</sup>). These results evidenced an important structuration of the nitrate phenotypes according to the populations.

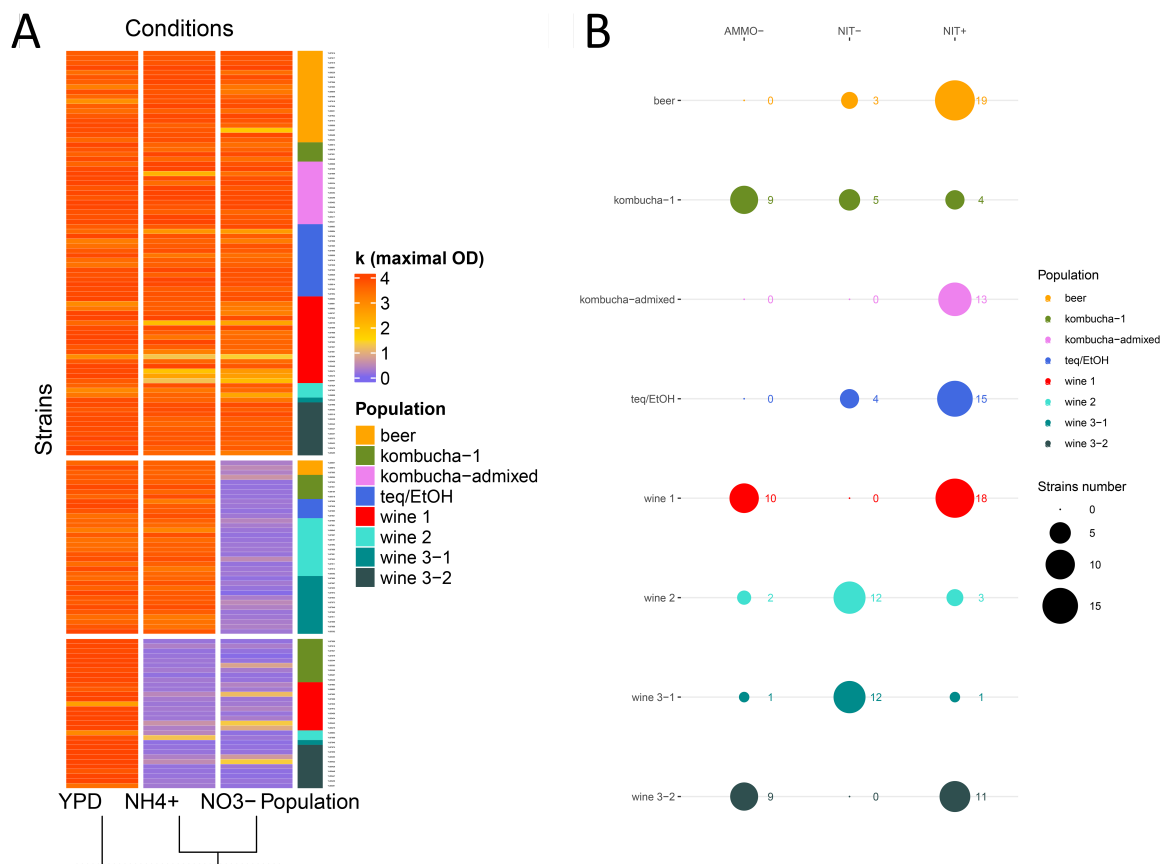


figure 2: A. Heat map of the maximal optical density (*k*) of 151 strains in three conditions (YPD, NH<sub>4</sub><sup>+</sup>, NO<sub>3</sub><sup>-</sup>). The colors on the right correspond to the different populations. The mean of the biological triplicates was used.

B. Distribution of the populations of *B. bruxellensis* in the three clusters (NIT+, NIT- and AMMO-). K<sub>hi</sub><sup>2</sup> test was performed (*p*-value = < 2.2 × 10<sup>-16</sup>) and showed significant variations between the distributions of the populations.

- NIT+
- \* NIT-
- AMMO-

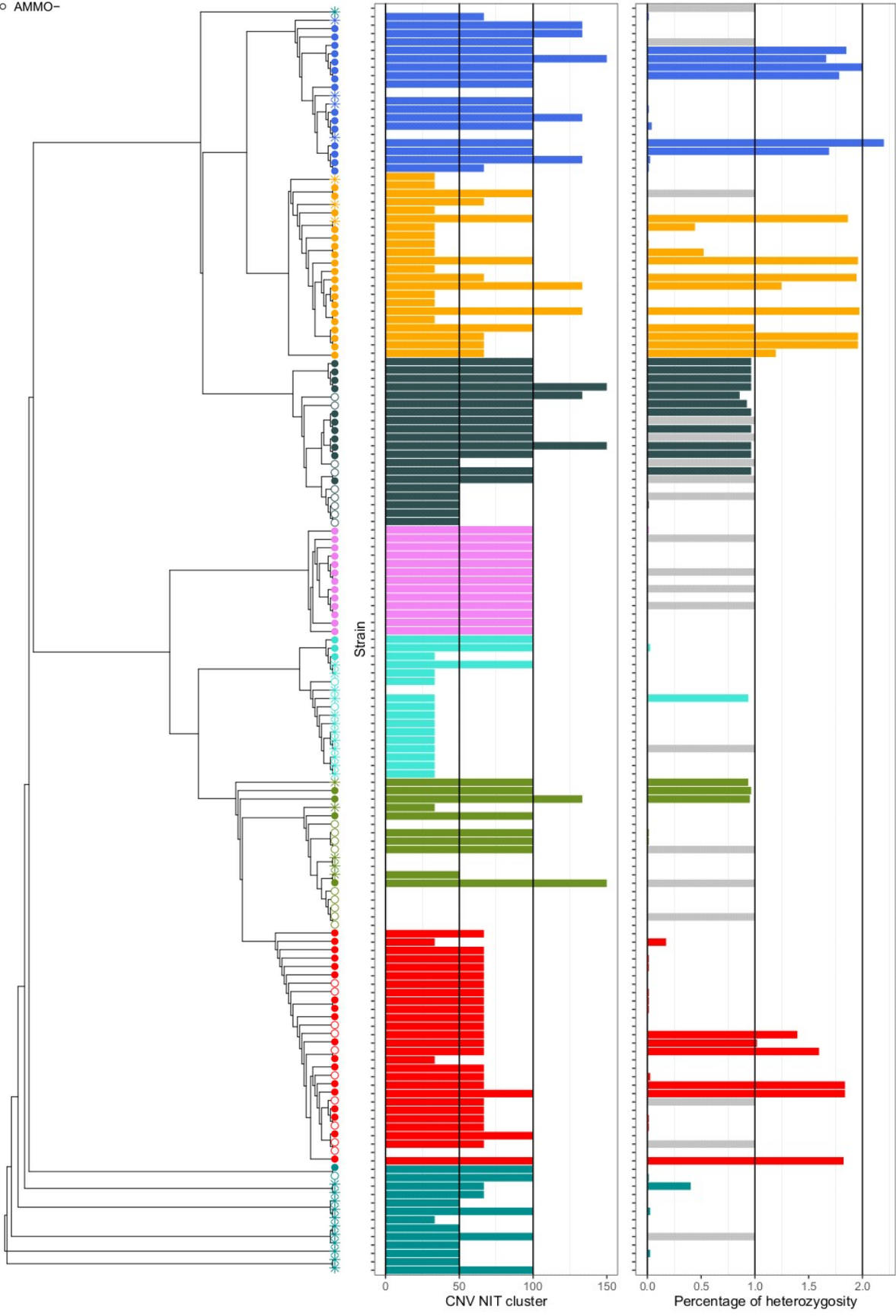


figure 3: Phylogenetic tree of the 151 strains, with the associated phenotype for nitrate assimilation, the copy number of NIT gene cluster and the percentage of heterozygosity. Full dots stand for NIT+ phenotype, stars stand for NIT- phenotype, and empty dots for AMMO- phenotype. The copy number variation of NIT cluster was normalized with the ploidy estimate of the strain, (100 meaning 2 copy for diploids, and 3 for triploids). The heterozygosity level is expressed in percentage. Grey bars stand for missing values, the colors are the same as in figure 2.

### 3. Genetic investigation of the NIT genes cluster

We next wanted to investigate if the phenotypic behaviours could be linked to genetic variations. The genomic sequences of the three NIT genes (*YNT1*, *YNR1*, *YNI1*) in the 151 strains were identified, and were located on the subtelomeric region of the fourth chromosome. The three genes were organized in tandem and thus the copy number was very similar for each strain (data not shown). The level of heterozygosity was assessed using SNPs matrix. The phylogenetic tree, the copy number variation (CNV) of the NIT cluster and the level of heterozygosity were integrated on figure 3.

Copy number of the NIT genes was compared to the genome basal level. For diploid strains, the NIT gene copy number was 0% for 10 strains (0 copy), 50% for 12 strains (1 copy), 100% for 31 strains (2 copies) and 150% for 3 strains (3 copies). For triploid isolates, the NIT gene copy number was 0% for 3 strains (0 copy), 33% for 28 strains (1 copy), 67% for 31 strains (2 copies), 100% 23 strains (3 copies) and 133% for 8 strains (4 copies). Most of the strain had copy number below the expected copy number in accordance to the ploidy level (97 out of 151 strains). *Beer*, *teq/EtOH*, *wine 3-1*, *wine 3-2* and *kombucha-1* groups showed variations into the copy number of the NIT genes. The *wine 1* group was quite homogenous in the copy number of the NIT genes (2 copies, 67%), as the *wine 2* (1 copy, 33%). The *kombucha-admixed* strains were homogenous, and showed the expected copy number of NIT cluster (2 copies, 100%). The heterozygosity level was also variable among isolates and showed a reduced heterozygosity level for the NIT genes for several strains (0 to

1% compared to ~1% for the whole diploid genomes, 0 to 2% compared to ~3% for the whole allotriploid genomes) (Eberlein et al., 2021). On the 98 strains harbouring at least 2 copies of the NIT genes, 48 strains were homozygous (<0.1% of heterozygosity). In particular, the diploid *kombucha-admixed*, *kombucha-1* and *wine 3-1* groups and the autotriploid *wine 2* group showed homozygosity for NIT genes. The allotriploids groups (*wine 1*, *beer*, *teq/EtOH*) encompassed strains which exhibited the highest degree of heterozygosity (around 2% in maximum), as expected for triploid strains exhibiting up to 3 haplotypes. Interestingly, all the NIT- strains (except two) showed a loss of heterozygosity in the NIT genes. A Multi-regression analysis was conducted to explore the impact of variables and factors on the *K* parameter (maximal Optical Density measured) in NO<sub>3</sub>- media for the NIT+ and NIT- strains (ANOVA, *p*-value<0.001, followed by Tukey post hoc analysis, *p*-value<0.05, figure 4). The copy number of the NIT genes and the populations were significant, explaining 12% and 55% and of the variance respectively. The level of heterozygosity was assessed, without significant impact, as well as the copy number for *YNA1* and *YNA2* genes. In details, between populations, *kombucha-admixed*, *wine 3-2*, *wine 1*, *beer* and *teq/EtOH* were different from *kombucha-1*, *wine 2* and *wine 3-1* (figure 4A). However, variation at strain level was also observed. For copy number of NIT genes, 150%, 133%, 100% and 66% were significantly different from 33% and from 50% and 0% (figure 4B). Investigations on copy number variation and heterozygosity level, explained at least in part the phenotypic variations of the nitrate capability. Nevertheless, some variations were yet unsolved, and therefore an analysis at the nucleotidic level was performed.

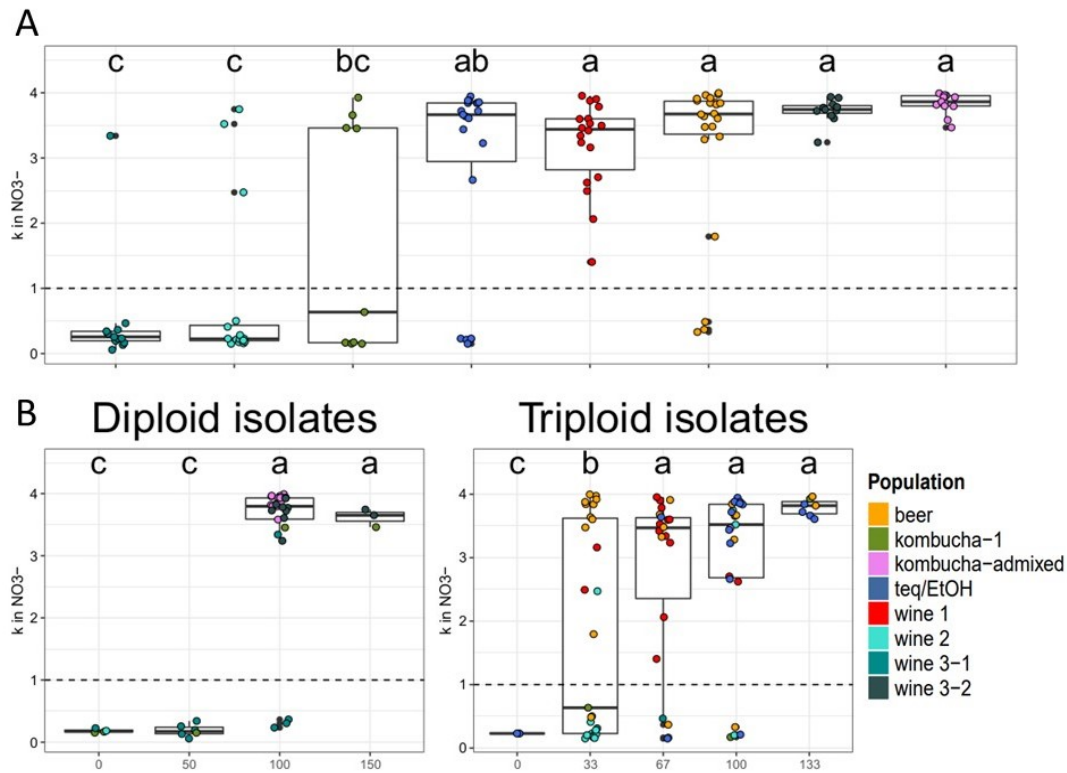


figure 4: Growth capacity on NO<sub>3</sub><sup>-</sup> medium of the NIT<sup>+</sup> and NIT<sup>-</sup> strains. Strains were classified as NIT<sup>-</sup> when their growth was inferior to OD<sub>600nm</sub>=1 (dotted line).

A: Strains were grouped by population. Upper letters represent significance groups (ANOVA followed by Tukey post-hoc analysis, p-value < 0.05).

B: Strains were separated depending on the ploidy level and the CNV of NIT cluster. Upper letters represent the significance groups (ANOVA followed by Tukey post-hoc analysis, p-value < 0.05).

#### 4. Investigation of the haplotype under selection

We next investigated sequence variations of the NIT genes. PCA (Principal Component Analysis) visualisation was performed using SNPs frequencies (for 103 strains sequences after filters, see Material and Method) (figure 5). On this PCA, most of the NIT<sup>-</sup> strains were clustered together, and the NIT<sup>+</sup> strains were more scattered. Three clusters of homozygous strains were evidenced. The first one encompassed diploid strains (cluster 1), and interestingly, most of the NIT<sup>-</sup> strains were present (20 NIT<sup>-</sup> strains, 13 NIT<sup>+</sup> strains). The second cluster was composed of diploid strains

from *kombucha-admixed* and *wine 3-2* populations (cluster 2). The last one was composed only of *wine 1* strains (cluster 3). Heterozygous diploids strains were localised between cluster 1 and cluster 2 and harboured either NIT<sup>+</sup> or AMMO<sup>-</sup> phenotype. Allotriploid heterozygous strains were localized between the three clusters. The *beer* and *teq/EtOH* heterozygous strains were located close to each other's, which was not the case of the *wine 1* heterozygous strains. The three homozygous clusters reflected three haplotypes. Two were present in diploids isolates (cluster 1 and cluster 2) and one (cluster 3) uniquely in the allotriploid group *wine 1*. The fact that only *wine 1* homozygous

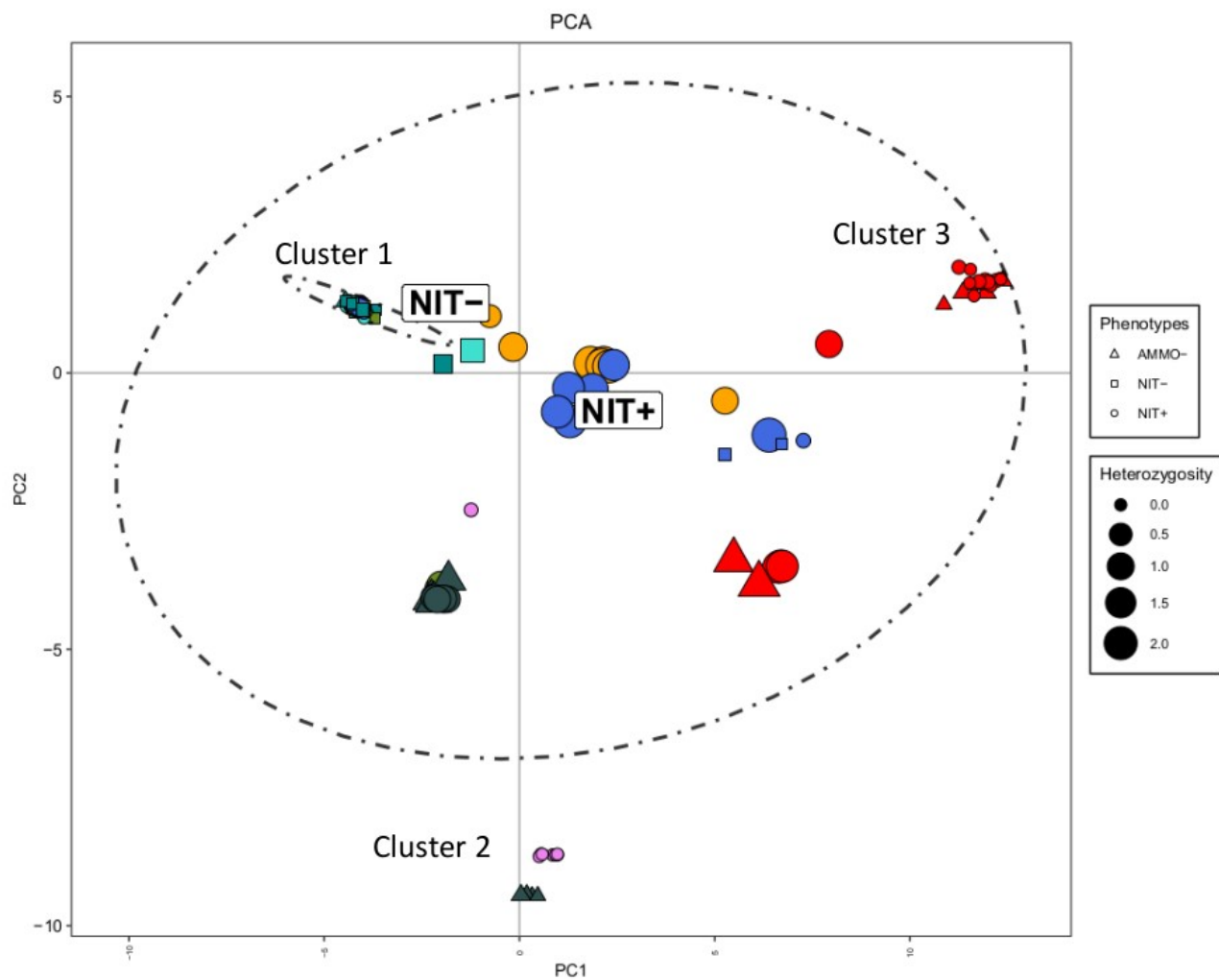


figure 5 : PCA (principal component analysis) of the NIT genes cluster for 103 strains. PC1 and PC2 axis account for 29.6% and 5.2% of the total variation, respectively. Colors correspond to the genetic populations, size point are correlated with the percentage of heterozygosity and the point shape are depending on the phenotype. Ellipses stand for NIT- and NIT+ phenotypes (90%).

strains contained the cluster 3 could be due to the maintaining of the additional haplotype from the haploid genome. Further studies are needed to determine which haplotype are retained in the *wine 1* population.

## Discussion

In the literature, *B. bruxellensis* yeast has been described as being able to consume nitrate, yet studies have shown that this ability may not be shared by all isolates (Borneman et al., 2014; Conterno et al., 2006; Serra Colomer et al., 2020b). In view of the recent genetic diversity highlighted, this study aimed to address a large strain screening for the ability to assimilate nitrate in the species *B. bruxellensis*. Three conditions were used, a rich medium as

positive control (YPD), a minimal medium with ammonium ( $\text{NH}_4^+$ ) as sole nitrogen source, and a minimal medium with nitrate ( $\text{NO}_3^-$ ).

### 1. Nitrate assimilation is under different selective pressure depending on the genetic population

At the same concentration, in aerobic condition, *B. bruxellensis* isolates grow without significant differences on ammonium or nitrate (for the main growth parameters studied for the NIT+ strains, e.g. maximal optical density, lag-phase and maximal growth rate). The growth ability of *B. bruxellensis* strains that were able to assimilate nitrate was comparable to the growth on ammonium in aerobic conditions. Regarding the ability to assimilate



nitrate, three main phenotypes were observed, strains able to grow in all three media, defined as being able to consume nitrate (NIT+), strains only able to grow in YPD and NH<sub>4</sub><sup>+</sup> defined as being not able to consume nitrate (NIT-) and finally strains only able to grow in YPD defined, as AMMO-. Among the genetic structure of the *B. bruxellensis* population, the distribution of these phenotypes was not random (figure 6). Three groups were principally NIT+ (beer, kombucha-admixed, teq/EtOH), two groups were NIT- (wine 3-1, wine 2), two groups were NIT+ or AMMO- (wine 1, wine 3-2) and the last group harboured strains belonging to the three phenotypic groups (kombucha-2). These phenotypic variations were explored at the genetic level with available genomes. The copy number and the level of heterozygosity of the NIT genes cluster was investigated. Strong variations of the copy number and heterozygosity level were observed, between populations but also at the strain level. At the population level, most of the strains showed a reduced copy number and heterozygosity of the NIT genes cluster compared to the expected in regard to the ploidy level (ranging from 0 to 4 for copy number, and 0 to 2% of heterozygosity) (figure 6). As expected, the highest values of heterozygosity were found in allotriploid isolates (around 2%) (Eberlein et al., 2021). On the 98 strains that have at least two copies of the NIT cluster, 48 have less than 0.05% of heterozygosity level. The global heterozygosity level expected for diploid groups is around 0.7% in the whole genome against 2-3% for the allotriploid groups (Gounot et al., 2020). The very low levels of heterozygosity observed for the NIT genes suggest loss of heterozygosity events (LOH). LOH is well documented and can arise after gene conversion or break-induced replication (Morales and Dujon, 2012; Smukowski Heil et al., 2017). These changes have been shown to play a major role for evolution of heterozygous yeast, and to shape *B. bruxellensis* genomes (Dutta et al., 2021; Eberlein et al., 2021). Moreover, the NIT genes cluster is localised at the extremity of a chromosome. The

subtelomeric regions are highly variable in eukaryotes and numerous subtelomeric genes are involved in secondary metabolism and/or key traits associated with adaptive evolution due to high variability in gene copy number (Liti 2005, Linardopoulou 2005). Thus, the decrease in CNV and LOH of the NIT genes cluster might be associated to its genomic location and resulted in the loss of the ability to use nitrate in peculiar populations. Additional works are needed to evaluate if LOH and genetic conversion arise multiple times or are restricted to rare events (unique in each population). The LOH event led to the presence of only one haplotype in some population. Two diploid haplotypes (common for the diploid populations) have been identified, with one that seemed to be linked to the NIT-phenotype. A third haplotype, present only for the wine 1 population, but present in each homozygous strains of this cluster has been observed. This third haplotype, specific of the wine 1 population could come from the haploid supplemental genome and seemed to be under selective pressure. Further studies are necessary to formerly identify the different haplotypes in each bpopulation.

## 2. Ecological relevance of nitrate assimilation in *B. bruxellensis* species

Nitrate is an important nitrogen source in nature. However, nitrate assimilation is restricted to a reduced number of yeast species (50/332 species) (Shen et al., 2018). These species share a common nitrate assimilation pathway, which strongly suggests that the pathway was present in the budding yeast common ancestor and was independently lost through the evolutionary diversification (Shen et al., 2018). We have shown in this study that most of *B. bruxellensis* strains were able to consume nitrate, but some individuals in some populations have independently lost this ability (84 NIT+, 36 NIT-).

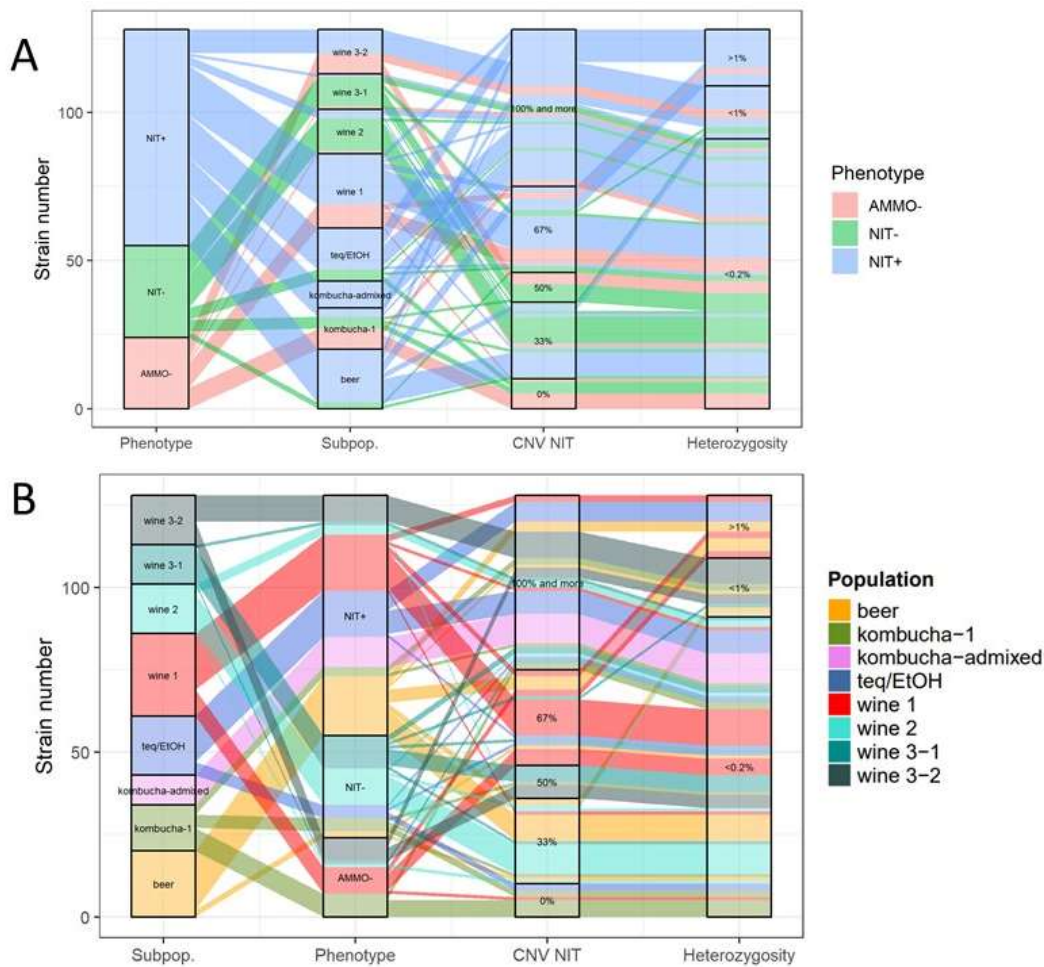


figure 6: A: Distribution of the three phenotypes into the population, copy number of NIT genes and level of heterozygosity.

B: Distribution of the different populations into the three phenotypes, copy number of NIT genes and level of heterozygosity.

This may result from the relaxation of the selective pressure on this trait and the degeneration of unused functions. The *B. bruxellensis* population is structured by the isolation niche (Avramova et al., 2018a). Therefore, the conservation of nitrate assimilation trait is dependent of the selective pressure present in the environment. So far, *B. bruxellensis* have been associated with anthropic environments exclusively (wine, beer, kombucha, bioethanol for example), and the natural reservoir of the species remains unknown (for review: Harrouard et al., 2022). The fact that nitrate assimilation was probably a species trait indicated that the ability to used nitrate was a trait under selective pressure in its natural niche and added clues to identify the

natural ecological niche of this food spoilage yeast.

Nitrates are present in variable concentration amongst the anthropized niches colonized by *B. bruxellensis*. In winemaking, the nitrate level is low, ranging from >2 mg/L to 50 mg/L and is dependent of the technical itinerary (Ough and Crowell, 1980; Smith and Divol, 2016). The maximal level of nitrate in beer (and in kombucha) is 50 mg/L, and originates from water and hops (Buiatti, 2008; Serra Colomer et al., 2020b). Nitrate in bioethanol production originate from the crops and fertilizers. The *teq/EtOH* (a genetic group mainly isolated in bioethanol process) shows a good conservation of the copy number NIT cluster, which could underline a positive selective pressure on it.

Moreover, it was suggested that nitrate assimilation of *B. bruxellensis* strains could explain its ability to overcome *S. cerevisiae* (Da Silva et al., 2016; de Barros Pita et al., 2011). In beer and wine, the fermentation stage is conducted by *Saccharomyces* species, unable to consume nitrate (Shen et al., 2018). *B. bruxellensis* spoilage usually occurs after the alcoholic fermentation, when ammonium and amino acids are scarce (Schifferdecker et al., 2014; Smith and Divol, 2016). Thus, nitrate consumption and the NIT cluster could be under positive selective pressure for wine-associated strains. The *wine 1* population has been studied for its increased sulphite tolerance in wine, and identified as the fittest group in winemaking conditions (Avramova et al., 2018b; Cibrario et al., 2019; Harrouard, in prep). Noteworthy, none of the 28 *wine 1* strains screened were NIT-.

### **3. Auxotrophic strains within *B. bruxellensis* are not so rare**

Finally, the strains showing AMMO- phenotype need further investigations. The inability to grow on minimum medium with ammonium could be due to the inability to use ammonium, which seems very unlikely, or due to amino acid synthesis defect (auxotroph strains). To our knowledge, no screening on the potential auxotrophic behaviour has been conducted in a large strain collection of *B. bruxellensis*. Auxotrophic strains have been already highlighted in response to anthropic pressures. However, these findings stand for bacterial species (Siezen et al., 2005; Steensels et al., 2019). In the light of the distribution of AMMO-isolates within the species, this phenotype arose several times independently. Further investigations are needed to understand the genetic basis of AMMO- phenotype, and whether degeneration of unused functions is at work in the evolution of anthropized *B. bruxellensis* strains.

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## Perspectives et poursuite des travaux

L'étude de la capacité d'assimilation des nitrates au sein de l'espèce *B. bruxellensis* a mis en évidence que cette capacité est un caractère de l'espèce *B. bruxellensis* qui est sans doute sous différentes pressions de sélection en fonction des milieux considérés. Trois haplotypes principaux ont été soulignés. Deux sont principalement présents chez les isolats diploïdes, dont un qui semble être associé à la perte de fonction des gènes NIT pour certains individus. L'étude des séquences est nécessaire et permettra de déterminer les potentielles causes de perte de fonction de gènes NIT (mutation non synonyme, insertions, délétions ...). L'identification sur les assemblages phasés (identification du génome core diploïde et du génome haploïde supplémentaire) de haute qualité des haplotypes des gènes NIT devrait permettre d'identifier si et quel haplotype est conservé dans chaque population. Le maintien chez la population *wine 1* d'un haplotype spécifique à cette population pourrait indiquer que l'haplotype des gènes NIT du génome supplémentaire est sélectionné et serait une signature d'adaptions apporté par l'hybridation interspécifique. Cette partie est en cours d'analyse.

Les nombreuses particularités génétiques des souches de *B. bruxellensis* concernant les gènes NIT (nombre de copies, perte d'hétérozygotie) semblent indiquer la présence d'évènements multiples et indépendants. L'étude du taux d'hétérozygotie dans les régions proximales des gènes NIT sont en cours et devrait permettre d'identifier les traces des modifications génétiques qui ont eu lieu dans la population. Les premiers éléments indiquent des évènements multiples et indépendants, ce qui pourrait indiquer une pression importante mais aussi récente (par rapport à l'apparition des population chez *B. bruxellensis*).

La capacité d'assimilation est corrélée en grande partie aux variations génétiques mises en lumière. Cependant, des approches de transformation génétiques sont nécessaires pour valider les différentes hypothèses. La transformation génétique de *B. bruxellensis* a déjà été réalisé au sein du laboratoire dans le cadre de cette thèse avec l'aide du laboratoire australien AWRI (Christian Varela) (résultats non montrés). La restauration du phénotype NIT+ d'une souche incapable d'assimiler les nitrates avec un haplotype compétent permettra de démontrer formellement le rôle des gènes nitrates, mais permettra aussi la validation/invalidation des hypothèses concernant l'impact des différents haplotypes sur le phénotype. De plus, il serait intéressant de tester si la capacité d'assimilation des nitrates présente une augmentation de la valeur adaptative dans différentes niches anthropisées telles que le vin, la bière, le kombucha, ou la fermentation de la molasse pour la production de bioéthanol. Des expériences de compétition (*in vitro*) pourraient être réalisées sur le même modèle que celles réalisées par Avramova et al., avec des concentrations d'azotes (nitrates/ammonium) variables (Avramova et al., 2019).

L'étude du phénotype « nitrate » a permis aussi de tester et de valider une approche GWAS réalisée par le laboratoire GMGM de Strasbourg (Victor Loegler et Anne Friedrich). Par cette approche, sur 151 souches, la détection de la variation de copies du cluster de gènes NIT a été associée de manière significative avec la variation phénotypique (60% de la variance) (Figure 24). Deux autres gènes à proximités du cluster NIT ont aussi été associés à la variation phénotypique ainsi qu'une variation nucléotidique unique (SNP). Les deux gènes associés codent pour une  $\beta$ -galactosidase et une  $\alpha$ -isomaltase. Il est possible que ces deux gènes soient associés au cluster NIT par phénomène d'auto-stop, et n'interviendraient pas sur l'assimilation des nitrates. Cependant, il est aussi possible que ces deux gènes soient sous pression de sélection fortes (fonctions importantes) et que les gènes du cluster



NIT se retrouvent dépendant (donc pris en auto-stop) des variations génétiques de ces deux gènes. Une étude génomique sur plus d'individus permettrait de tester cette hypothèse.

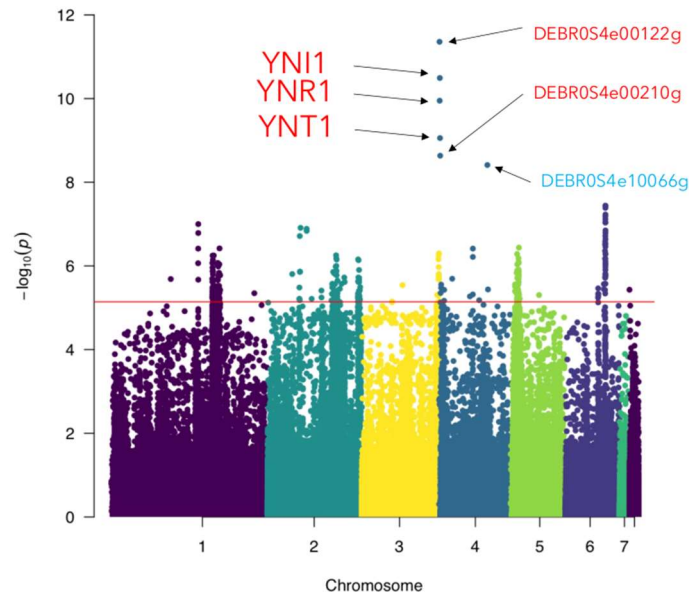


Figure 24: Manhattan plot présentant les résultats de l'analyse GWAS (fournie par Victor Loegler, thèse GMGM Strasbourg). Les différents chromosomes sont représentés sur l'axe des abscisses, et la p-value sur l'axe des ordonnées. Les gènes affectés par la variation de nombre de copies sont identifiés en rouge, les gènes affectés par des variations nucléotidiques sont en bleus. L'analyse a été réalisée sur les données d'aire sous la courbe (AUC) de croissance sur milieu nitrate calculée pour les souches NIT+ et NIT-.

Pour finir, un troisième phénotype a été mis en avant, appelé « AMMO- » dans l'article. Ce phénotype correspond probablement à des souches auxotrophes. Des expériences complémentaires ont été réalisées sur ces souches, avec l'ajout de plusieurs acides aminés (18) (données non montrées). De manière surprenante, l'addition d'acides aminés soufrés (méthionine et cystéine) restaure la croissance de la grande majorité des souches avec pour seule source d'azote l'ammonium. Ces résultats qui nécessitent de plus amples investigations, pourraient indiquer de potentielles convergences vers la perte de la capacité à néo-synthétiser les acides aminés soufrés. La néo-synthèse des acides aminés soufrés dans un milieu œnologique est possible par deux voies chez *S. cerevisiae*, la voie des sulfates et celle des sulfites. La voie des sulfates induit, une production de sulfure d'hydrogène ( $H_2S$ ) (Figure 25) (Dzialo et al., 2017; Jiranek et al., 1995; Swiegers and Pretorius, 2007). La production d' $H_2S$  a été évaluée dans la collection des 151 souches de *B. bruxellensis* et les souches possédant le phénotype « AMMO- » ne produisent pas d' $H_2S$ , en accord avec l'hypothèse de la perte de la voie des sulfates pour la synthèse des acides aminés soufrés. La seconde voie de synthèse des acides aminés soufrés fait intervenir les sulfites comme précurseur. La perte de la voie des sulfates pourrait entraîner une augmentation de l'utilisation de la voie des sulfites pour réduire la toxicité du milieu lorsque les sulfites sont présents. Le phénotype « AMMO- » est surreprésenté dans la population *wine 1*, population résistante aux sulfites. Ces premiers résultats et ces hypothèses nécessitent d'autres expériences pour être validées mais semblent intéressantes dans l'identification de phénomènes d'adaptation évolutive à des milieux anthropisés.

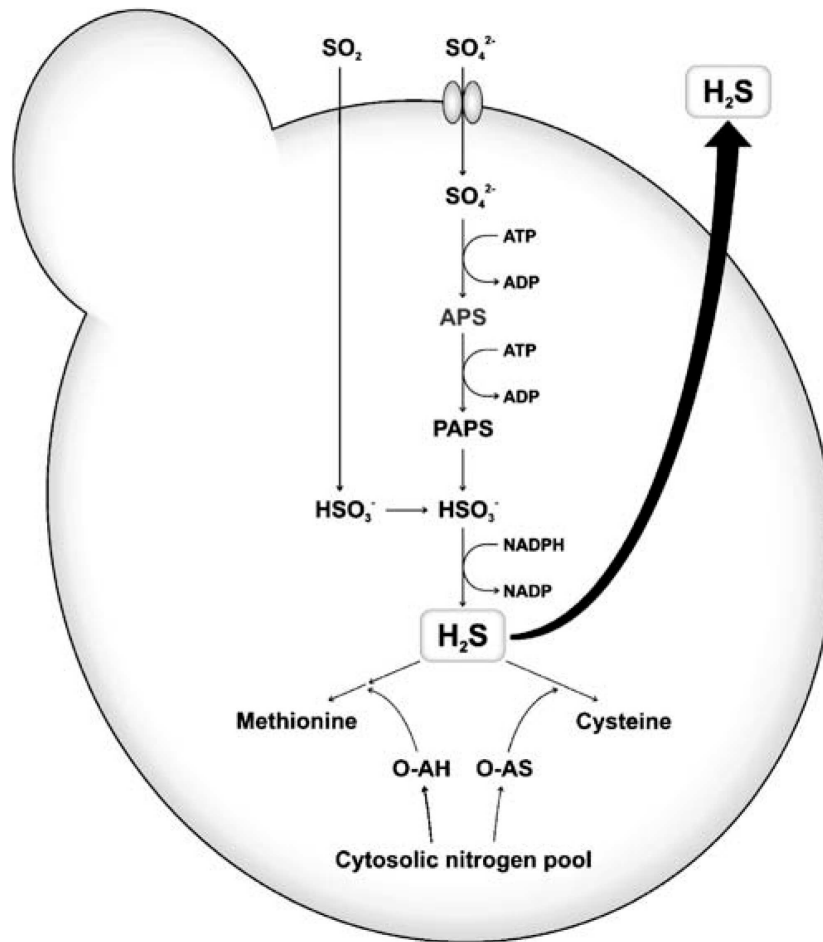


Figure 25: Voie métabolique de la synthèse des acides aminés soufrés. D'après Swiegers and Pretorius, (2007).

## Chapitre 4 : Procédés physiques innovants de stabilisation du vin

## Introduction

La filière viti-vinicole doit faire face à de nombreux changements dont notamment la volonté des consommateurs d'avoir accès à des vins plus respectueux de l'environnement et de leur santé. Cette tendance est visible particulièrement sur les vins biologiques et en biodynamie (+15% par an depuis 10 ans) (Ugaglia et al., 2019). Cette demande des consommateurs interroge évidemment sur l'utilisation des sulfites dans l'élaboration des vins. En effet, il est l'intrant majoritairement utilisé, pour ses propriétés antiseptiques, antioxydantes, antioxydasiques. En plus de la tendance du marché, l'utilisation du SO<sub>2</sub> est questionnée par la diminution de l'acidité des vins (due au changement climatique) ainsi que par l'augmentation de la proportion de souches de *B. bruxellensis* tolérantes à sa présence, réduisant d'avantage son intérêt (van Leeuwen and Darriet, 2016). Ces changements poussent la filière à étudier de nouveaux procédés de contrôle des populations microbiennes et notamment à regarder ce qui est utilisé dans d'autres filières alimentaires.

Dans ce cadre, en collaboration avec des collègues spécialisés en génie des procédés de notre unité de recherche, l'efficacité de deux méthodes physiques innovantes de stabilisation microbiologique, les UV-C et la lumière pulsée (PL), a été caractérisée. Ces méthodes de stabilisation sont déjà utilisées pour des traitements de surface de denrées alimentaires (légumes, viandes), pour le traitement de matériel médical, ainsi que pour le traitement de liquide (eau, jus de fruit). Leur utilisation dans la filière viti-vinicole n'est pas encore approuvée par l'Organisation Internationale de la Vigne et du Vin (OIV).

Pour la caractérisation des deux méthodes de stabilisation, une stratégie similaire a été mise en place. Une première approche de criblage sur boîte de Pétri pour évaluer l'impact des traitements sur la capacité des micro-organismes à se développer a été mise au point, permettant la caractérisation de la sensibilité d'un grand nombre de souches et d'espèces de levures inféodées à l'œnologie. Dans un second temps, sur un nombre restreint d'individus, un traitement sur du vin a été conduit grâce à des pilotes disponibles au laboratoire. Concernant l'utilisation des traitements PL, il s'agit à notre connaissance de la première étude appliquée sur vin rouge.

Les résultats obtenus ont été valorisés scientifiquement par la publication de deux articles scientifiques dans le journal *Food Microbiology* et sont présentés dans la suite de cette thèse. Les données supplémentaires des articles sont présentées dans la partie annexe de la thèse.



## Wine yeast species show strong inter- and intra-specific variability in their sensitivity to ultraviolet radiation

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### ABSTRACT

While the trend in winemaking is toward reducing the inputs and especially sulphites utilization, emerging technologies for the preservation of wine is a relevant topic for the industry. Amongst yeast spoilage in wine, *Brettanomyces bruxellensis* is undoubtedly the most feared. In this study, UV-C treatment is investigated. This non-thermal technique is widely used for food preservation. A first approach was conducted using a drop-plated system to compare the sensitivity of various strains to UV-C surface treatment. 147 strains distributed amongst fourteen yeast species related to wine environment were assessed for six UV-C doses. An important variability in UV-C response was observed at the interspecific level. Interestingly, cellar resident species, which are mainly associated with wine spoilage, shows higher sensitivity to UV-C than vineyard-resident species. A focus on *B. bruxellensis* species with 104 screened strains highlighted an important effect of the UV-C, with intra-specific variation. This intra-specific variation was confirmed on 6 strains in liquid red wine by using a home-made pilot.  $6624 \text{ J.L}^{-1}$  was enough for a reduction of  $5 \log_{10}$  of magnitude for 5 upon 6 strains. These results highlight the potential of UV-C utilization against wine yeast spoiler at cellar scale.

### 1. Introduction

In food industry, microbial stabilization is crucial to ensure good storage and aging. In the winemaking industry, wine stabilization is historically managed by using sulphite ( $\text{SO}_2$ ) that has both antioxidant and antimicrobial properties. However, there is a trend towards reducing the doses of sulphites in wines (Santos et al., 2012). This can be explained by (i) health concerns and a growing consumer preference for wines with the least possible added input; (ii) the fact that strongest sulphite doses are required for an optimal efficiency due to the increase of wine's pH (van Leeuwen and Darriet, 2016) and (iii) because of the emergence of sulphite resistant strains amongst spoilers (Curtin et al., 2012a). Microfiltration and flash-pasteurization are the main technologies used in conventional winemaking even if they are both expensive, highly energy consuming and known to have potential negative impact on both colour and aromatic profile of wines. That is why other chemical and physical control methods are intensively studied (reviewed by Pinto et al., 2020; Tubia et al., 2018; Lisanti et al., 2019)).

Recent studies suggested that UV-C treatment (253.7 nm) could be an alternative technology to inactivate microorganisms in grape juices and is already used for surface equipment disinfection (Fredericks et al., 2011; Diesler et al., 2019; Junqua et al., 2020; Durner et al., 2017). This technology has been widely investigated for two decades and is regarded as a promising approach for fruit juice stabilization with minimal negative impacts (Pala çığdem and Toklucu, 2013; Gabriel, 2012; Abdul Karim Shah et al., 2016). Microbial inactivation caused by UV-C radiation is based on the rearrangement of the microorganism's nucleic acid and DNA-protein cross link (Cyclobutane Pyrimidine Dimers, CPDs) which directly interferes with the ability of microorganisms to reproduce (Gabriel and Nakano, 2009; Bintsis et al., 2000; Friedberg et al., 2005; Tran and Farid, 2004). Different studies showed a wide spectrum of inactivation of wine-associated microorganisms in different types of wines and grape juices. Fredericks et al., Diesler et al., and Junqua et al. demonstrated a reduction of 5–6  $\log_{10}$  of magnitude in yeast and bacteria species in various wines and grape musts (Fredericks et al., 2011; Diesler et al., 2019; Junqua et al., 2020). Those studies showed a

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positive correlation between UV-C dosage and microbiological stabilization, with the treatment efficiency depending on physical properties of the matrix (e.g. optical density at 254 nm, turbidity), the considered species and the population level. For example, to obtain a reduction of 6 log<sub>10</sub> of magnitude of *S. cerevisiae*, 800 J.L<sup>-1</sup> are required for rosé and white wines ( $\alpha_{254\text{nm}} < 10 \text{ cm}^{-1}$ ) when 5000 J.L<sup>-1</sup> is necessary for red wine ( $\alpha_{254\text{nm}} = 47 \text{ cm}^{-1}$ ) (Junqua et al., 2020). To obtain a reduction of 6 log<sub>10</sub> of magnitude of *S. cerevisiae*, 600 J.L<sup>-1</sup> are required for clear Riesling must (turbidity 26.7 NTU) when 800 J.L<sup>-1</sup> is necessary for Müller-Thurgau must (turbidity 36 NTU) (Diesler et al., 2019). Regarding population level, 600 J.L<sup>-1</sup> allow the stabilization of Müller-Thurgau must inoculated at 10<sup>4</sup> cell.mL<sup>-1</sup> initial cell count, when 1400 J.L<sup>-1</sup> is not sufficient for 10E08 initial cell count (Diesler et al., 2019). Considering the bacteria and yeast, 200J.L<sup>-1</sup> are sufficient to inactivate *Acetobacteria aceti* when 400J.L<sup>-1</sup> are required for *Brettanomyces bruxellensis* and 600J.L<sup>-1</sup> for *S. cerevisiae* (Junqua et al., 2020). However, these studies compared only a few number of species and strains and did not take into account the intraspecific variability.

In winemaking, a plethora of yeasts are present, originating from the vineyard (e.g. present on the grape surface) or from the winery. These species can be considered as positive, neutral or negative from a winemaking viewpoint (Loureiro and Malfeito-Ferreira, 2003). Wine yeast spoilage is problematic due to contamination by *Brettanomyces bruxellensis*, *B. anomalous*, *Schizosaccharomyces pombe*, *Zygosaccharomyces rouxii*, *Z. bailii*, *Trigonopsis cantarellii*, etc. (Loureiro and Malfeito-Ferreira, 2003; Cocolin et al., 2004; Portugal et al., 2015). Among them, *B. bruxellensis* is considered as the major contaminant in wine due to its ability to produce volatile phenols (4-ethylphenol, 4-ethylguaiaicol and 4-vinylphenol) causing the 'Brett' taint described as barnyard, horse sweat or burnt plastic (Chatonnet et al., 1992). *B. bruxellensis* can spoil up to 25–30% of red wine production (Gerbaux et al., 2000; Romano et al., 2008). This species displays a huge genetic and phenotypic diversity (Avramova et al., 2018a; Serra Colomer et al., 2020; Gounot et al., 2020; Crauwels et al., 2017). Isolates cluster in six genetic groups depending on the ploidy level, the substrate of isolation and geographical niches (Avramova et al., 2018a; Serra Colomer et al., 2020; Gounot et al., 2020). *B. bruxellensis* strains isolated from wine mainly belong to three genetic groups defined as 1st Wine 3N (or AWRI1499-like), Wine 2N (or CBS2499-like) and Wine/Beer 3N (or AWRI1608-like) which respectively encompass 39%, 37% and 16% of isolates (Avramova et al., 2018a; Cibrario et al., 2019). Concerning control methods for wine production, *B. bruxellensis* is an actual challenge (Pinto et al., 2020): up to 45% of wine isolates are sulphite tolerant or resistant (Avramova et al., 2018b). Tolerant/resistant strains mainly belong to the 1st Wine 3N group. In addition, *B. bruxellensis* isolates exhibit variable sensitivity to other treatments such as chitosan (Bağder Elmacı et al., 2015; Petrova et al., 2016). Recently, Paulin et al. showed that, in a representative collection of 53 *B. bruxellensis* isolates, 41% of strains were sensitive to chitosan, 13% of tolerant and 46% with intermediary behaviour (Paulin et al., 2020). Thus, it is necessary to take into account the phenotypic variability of *B. bruxellensis* in order to properly assess its UV-C sensitivity to microbial treatment, by using a representative panel of strains belonging to the different genetic cluster of this species.

In this work, we first evaluated the efficiency of UV-C treatment using a plate screening (surface) approach. We tested 14 yeast species and 147 strains associated with winemaking, in order to appreciate the relative interspecific variability. We particularly focused on *B. bruxellensis* species for which 104 representative isolates were tested. Considering the first results, we selected 6 strains of *B. bruxellensis* belonging to the three main genetic groups found in wine and harbouring different sensitivity to UV-C on solid medium in order to assess the efficiency of UV-C treatment in (liquid) red wine.

## 2. Materials and method

### 2.1. Yeast strains

The 147 strains from 14 species used in this study were collected from different origins: the CRB Oenologie collection (Centre de Ressources Biologiques Oenologie, Institut des Sciences de la Vigne et du Vin, France) or other laboratories/collections (Table 1). Strains were grown and maintained in YPD plates at 24 °C (10 g.L<sup>-1</sup> yeast extract, 10 g.L<sup>-1</sup> peptone, 20 g.L<sup>-1</sup> glucose, 20 g.L<sup>-1</sup> agar) (see Table 2).

### 2.2. Surface UV-C treatment of yeast

Strains were grown in liquid media (YPD 10 g.L<sup>-1</sup> yeast extract, 10 g.L<sup>-1</sup> peptone, 20 g.L<sup>-1</sup> glucose) for 24 h and the population was estimated by optical density (FLUOstar Omega, MNGLabtech, France). Two µl of serial dilutions (0.5, 0.05 and 0.005 DO) were spotted onto solid medium (YPD), aiming at 3 different densities (around 1000, 100 and 10 CFU/drop). Drops were performed in triplicate for each condition. Spotted plates were exposed to increasing UV-C doses using an apparatus made at the laboratory. The UV-C treatment unit consists of a box containing two 45W@UV-C low-pressure mercury lamps placed 15 cm above the plates. Six increasing UV-C doses (0, 2000, 4000, 6000, 7500 and 10 000 µJ cm<sup>-2</sup>) were applied by varying the exposure times. UV-C doses were monitored (in µJ.cm<sup>-2</sup>) using an UV-C sensor (HD2102.2 with LP 471 UV-C probe from DeltaOHM, Italy).

**Table 1**

Strains used in this work. The reference, collection of these strains are presented in supplemental Table 1.

Species	Number and strains ID
<i>B. anomalous</i>	3 strains: BR 23-4; CLIB 304; NRRL Y-17522 T
<i>B. bruxellensis</i>	1st Wine 3N - 22 strains: 20T13_02; 33_2; AWRI1499; GB12; GSP1509; CRBO L0417; CRBO L14155; CRBO L14174; CRBO L14175; CRBO L14194; CRBO L1703; CRBO L17111; CRBO L1727; YJS5408; YJS5434; YJS5445; YJS5459; YJS5469; YJS5473; YJS5476; YJS5478; YJS5487 Wine/Kombucha 2N - 19 strains: 15_1; ISA1601; YJS5301; YJS5310; YJS5334; YJS5340; YJS5344; YJS5349; YJS5363; YJS5368; YJS5384; YJS5398; YJS5402; YJS5406; YJS5407; YJS5413; YJS5417; YJS5420; YJS5431 Wine 2N - 30 strains: 1961_MX_M1_E2; CBS 2499; ISA2150; CRBO L0469; CRBO L0614; CRBO L14163; CRBO L1714; CRBO L1751; YJS5302; YJS5319; YJS5320; YJS5345; YJS5347ww; YJS5357; YJS5373; YJS5385; YJS5392; YJS5416; YJS5422; YJS5426; YJS5440; YJS5447; YJS5449; YJS5453; YJS5456; YJS5458; YJS5461; YJS5463; YJS5479; YJS5485 Wine/Beer 3N - 21 strains: 20T13_05; 20T13_07; 20T14_01; 20T14_03; AWRI1608; CDR222; GB62; ISA2397; CRBO L17112; CRBO L1741; CRBO L1749; CRBO L1771; LB15107g; LB15110g; NL045; NL059; VP1519; YJS5396; YJS5397; YJS5400; YJS5454 Tequila/bioethanol 3N - 5 strains: CRBO L14169; CRBO L17108; CRBO L1715; SJ12_4; UWOPS_92_298_4 2nd Wine 3N - 7 strains: ISA2211; CRBO L0308; CRBO L1733; CRBO L1782; VP1539; VP1544; YJS5382
<i>H. uvarum</i>	3 strains: L1433; NZ15; Y-1614
<i>L. thermotolerans</i>	3 strains: 18; AEB; CLIB292
<i>M. pulcherrima</i>	3 strains: L0675; NZ268; Y-7111
<i>S. cerevisiae</i>	10 strains: A24; Fx 10; GN; CRBO L0431; CRBO L0432; CRBO L0433; CRBO L0437; SB; X5; Y7327
<i>S. uvarum</i>	2 strains: U1; U3
<i>S. cerevisiae</i> x <i>S. uvarum</i>	2 strains: DU23; EU23
<i>Schizo. pombe</i>	3 strains: CRBO L0442; Y-11791; Y-12796
<i>Starm. bacillaris</i>	3 strains: 10_372; CRBO L0473; NZ12
<i>T. delbrueckii</i>	3 strains: B172; CLIB 230; CRBO L0705
<i>Tri. cantarellii</i>	3 strains: CRBO L0412; CRBO L0416; CRBO L0419
<i>Zygo. bailii</i>	3 strains: CLIB 213; CRBO L0446; CRBO L0536
<i>Zygo. rouxii</i>	2 strains: CLIB 233; CRBO L0314

**Table 2**

K-means classes from Normalized AUC with number of strains per species or genetic groups for the *B. bruxellensis* species.

UV-C response (K-means clustering)	Species//genetic groups	Number of strains
Class 1	<i>B. bruxellensis</i> //Wine/Kombucha 2N	3/19
	<i>B. bruxellensis</i> //1st Wine 3N	7/22
	<i>B. bruxellensis</i> //Wine/Beer 3N	5/21
	<i>B. bruxellensis</i> //Tequila/Bioethanol 3N	2/5
Class 2	<i>B. bruxellensis</i> //Wine 2N	6/30
	<i>B. bruxellensis</i> //Wine/Kombucha 2N	10/19
	<i>B. bruxellensis</i> //1st Wine 3N	15/22
	<i>B. bruxellensis</i> //Wine/Beer 3N	14/21
	<i>B. bruxellensis</i> //Tequila/Bioethanol 3N	2/5
	<i>B. bruxellensis</i> //Wine 2N	17/30
	<i>B. bruxellensis</i> //2nd Wine 3N	6/7
	<i>B. anomalus</i>	3/3
Class 3	<i>Tri. cantarellii</i>	3/3
	<i>Zygo. rouxii</i>	1/2
	<i>B. bruxellensis</i> //Wine/Kombucha 2N	6/19
	<i>B. bruxellensis</i> //1st Wine 3N	1/22
	<i>B. bruxellensis</i> //Wine/Beer 3N	2/21
	<i>B. bruxellensis</i> //Tequila/Bioethanol 3N	1/5
	<i>B. bruxellensis</i> //Wine 2N	7/30
	<i>B. bruxellensis</i> //2nd Wine 3N	1/7
	<i>H. uvarum</i>	1/3
	<i>L. thermotolerans</i>	1/3
	<i>S. cerevisiae</i>	9/11
Class 4	<i>Saccharomyces cerevisiae</i> x <i>Saccharomyces uvarum</i>	2/2
	<i>Zygo. bailii</i>	2/3
	<i>H. uvarum</i>	2/3
	<i>L. thermotolerans</i>	2/3
	<i>M. pulcherrima</i>	3/3
	<i>S. cerevisiae</i>	2/11
	<i>S. uvarum</i>	3/3
	<i>Schizo. pombe</i>	3/3
	<i>Starm. bacillaris</i>	3/3
	<i>T. delbrueckii</i>	3/3
	<i>Zygo. bailii</i>	1/3
	<i>Zygo. rouxii</i>	1/2

### 2.2.1. Growth monitoring from agar-plates

After treatment, plates were incubated at 24 °C in the dark. The growth was monitored every day: plates were imaged from an illuminated desk to avoid light gleam (model DMC-FS7, Panasonic Corporation, Japan, see Fig. 2A, D, G, J). Growth data was analysed with custom-made scripts in R (R Development Core Team, 2013). Briefly, plate images were imported on R using the *OpenImageR* package. The images of the plates were cropped for superimposition and the position of the drops was determined by manual clicking using the *grid* package and the *grid.locator* function. The area of each drop (in pixel) was calculated using automatic background subtraction. The dataset (around 90 000 measures including triplicates) was used to represent growth kinetics (Fig. 2B, E, H, K).

In order to analyse and normalize the obtained dataset, the growth kinetics were expressed as AUC (Area Under Curve). This unique parameter takes into account variations in lag-phases, exponential phases and the stationary phases. For each treatment and strain, growth kinetics were fitted using a three-parameter log-logistic distribution, AUCs were calculated and then normalized against AUCs without UV-C treatment (*Normalized AUC*, see Fig. 2C, F, I, L). AUC curves were subsequently analysed using K-means clustering (*cutRepeatedKmeans* function from *ClassDiscovery* package). Multi-way analyses of variance (ANOVA) were performed followed by post-hoc Tukey tests (*HSD.test* function from *agricolae* package).

### 2.3. UV-C treatment from liquid red wine

100 liters of SO<sub>2</sub> free 2018 Cabernet Sauvignon wine were used in this study for both yeasts adaptation and UV-C treatments. Wine's absorption coefficient at 254 nm ( $\alpha_{254}$ ) and turbidity were equal to 31.6 cm<sup>-1</sup> and 170 NTU respectively. The wine was pasteurised and kept at 10 °C until UV-C treatment. Wine sterility was controlled after pasteurization and before inoculation by plating on Total yeasts, Non-Saccharomyces yeasts, Lactic Acid bacteria and Acetic bacteria medias.

AWRI1608, L1735, L1737, CBS2499, AWRI1499 and L1746 *B. bruxellensis* strains were selected for this liquid assay. Those strains were firstly grown on YPD plates, and then inoculated in YPD liquid medium (24 °C). Yeasts were adapted to wine by successive transplantings in pasteurised wine. Populations were monitored by counting on Malassez cell with addition of methylene blue. Each strain was inoculated in pasteurised red wine for test at a concentration of 10<sup>4</sup> CFU mL<sup>-1</sup>.

#### 2.3.1. UV-C module treatment

The UV-C module consisted of a low-pressure mercury amalgam germicidal lamp, 78 cm long with maximum peak radiation at 254 nm, surrounded by a quartz sleeve (Suzhou Xicheng Water Treatment Equipment, China). A food grade fluorinated ethylene propylene (FEP) tube (Serto S.A.R.L, France), diameter 8/10 mm (inner/outer), chosen for its physical properties (flexibility and good UV-C transmittance), was coiled around the quartz sleeve (Fig. 1).

Due to the curvature of the pipe and hydrodynamic conditions applied (Junqua et al., 2020; Mishra and Gupta, 1979), Dean Vortices are generated allowing an homogenous treatment. Wine flow rate in the pipe was 200 L h<sup>-1</sup> and the applied dose for a single cycle of treatment was 1656 J.L<sup>-1</sup>. Wine samples were collected before UV-C treatment (UV0), after four cycles in the pilot with lamps turned off (OFF), after one, two, three of four cycles (UV1 = 1656 J.L<sup>-1</sup>, UV2 = 3312 J.L<sup>-1</sup>, UV3 = 4968 J.L<sup>-1</sup>, and UV4 = 6624 J.L<sup>-1</sup>). Between each cycles, the reactor was disinfected using 95% ethanol. Wine samples (50 mL) were collected and stored at 4 °C until microbiological analyses were performed. Each trial was conducted in triplicate.

#### 2.3.2. Microbiological analysis

Microbial counts were determined in triplicates by plating serial 10-fold dilutions of the samples and 100 µL was plated in 9 cm diameter petri dishes. Yeasts were enumerated on YPD plates with chloramphenicol (0.1 mg mL<sup>-1</sup>), biphenyl (0.15 mg mL<sup>-1</sup>) and actidione (0.5 mg mL<sup>-1</sup>) after 7 days of incubation at 27 °C. All incubations were performed in the dark, as DNA repair mechanisms are known to be less effective in dark conditions (Salcedo et al., 2007). The number of colonies counted was expressed in CFU.mL<sup>-1</sup> and the limit of detection was 1 CFU mL<sup>-1</sup>. Multi-way analyses of variance (ANOVA) were performed followed by post-hoc Tukey tests (*HSD.test* function from *agricolae*

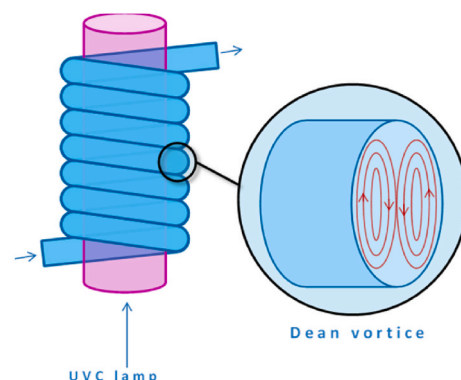
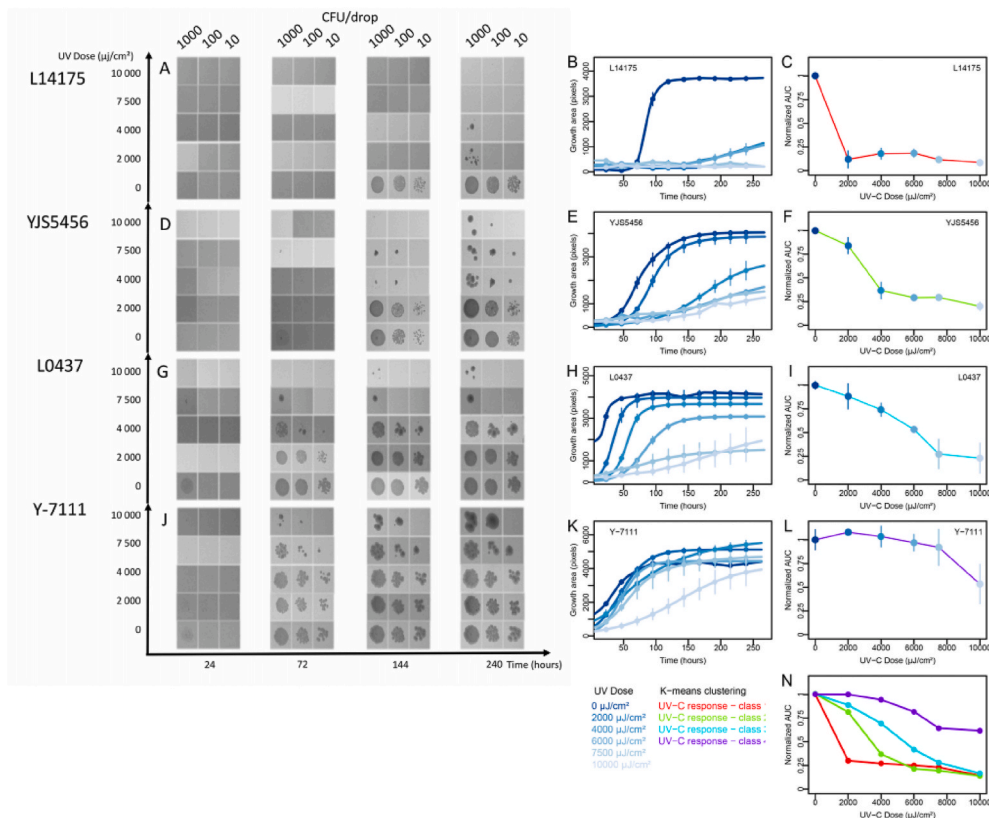


Fig. 1. Schematic representation of the coiled UV-C reactor.



**Fig. 2.** Impact of UV-C treatment on growth of four yeast strains. L14175 (*B. bruxellensis*, A,B,C), strain YJS 5456 (*B. bruxellensis*, D,E, F), L0437 (*S. cerevisiae*, G,H,I) and Y-7111 (*M. pulcherrima*, J,K,L). A, D, G, J are images from the three dilutions made on petri dishes with different UV-C doses. B, E, H, K show growth area (in pixels) over time for increasing UV-C treatment for the same density (1000 cells/drop). Growth kinetics were used to calculate AUC (Area Under Curve), normalized so that AUC equals 1 for UV = 0  $\mu\text{J cm}^{-2}$ . C, F, I, L show normalized AUC depending on UV-C treatment. Colours (red, green, turquoise and violet) represent the different AUC-UV-C dose response classes determined by K-means clustering (N). L14175 and L0437 stand for CRBO L14175 and CRBO L0423 respectively.

package, R software).

### 3. Results

#### 3.1. Surface UV-C treatment

##### 3.1.1. UV-C treatment and interspecific variability

In this work, UV-C sensitivity was assessed for 147 strains from fourteen yeast species associated with grape must and wine (Table 1). Besides some *Saccharomyces* species (*S. cerevisiae*, *S. uvarum* and some of their hybrids), several non-*Saccharomyces* species considered as beneficial from an oenological viewpoint were tested: *L. thermotolerans*, *T. delbrueckii* and *M. pulcherrima*. Abundant species associated with grapes or pre-fermentative stages such as *H. uvarum* or *S. bacillaris* were also included, as well as six species mostly considered as wine spoilers: *B. bruxellensis*, *B. anomalus*, *Tri. cantarelli*, *S. pombe*, *Z. rouxii* and *Z. bailii*.

Drop-plating was used to study UV-C sensitivity of the 147 yeast strains. Six increasing UV-C doses were applied (0, 2000, 4000, 6000, 7500 and 10 000  $\mu\text{J cm}^{-2}$ ) for three densities (number of initial cells per drop around 10, 100 or 1000). Petri dishes were pictured almost every day during ten days and the growth area was measured using home-made R-script. The dataset (around 90 000 measures with triplicates) was used to represent growth kinetics with time (Fig. 2).

Four different strains, representative of typical behaviours observed, are shown in Fig. 2 (two *B. bruxellensis*, one *S. cerevisiae*, one *M. pulcherrima*). In general, higher UV-C doses increased the lag-phase and decreased the growth rate and the maximal population (Fig. 2B, E, H, K). Growth curves were then used to calculate the normalized AUC (Area Under Curve) represented in Fig. 2C, F, I, L. Strain L14175 (*B. bruxellensis*) was highly sensitive to UV-C and showed a strong growth decrease for low UV-C dose (<0.25 of Normalized AUC at 2 000  $\mu\text{J cm}^{-2}$ , Fig. 2C). Strains YJS5456 and L0437 (*B. bruxellensis* and *S. cerevisiae* respectively, Fig. 2E and H) were sensitive to higher dose of UV-C (4000 and 7500  $\mu\text{J cm}^{-2}$ ). By contrast, some strains appeared

poorly impacted by low UV-dose: the growth of *M. pulcherrima* strain Y-7111 (Fig. 1G & H) was poorly affected by UV-C-dose lower than 7500  $\mu\text{J cm}^{-2}$ .

K-means clustering was used to sort AUC curves in different profiles, and resolved into four classes (Fig 2N). The fourteen tested species were sorted into one or two classes except for *B. bruxellensis*, which was distributed in three classes (Table 3). K-means class 1 corresponded to the most UV-C sensitive strains, and contained only *B. bruxellensis* strains. By contrast, all tested strains from *S. pombe* or *S. bacillaris* were sorted into class 4, corresponding to less UV-C sensitive behaviour.

In order to determine whether yeast growth was mostly affected by UV-C treatment, density and/or species, we performed a three-way ANOVA (Table 3, 1st ANOVA). All three factors (Species, Drop density and UV-C dose) were strongly significant, with very low p-values (<0.001). UV-C dose explained most of the percentage of variation of the data (59.99%) followed by species and density factors (8.93% and

**Table 3**

Impact of various factors on yeast growth. Analysis of variance was performed either on the whole dataset (147 strains distributed in 14 species) or only on the *B. bruxellensis* strains (104).

1st ANOVA			
Factors	Degree of Freedom	Percentage of Variation	P-values
Species	13	8.93%	<2e <sup>-16</sup>
Drop density	2	1.71%	6.71e <sup>-127</sup>
UV-C dose	5	59.99%	<2e <sup>-16</sup>
Residuals	10262	29.37%	
2nd ANOVA			
Factors	Degree of Freedom	Percentage of Variation	P-values
Genetic group	5	0.51%	5.39e <sup>-32</sup>
Drop density	2	1.47%	1.07e <sup>-96</sup>
UV-C dose	5	74.83%	<2e <sup>-16</sup>
Residuals	7197	23.18%	



1.71% respectively). Post-hoc Tukey tests were performed to determine significance groups (Fig. 3): the different UV-C dose were significantly separated from one another, with 10 000  $\mu\text{J cm}^{-2}$  associated to the lower Normalized AUC (ie lower yeast growth) (Fig. 3A). For drop density, higher initial densities were associated with higher growth (Fig. 3B). Finally, Tukey tests revealed significant differences between species, with *Schizo. Pombe* and *M. pulcherrima* showing highest Normalized AUC at high UV-C doses, and *Tri. Canterellii* and *B. anomalus* showing the lowest Normalized AUC (Fig. 3C) in accordance with K-means clustering. In particular, *Schizo. pombe*, *M. pulcherrima*, *Starm. bacillaris* and *L. thermotolerans* display a poor sensitivity to the two first UV-C doses used (2 000, 4000  $\mu\text{J cm}^{-2}$ , Fig. 3C) with a low reduction of the Normalized AUC. For the others species, all UV-C doses affected the yeast growth.

### 3.1.2. *B. bruxellensis* sensitivity

In winemaking, the most of the spoilage treat is due to *B. bruxellensis*. Here, we included 104 strains of *B. bruxellensis* distributed in all defined genetic groups (Lisanti et al., 2019) to assess their sensitivity to UV-C treatment (Table 1). K-means clustering sorted *B. bruxellensis* in three classes, suggesting a large intra-specific variability. To determine whether UV-C treatment, density and/or genetic group affected strain growth, we performed a three-way ANOVA followed by Tukey test (Table 3, 2nd ANOVA). All three factors significantly affected *B. bruxellensis* growth (p-values < 0.001), with UV-C dose, density and genetic group explaining respectively 74.83, 1.47 and 0.51% of the total variation. The higher the UV-C dose, the lower the growth (Fig. 4A) while lower drop densities were associated with lowest growth (Fig. 4B). When considering the effect of the genetic group, significant differences were recorded for 2000, 4000, 6000 and 7500  $\mu\text{J cm}^{-2}$  UV-C doses. At 2000  $\mu\text{J cm}^{-2}$ , three groups (2nd Wine 3N, Wine/Kombucha 2N, Wine 2N) showed higher normalized AUC (Tukey test 'a') than Wine/Beer 3N, 1st Wine 3N and Tequila/Bioethanol 3N (Tukey 'b'). For higher UV-C doses, the rank of the genetic groups changed marginally, but in general, the Wine/Kombucha 2N group was one of the less sensitive group,

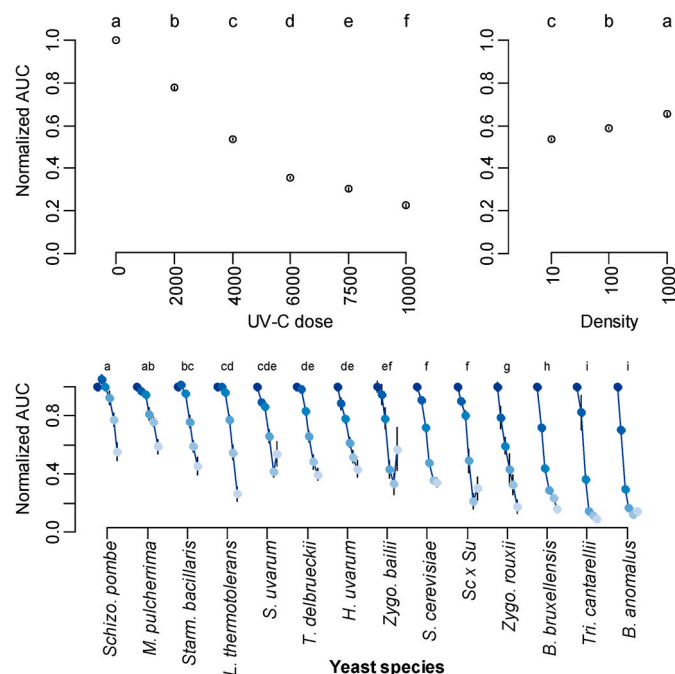


Fig. 3. Impact of UV-C treatment (A), drop density (B) and species (C) on yeast growth. Yeast growth was assessed using Normalized AUC. The mean of repetitions  $\pm$  standard errors were represented for each factor. Upper letters represent significance groups (ANOVA followed by Tukey's post-hoc test,  $\alpha = 5\%$ ). *Sc x Su* stands for *S. cerevisiae* x *S. uvarum* hybrids.

while the 1st Wine 3N was systematically the most impacted by UV-C.

Finally, at the higher UV-C dose tested (10000  $\mu\text{J cm}^{-2}$ ), the normalized AUCs were below 0.2 for all genetic groups without significant difference, indicating that UV-C treatment is efficient for all *B. bruxellensis* strains.

Yeast growth was assessed using Normalized AUC. The mean of repetitions  $\pm$  standard errors were represented for each factor. Upper letters represent significance groups (ANOVA followed by Tukey's post-hoc test,  $\alpha = 5\%$ ).

### 3.2. UV-C and liquid media

The impact of UV-C treatment on *B. bruxellensis* strains in liquid wine was evaluated. The UV-C pilot was designed for the treatment of large volumes of wine, and 100 liters of red wine were used to evaluate the impact of different UV-C doses on a few strains. Six strains of *B. bruxellensis* were studied, belonging to the three main genetic groups associated with winemaking (Wine 2N, 1st Wine 3N and Wine/Beer 3N).  $\text{Log}_{10}$  reduction has been used as the factor describing the strain response to UV-C treatments in wines (Fig. 5). A two-way ANOVA was performed to determine the effect of UV-C dose and strain on the treatment efficiency. Both factors were strongly significant (p-value < 0.001) and explained most of the percentage of data variation, with 74.4% for UV-C dose and 3.7% for strain.

No significant difference was recorded between UV0 and OFF modalities indicating that pumping through the UV-C reactor did not induce cell mortality. UV1 (1656 J.L<sup>-1</sup>), UV2 (3312 J.L<sup>-1</sup>), UV3 (4968 J.L<sup>-1</sup>) and UV4 (6624 J.L<sup>-1</sup>) treatments were all significantly different from both controls (UV0 and OFF) and between each other, with the greater the UV-C dose applied, the greater the logarithmic reduction achieved (Fig. 5A).

UV3 treatment (4968 J.L<sup>-1</sup>) was enough to achieve 4.70 and 5.17  $\text{log}_{10}$  reduction for AWRI1499 and L1746 strains (1st Wine 3N sulphite resistant) respectively, resulting in populations lower than 1 CFU mL<sup>-1</sup>. UV4 (6624 J.L<sup>-1</sup>) treatment was required to achieve the same level of population (< 1 CFU mL<sup>-1</sup>) for CBS2499, AWRI1608 and L1735 strains with respectively 5.33, 4.97 and 5.18  $\text{log}_{10}$  reduction. L1737 strain was the only one which did not achieve a cell population lower than 1 CFU mL<sup>-1</sup> even after UV4 treatment and with the lowest initial cell population.

Considering the genetic groups of the strains (Fig. 5B), the 1st Wine 3N group (L1746 and AWRI1499) was the more sensitive to UV-C treatment, the Wine 2N group (CBS2499 and L1737) was the less sensitive to UV-C while the Wine/Beer 3N group (AWRI1608 and L1735) was found to be intermediary.

## 4. Discussion

### 4.1. Surface and liquid assays are complimentary approaches to assess the impact of UV-C

The present study aimed to evaluate the sensitivity to UV-C treatment of yeast species related to winemaking with a focus on *B. bruxellensis* species. For this, a large plate screening method was developed to test 147 strains of 14 different species with a focus on *B. bruxellensis* (104 strains). We then confirmed the impact of UV-C treatment in red wine. Due to practical constraints (100 liters of wine necessary for 6 strains), only strains of *B. bruxellensis*, the main wine spoiler, were tested. Regarding plate screening method, all the tested species were significantly impacted by increasing UV-C doses. The number of cells per drop was also a significant factor indicating an impact of the initial cell densities. The density effect could be explained by a shadowing effect, the cells acting as absorbance particles and decreasing UV-C transmittance. This cell density impact was also observed in grape must (Diesler et al., 2019). The impact of UV-C treatment was congruent with previous studies at similar UV-C doses

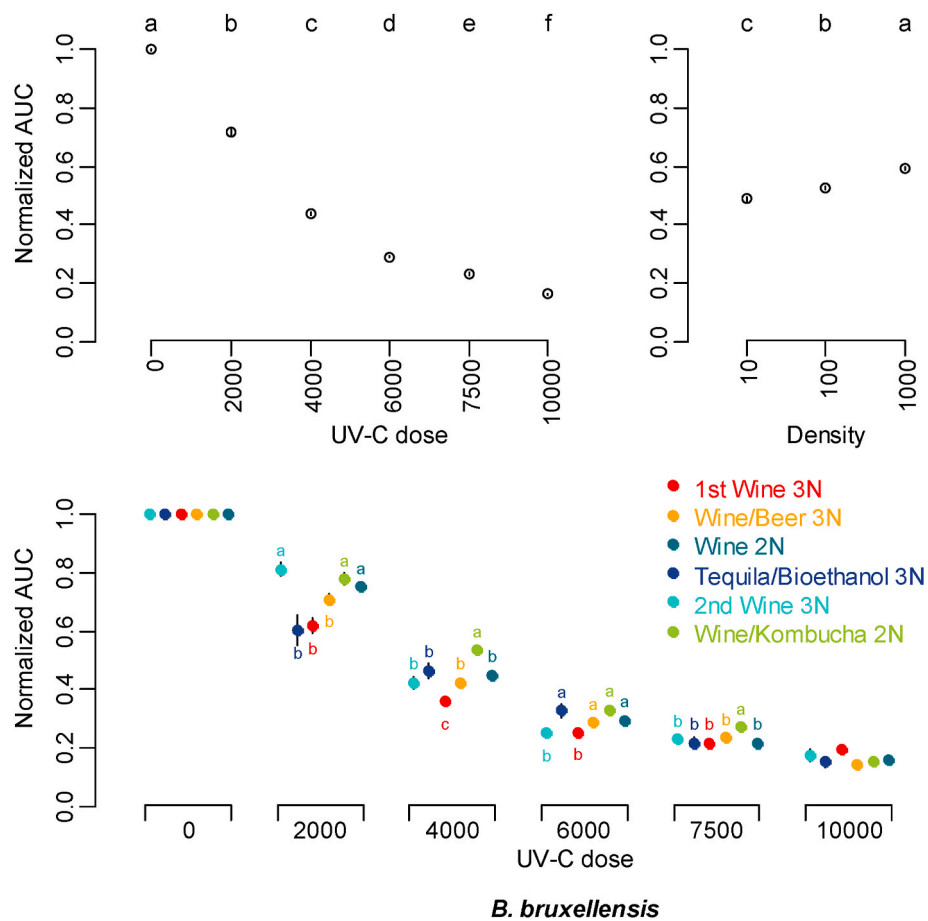


Fig. 4. Impact of UV-C treatment (A), drop density (B) and species (C) on yeast growth regarding the genetic groups of 104 strains of *B. bruxellensis*.

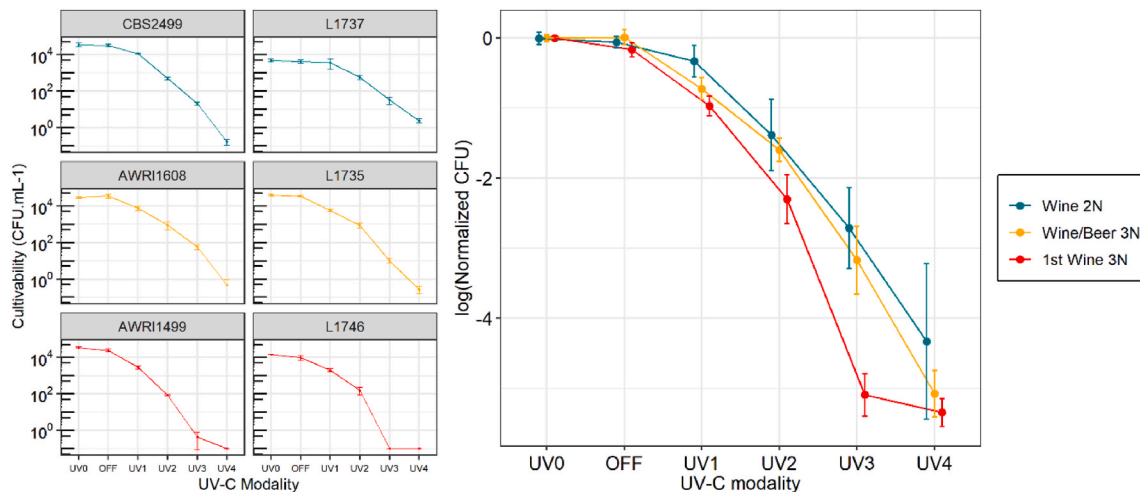


Fig. 5. Survival curves of *B. bruxellensis* strains for UV-C treatment in red wine. Cultivability is expressed in  $\text{CFU.mL}^{-1}$  (A). Normalized survival curves of *B. bruxellensis* depending on the genetic groups of the strains (B). For each measure,  $\text{CFU.mL}^{-1}$  were normalized to the mean of the corresponding UV0 modality (Normalized CFU), the mean of triplicates  $\pm$  standard errors were represented for each modality. UV0 correspond to the wine samples collected before UV-C treatment, OFF correspond to four cycles in the pilot with lamps turned off and UV1, UV2, UV3, UV4 after one, two, three and four cycles (UV1 =  $1656 \text{ J.L}^{-1}$ , UV2 =  $3312 \text{ J.L}^{-1}$ , UV3 =  $4968 \text{ J.L}^{-1}$ , and UV4 =  $6624 \text{ J.L}^{-1}$ ). The colours correspond to the genetic groups of the strains. L1737, L1735, L1746 stand for CRBO L1737, CRBO L1735 and CRBO L0423 respectively.

(Pulschen et al., 2015; Longan et al., 2017; Kim et al., 2017). Surface UV-C assays revealed strong difference of sensitivity between wine species. Similar results were obtained in apple juice: *S. cerevisiae* and *Z. bailii* were less sensitives than *B. bruxellensis* and *B. anomalous* (Gouma

et al., 2015). Moreover, the surface plate screening revealed intraspecific variation within *B. bruxellensis*, which was confirmed by our liquid trials. Although our plate screening did not allow the determination of the log reduction after treatment, it allowed the screening of a large

number of species (14 yeasts) and strains (147 distinct isolates), unachievable with liquid assays. Moreover, UV-C surface treatment could be useful for oenological equipment disinfection and such plate-screening approach is pertinent to assess its relevance. The UV4 modality ( $6624 \text{ J.L}^{-1}$ ) achieved the reduction of cell population lower than  $1 \text{ CFU mL}^{-1}$  except for L1737 strain. Those results confirmed the efficiency of UV-C treatment even for a very absorbent red wine ( $\alpha_{254} = 31.6 \text{ cm}^{-1}$ ) contaminated by high populations ( $10^4 \text{ CFU mL}^{-1}$ ) of *B. bruxellensis* and valid our large plate screening and data analysis approach as an efficient tool for the assessment of a control method.

#### 4.2. Wine yeast species show strong sensitivity variation to UV-C treatments

Although all yeast species showed sensitivity to UV-C, strong interspecies variations were observed within the dose range used. Numerous hypotheses can be made to explain interspecific sensitivity variation. Secondary metabolites such as photoprotective pigments (carotenoid, melanin) or mycosporine-like amino acids (MAAs) are known to protect from UV-C radiation in bacteria and fungi (Pulschen et al., 2015; Wang and Casadevall, 1994; Gao and Garcia-Pichel, 2011; Blachowicz et al., 2020; Singaravelan et al., 2008; Libkind et al., 2009). The UV-C radiations are known to impact DNA by creating CPDs (Cyclobutane Pyrimidine Dimers) leading to the non-transcription and non-replication of DNA, ultimately inducing deformation of the DNA helix and occasioning double strand breaks (Ikehata and Ono, 2011). DNA damages caused by UV-C treatment were shown to be the primary factors affecting microorganism death (Kim et al., 2017). To repair CPDs, two DNA repair mechanisms are described, the photoreactivation performed by photolyases (Fernández Zenoff et al., 2006; Zhang et al., 2017) and the Nucleotide Excision Repair (NER) (Yu et al., 2011). The first mechanism is highly species dependent, with some species lacking photolyase genes (Lucas-Lledó and Lynch, 2009; Mei and Dvornyk, 2015) indicating a low selective pressure on this function. Subsequent works will be necessary to identify the molecular mechanism(s) explaining variation in UV-C sensitivity amongst wine yeast species.

Among the considered species, *Schizo. pombe*, *M. pulcherrima* and *Starm. bacillaris* were found to be the less affected by UV-C radiations. *L. thermotolerans*, *S. uvarum*, *T. delbrueckii*, *H. uvarum* and *S. cerevisiae* exhibited an intermediary phenotype while *Z. bailii*, *Z. rouxii*, *B. bruxellensis*, *T. cantarellii* and *B. anomalus* presented the most sensitive phenotype. Our results are mostly in accordance with a previous study reporting that *M. pulcherrima* was less sensitive than *S. cerevisiae* and *H. uvarum*, and was still viable after  $1.0 \text{ kJ.L}^{-1}$  UV-C dose in red must (Diesler et al., 2019). The same authors showed that *S. cerevisiae* and *H. uvarum* sensitivities in grape must were similar and resulted in a total loss of detectable cultivability with  $600 \text{ J.L}^{-1}$  of UV-C dose.

Interestingly, some of the less sensitive species (*Schizo. pombe*, *Starm. bacillaris*, *M. pulcherrima*) are known to be vineyard resident, frequently isolated from grapes or other fruit surfaces (Sipiczki, 2016; Tristezza et al., 2013; Masneuf-Pomarede et al., 2015; Wang et al., 2015). Intermediate species (*L. thermotolerans*, *S. uvarum*, *T. delbrueckii*, *H. uvarum* and *S. cerevisiae*) are ubiquitous, frequently isolated from vineyards but also from the cellar environment (fermentative grape must, wine, equipment, etc.) (Sipiczki, 2016; Tristezza et al., 2013; Wang et al., 2015; van Breda et al., 2013; Albertin et al., 2014; Zott et al., 2008; Goddard and Greig, 2015). By contrast, the more sensitive species (*Z. bailii*, *Z. rouxii*, *B. bruxellensis*, *T. cantarellii* and *B. anomalus*) are mainly cellar-resident, adapted to wine and/or to anthropic environments and scarcely isolated from grapes or natural environments (Portugal et al., 2015; Zuehlke et al., 2013; Oro et al., 2019). On earth, stratospheric ozone layer absorbs UV-C radiation and terrestrial UV radiations are mostly composed of UV-A and UV-B that can create the same types of damages (Sinha and Häder, 2002). These UV radiations are known to influence and modulate yeast community composition on grapes (Longan et al., 2017; Valero et al., 2007). A weaker selective

pressure on UV-C tolerance mechanisms at cellar scale could explain from an evolutionary viewpoint the differences between wine yeast species. Beside *Saccharomyces* species used to control and complete wine alcoholic fermentation, none conventional yeasts (*T. delbrueckii*, *L. thermotolerans* and *M. pulcherrima*) can be used as technological auxiliaries by winemakers for a wide range of applications as acidification, biocontrol agent or to improve aromas (Roudil et al., 2019). It could be interesting to evaluate the impact of UV-C treatment in mix-inoculated wine or must on yeasts spoilers and auxiliaries in order to target specifically unwanted ones. In any case, this observation highlights the potentiality of UV-C treatment to eliminate cellar-residents species, found on oenological equipment and in wines.

#### 4.3. *B. bruxellensis* species shows low but significant intra-specific variation to UV-C sensitivity

Regarding *B. bruxellensis*, which is highly sensitive to UV-C treatment, some intraspecific variability, low yet significant, was recorded. *B. bruxellensis* is known to be phenotypically versatile. When focusing on the main genetic groups associated with wine, the 1st Wine 3N group is the more sensitive to UV-C, followed by Wine/Beer 3N and Wine 2N. Comparable results were obtained in our red wine trials. Some of *B. bruxellensis* strains were shown to be resistant to sulphite addition in wine (Curtin et al., 2012b; Varela et al., 2019). Recently, a study linked this peculiar ability to a specific genetic group, 1st Wine 3N (Avramova et al., 2019). This group being the most sensitive to UV-C treatment in both surface and liquid trials, UV-C stabilization could emerge as a pertinent method to control *B. bruxellensis* in winemaking.

*B. bruxellensis* is a diplo-triploid species complex, resulting in diploid and triploid (comprising diploid genome and one divergent haploid genome). Polyploidy can be involved in UV-C sensitivity, by increasing the redundancy of essential genes, increasing the cell volume and organites. In *S. cerevisiae*, it was shown that polyploids as a better survival rates compared to diploid with UV-C treatment (Lidzbarsky et al., 2009; Sasaki, 1992). In our case, the triploids strains (that have probable hybrid origin) belong to the more sensitive genetic groups (1st Wine 3N, Wine/Beer 3N). We can hypothesize that interspecific hybrids have less-efficient repair systems due to the combination of divergent genomes, resulting in accrued UV-C sensitivity.

## 5. Conclusion

The main yeast species encountered in winemaking were compared with respect to their sensitivity to UV-C radiation first with a plate screening approach. Strong interspecific variation was observed. Interestingly, UV-C sensitivity was associated with the ecological niche of the yeast species, with the cellar resident species being more sensitive to UV-C treatment than the vineyard-resident ones. Amongst spoilers, *B. bruxellensis* was highly sensitive to UV-C compared to others species. Intraspecific variability was observed, depending on genetic groups and was confirmed in liquid trials. The strains from the 1st Wine 3N genetic group were more sensitive compared to the strains from the Wine/Beer 3N and Wine 2N groups. Indeed, 1st Wine 3N strains required 25% less energy than other strains to achieve 5  $\text{Log}_{10}$  reduction. Strains from this group were shown to be sulfite tolerant/resistant, thus, this support the interest of UV-C treatment in wine context. Overall, wine treatments were effective, proving that UV-C could be used to control *B. bruxellensis* even at high levels of cell population and in absorbent red wine.

This study does not address the possible wine modification induced by the process but additional work is underway to characterize the impact on organoleptic qualities at UV-C doses required for microbiological stabilization.

#### Declaration of competing interest

There is no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fm.2021.103864>.

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## Evaluating the influence of operational parameters of pulsed light on wine related yeasts: focus on inter- and intra-specific variability sensitivity

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### ABSTRACT

In oenology, there is a growing demand by consumers for wines produced with less inputs (such as sulphite, frequently used for microbial control). Emerging control methods for managing microorganisms in wine are widely studied. In this study, the efficiency of pulsed light (PL) treatment was investigated. A drop-platted system was used to evaluate the impact of three PL operational parameters: the fluence per flash, the total fluence and the flash frequency. Fluence per flash appeared to be a key parameter prior to total fluence, thus demonstrating the importance of the effect of peak voltage during PL treatments. The efficiency of PL treatment was assessed on 198 strains distributed amongst fourteen yeast species related to wine environment, and an important variability in PL response was observed. *Brettanomyces bruxellensis* strains were strongly sensitive to PL, with intraspecific variation. PL was then applied to red wines inoculated with 9 strains of *B. bruxellensis*, *Saccharomyces cerevisiae* and *Lachancea thermotolerans*. Results confirmed interspecific response variability and a higher sensitivity of *B. bruxellensis* species to PL. Wine treatments with a total fluence of 22.8 J cm<sup>-2</sup> resulted in more than 6 log reduction for different *B. bruxellensis* strains. These results highlight the potential of PL for wine microbial stabilization.

### 1. Introduction

Wide varieties of food preservation methods are available including heating, freezing, filtration and addition of preservatives. However, these methods are cost effective and can impair food quality and therefore major efforts have been made to develop alternative technologies with a lower impact on food quality. Pulsed light (PL) is a non-thermal technology used to decontaminate surfaces or liquid by inactivating microorganisms using short-time pulses of an intense broad-spectrum light (John and Ramaswamy, 2018; Kramer et al., 2017a; Pirozzi et al., 2021). PL relies on a wide wavelength range of 200–1100 nm, which includes ultraviolet (UV, 200–400 nm), visible light (VIS, 400–700 nm), and near-infrared region (IR, 700–1100 nm). PL treatment is based on the accumulation of high discharge voltage in a capacitor where the stored electrical energy is delivered as intermittent

short pulses through a light source filled with xenon gas. This xenon-light source emits a broad-spectrum light flash (typically 1 to 10 pulses per second) with approximately 25% in the UV range (Keener and Krishnamurthy, 2014). Microbial inactivation is commonly attributed to photochemical damage of DNA caused by UV wavelengths, enhanced by photothermal effect due to local overheating and photophysical effect on proteins and membranes (Elmnasser et al., 2007; Ikehata and Ono, 2011). Photophysical effect is responsible for membrane disruption and vacuole extension observed in yeast (*Saccharomyces cerevisiae*) and bacterial cells (*Bacillus subtilis*) (Clair et al., 2020; Ferrario and Guerrero, 2017; Nicorescu et al., 2013; Takeshita et al., 2003). The concomitant action of these three mechanisms (e.g. photochemical, photothermal and photophysical effects), which may act synergistically, as well as the high peak power involved, can explain the generally reported higher decontamination effectiveness of PL in comparison with

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continuous wave UV light treatment (Clair et al., 2020). The main process parameters governing PL efficiency are the fluence ( $\text{J}\cdot\text{cm}^{-2}$ ) over exposure time (s), the frequency (Hz), the number of pulses applied (n), the pulse width ( $\tau$ ) and the peak power (W) (Barbosa-Canovas et al., 2000; Pataro et al., 2016).

Since the first works on disinfection with flash lamps performed in the late 1970s in Japan, PL has been widely explored. Various applications were developed, ranging from the decontamination of surfaces (packaging, processing equipment, medical devices, and solid foods) to the microbial stabilization of liquid (disinfection of air, water, food liquids) (Aguirre et al., 2014; Ferrario and Guerrero, 2017; Hwang et al., 2015; Levy et al., 2012; Pataro et al., 2011; Proulx et al., 2017). In liquid application, PL efficiency may be restricted by its low penetrance. This difficulty is usually overcome via the design of specific reactors maximising microorganism exposure to light (Junqua et al., 2020).

This innovative non-thermal control method was started to be evaluated only recently in oenology. For example, PL treatment was shown to be effective in a limited extent, and reduced the microbial density (e.g. yeast and bacteria) on red grape surfaces, by about  $1.2 \log_{10} \text{CFU mL}^{-1}$  with  $10.7 \text{ J cm}^{-2}$  (Escott et al., 2017, 2021). A reduction of  $4.89 \log_{10} \text{CFU mL}^{-1}$  of *Escherichia coli* was found upon the application of 80 pulses ( $0.66 \text{ J cm}^{-2}$ , pulse $^{-1}$ ) to red grape juice (Xu et al., 2019). During winemaking, yeasts management is essential to ensure efficient fermentations and to limit the risk of organoleptic changes. Indeed, while *Saccharomyces* yeasts are essential for must fermentation, some species are considered as spoilers (e.g. *Brettanomyces bruxellensis*, *B. anomalus*, *Trigonopsis cantarellii*, *Zygosaccharomyces rouxii*, *Z. bailii*, *Schizosaccharomyces pombe*, etc.). Those species are able to affect the fermentation kinetics and/or to produce off-flavours and/or to modify wine's physical properties, lowering the quality of the final product (Loureiro and Malfeito-Ferreira, 2003; Padilla et al., 2016). Among them, *Brettanomyces bruxellensis* is certainly the most feared spoiler, damaging up to 25% of the world red wine production (Alston et al., 2021; Oro et al., 2019; Romano et al., 2008). *B. bruxellensis* yeasts are able to produce volatile phenols such as 4-vinylphenol, 4-vinylguaiacol, 4-ethylphenol and 4-ethylguaiacol, whose aromatic notes are unpleasant and described as animal, leather, horse, stable or pharmaceutical (Chatonnet et al., 1992; Oelofse et al., 2008; Schopp et al., 2013). Several recent studies have shown both high genetic and phenotypic diversity of *B. bruxellensis* in different wine regions (Avramova et al., 2018a; Cibrario et al., 2020; Eberlein et al., 2021; Gounot et al., 2020). This important diversity is reflected in the relative sensitivity of different strains to existing microbial stabilization methods used during winemaking. For instance, genetic groups resistant to sulphite addition or expressing lower sensitivity to chitosan or to UV-C treatment have been identified (Avramova et al., 2018b; Paulin et al., 2020; Pilard et al., 2021). Preliminary results on wine microbial stabilization with PL were obtained in white wine with a maximal reduction of  $2.10 \log$  of *B. bruxellensis* (Pérez-López et al., 2020). PL could thus be interesting in the wine industry to control the risk associated with *Brettanomyces* yeasts contamination.

The aim of this research was to investigate the PL sensitivity of fourteen yeast species associated with winemaking, with a focus on *Saccharomyces cerevisiae* and *Brettanomyces bruxellensis* to identify inter- and intra-specific variability. Specifically, this study explored the dose-effect relationship between PL total fluence, fluence per flash, frequency, and inactivation of microorganisms naturally present in wine. A plate-based screening approach was applied to a large collection of yeast species and strains (14 species and 198 strains). Different PL treatment's modalities (with varying fluence per flash, frequency, and total fluence) were considered and the variability within *B. bruxellensis* and *S. cerevisiae* was compared. In a second step, six *B. bruxellensis*, two *S. cerevisiae* and one *L. thermotolerans* strains were selected to evaluate PL potential to inactivate those yeasts in a highly absorbent red wine. In this part, the inoculated red wine was treated with several PL intensities using a lab-scale continuous flow PL apparatus.

## 2. Materials and methods

### 2.1. Yeast strains

The 198 strains from 14 species used in this study were collected from different laboratories or collections (Table 1, Supplemental Table 1). Strains were grown and maintained in Yeast extract peptone dextrose (YPD) plates at  $24^\circ\text{C}$  ( $10 \text{ g}\cdot\text{L}^{-1}$  yeast extract,  $10 \text{ g}\cdot\text{L}^{-1}$  peptone,  $20 \text{ g}\cdot\text{L}^{-1}$  glucose,  $20 \text{ g}\cdot\text{L}^{-1}$  agar, Fisher Scientific, Hampton, New

**Table 1**

List of the yeast strains used in this work.

Species	Strains
<i>B. anomalus</i>	3 strains: BR 23-4; CLIB 304; NRRL Y-17522 T
<i>B. bruxellensis</i>	Wine 2N - 34 strains: 1961_MX_M1_E2; CBS 2499; ISA2150; CRBO L0469; CRBO L0614; CRBO L14163; CRBO L1703; CRBO L17111; CRBO L1714; CRBO L1715; CRBO L1727; CRBO L1751; YJS5302; YJS5319; YJS5320; YJS5345; YJS5347; YJS5357; YJS5373; YJS5385; YJS5392; YJS5416; YJS5422; YJS5426; YJS5440; YJS5447; YJS5449; YJS5453; YJS5456; YJS5458; YJS5461; YJS5463; YJS5479; YJS5485 Wine/Kombucha 2N - 19 strains: 15_1; ISA1601; YJS5301; YJS5310; YJS5334; YJS5340; YJS5344; YJS5349; YJS5363; YJS5368; YJS5384; YJS5398; YJS5402; YJS5406; YJS5407; YJS5413; YJS5417; YJS5420; YJS5431 Wine/Beer 3N - 25 strains: 20T13_05; 20T13_07; 20T14_01; 20T14_03; AWRI1608; CDR222; GB62; ISA2397; CRBO L0417; CRBO L14155; CRBO L14194; CRBO L17112; CRBO L1741; CRBO L1749; CRBO L1771; LB15107g; LB15110g; MLC_296_2014_9; NL045; NL059; VP1519; YJS5396; YJS5397; YJS5400; YJS5454 1st Wine 3N - 19 strains: 12 LT_VGC3_c_10; 20T13_02; 33_2; AWRI1499; CDR217; GB12; GSP1509; CRBO L14174; CRBO L14175; NL050; YJS5408; YJS5434; YJS5445; YJS5459; YJS5469; YJS5473; YJS5476; YJS5478; YJS5487 Tequila/bioethanol 3N - 6 strains: CBS 5512; CBS 6055; CRBO L14169; CRBO L17108; SJ12_4; UWOPS_92_298_4 2nd Wine 3N - 8 strains: ISA2211; CRBO L0308; CRBO L14190; CRBO L1733; CRBO L1782; VP1539; VP1544; YJS5382 3 strains: CRBO L1433; NZ15; Y-1614 3 strains: 18; AEB; CLIB292 3 strains: CRBO L0675; NZ268; Y-7111 Beer - 10 strains: 227; 382; 650; Nottingham; premium gold; S-04; US-56; Windsor; Y7327; Y7328 Bread - 10 strains: 215; 319; 324; 646; Hironnelle; SBA; SBB; SBC; SBD; SBE Dist - 5 strains: A24; alcotec 48; Y-963; YB-427; YB-428 Food - 2 strains: Y-767; YB-360 Fruit - 5 strains: Y-6678; Y-7568; Y-965; YB-210; YB-4081 Fruit juice - 4 strains: Y-129; Y-2230; Y-6275; Y-6278 Nature - 2 strains: Y-35; Y-7567 Wine - 13 strains: 154; 157; 328; 479; Fx 10; GN; CRBO L0431; CRBO L0432; CRBO L0433; CRBO L0437; SB; X5; Y-1301 2 strains: U1; U3 2 strains: DU23; EU23 3 strains: CRBO L0442; Y-11791; Y-12796 3 strains: 10_372; CRBO L0473; NZ12 3 strains: B172; CLIB 230; CRBO L0705 3 strains: CRBO L0412; CRBO L0416; CRBO L0419 2 strains: CLIB 213; CRBO L0536 2 strains: CLIB 233; CRBO L0314
<i>H. uvarum</i>	3 strains: CRBO L1433; NZ15; Y-1614
<i>L. thermotolerans</i>	3 strains: 18; AEB; CLIB292
<i>M. pulcherrima</i>	3 strains: CRBO L0675; NZ268; Y-7111
<i>S. cerevisiae</i>	Beer - 10 strains: 227; 382; 650; Nottingham; premium gold; S-04; US-56; Windsor; Y7327; Y7328 Bread - 10 strains: 215; 319; 324; 646; Hironnelle; SBA; SBB; SBC; SBD; SBE Dist - 5 strains: A24; alcotec 48; Y-963; YB-427; YB-428 Food - 2 strains: Y-767; YB-360 Fruit - 5 strains: Y-6678; Y-7568; Y-965; YB-210; YB-4081 Fruit juice - 4 strains: Y-129; Y-2230; Y-6275; Y-6278 Nature - 2 strains: Y-35; Y-7567 Wine - 13 strains: 154; 157; 328; 479; Fx 10; GN; CRBO L0431; CRBO L0432; CRBO L0433; CRBO L0437; SB; X5; Y-1301 2 strains: U1; U3 2 strains: DU23; EU23 3 strains: CRBO L0442; Y-11791; Y-12796 3 strains: 10_372; CRBO L0473; NZ12 3 strains: B172; CLIB 230; CRBO L0705 3 strains: CRBO L0412; CRBO L0416; CRBO L0419 2 strains: CLIB 213; CRBO L0536 2 strains: CLIB 233; CRBO L0314
<i>S. uvarum</i>	3 strains: CRBO L1433; NZ15; Y-1614
<i>Sc x Su (S. cerevisiae x S. uvarum synthetic hybrids)</i>	2 strains: DU23; EU23
<i>Schizo. pombe</i>	3 strains: CRBO L0442; Y-11791; Y-12796
<i>Starm. bacillaris</i>	3 strains: 10_372; CRBO L0473; NZ12
<i>T. delbrueckii</i>	3 strains: B172; CLIB 230; CRBO L0705
<i>Tri. cantarellii</i>	3 strains: CRBO L0412; CRBO L0416; CRBO L0419
<i>Zygo. bailii</i>	2 strains: CLIB 213; CRBO L0536
<i>Zygo. rouxii</i>	2 strains: CLIB 233; CRBO L0314

PL treatment of yeast on YPD solid medium.

Hampshire, USA). These species were selected for their relevance in the winemaking process.

Strains were grown in liquid YPD media (10 g.L<sup>-1</sup> yeast extract, 10 g.L<sup>-1</sup> peptone, 20 g.L<sup>-1</sup> glucose) for 24 h and the population was estimated by optical density (FLUOstar Omega, MNGLabtech, France). Droplets of 2 µL of serial dilutions (0.5, 0.05, 0.005 and 0.0005 Optical Density at 600 nm, corresponding to concentrations around 10<sup>6</sup>, 10<sup>5</sup>, 10<sup>4</sup> and 10<sup>3</sup> cells.mL<sup>-1</sup>) were spotted onto solid medium (YPD with 20 g.L<sup>-1</sup> agar, Ø4mm-diameter drops), aiming at obtaining 4 different densities (around 1000, 100, 10, 1 CFU.drop<sup>-1</sup>). Drops were produced in triplicate for each condition on square Petri dishes (12\*12 cm). Spotted plates were then placed in a PL. Box (Sanodev, France), at 30 cm distance from the flash lamp and treated with different modalities (Table 2). First, modalities 1, 5, 6, 8, 10, 11 and 12 were designed to study the impact of an increasing fluence with the same flash frequency (2 Hz) and fluence per flash (42 mJ cm<sup>-2</sup>). Then, modalities 2, 3, 4, 7, 8, 9, 13, 14 and 15 were designed to evaluate the impact of both the fluence per flash (22 mJ cm<sup>-2</sup>, 42 mJ cm<sup>-2</sup> and 87 mJ cm<sup>-2</sup>, according to the manufacturer) and the flash frequency (1 Hz, 2 Hz and 5 Hz) at equivalent total fluences. Total fluence for the different treatment modalities was calculated according to the number of applied flashes and the fluence per flash (mJ.cm<sup>-2</sup>). According to the manufacturer, the fluence was homogeneous on the 15 cm\*15 cm surface 30 cm under the flash lamp and no statistical impact of the drop positions was evidenced (data not shown).

After PL treatment, plates were incubated in the dark at 24 °C, a temperature chosen to support the growth of all yeast species tested. The growth was monitored every day for 10 days: plates were imaged from an illuminated desk to avoid light gleam (model DMC-FS7, Panasonic Corporation, Japan). Growth data were analysed with custom-made scripts in R (R Development Core Team, 2013) as previously described (Pilard et al., 2021): plate images were imported on R using the *OpenImageR* package (<https://cran.r-project.org/web/packages/OpenImageR/index.html>). The images of the plates were cropped for superimposition and the position of the drops was determined by manual clicking using the *grid* package and the *grid.locator* function. The area of each drop (in mm<sup>2</sup>) was calculated using automatic background subtraction. All statistical analyses were performed using R home-made scripts. In particular, growth dynamics were analysed using K-means clustering (*cutRepeatedKmeans* function; *ClassDiscovery* R package). From 2 to 15 clusters were tested, and the optimal number of clusters (4) was determined visually

## 2.2. PL treatment of yeasts inoculated in red wine

For both yeasts adaptation and PL treatments of yeasts inoculated in red wine, 18 L of Bordeaux red wine (Merlot and Cabernet Sauvignon

blend of the 2019 vintage) were used. This wine was chosen for its high UV absorbance ( $\alpha_{254nm} = 49 \text{ cm}^{-1}$ ) and its low free SO<sub>2</sub> content (<15 mg.L<sup>-1</sup>). To facilitate yeast strains adaptation and growth into the wine, few drops of hydrogen peroxide were added to the wine until free SO<sub>2</sub> content reached zero. Then, to ensure complete sterilization, wine was pasteurized (80 °C for 40 min) and then kept at 4 °C. *B. bruxellensis* (CRBO L1735, CRBO L1737, CRBO L1746, AWRI1499, AWRI 1608, CBS2499), *S. cerevisiae* (FX10, CRBO L0437), *L. thermotolerans* (CLIB292) strains were selected for these experiments. They were firstly grown on YPD plates, then inoculated in YPD liquid medium (24 °C) and finally in sterile red grape juice. To lower the lethality due to the inoculation in the wine during PL treatments, the proportion of pasteurized wine in the culture medium was gradually increased until it reached 90%. Populations were counted using Malassez cell with addition of methylene blue (Ribéreau-Gayon et al., 2006). Just before PL treatments, each strain was inoculated at a final concentration of 10<sup>5</sup>–10<sup>6</sup> CFU mL<sup>-1</sup> in three (triplicates) 500 mL batches of pasteurized red wine. The set-up used in this study was a lamp with 8 mm of inner diameter and 10 mm of outside diameter filled with 600 mbar of xenon. The distance between each electrode was 200 mm. Wine treatments were realized in continuous flow at 9 L h<sup>-1</sup> in a home-made reactor, which consists of a 2 mm inner diameter FEP tubing (16.5 m length) coiled around PVC tubing (8 cm inner diameter, 25 cm length). The reactor was placed horizontally, at 15 cm distance from the lamp in the PL treatment apparatus (LP.Box, Sanodev, France) (Fig. 1). This type of reactor was chosen to limit the thickness of the liquid treated and to homogenize the

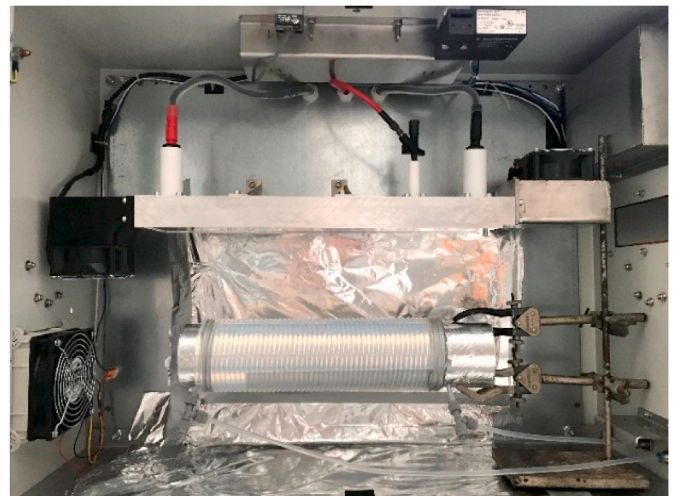


Fig. 1. Experimental setup for PL treatment of wine.

Table 2

List of the 15 modalities of PL used for the plate screening.

Modality	Lamp Voltage (V)	Fluence/Flash (mJ.cm <sup>-2</sup> )	Flash Number	Treatment Time (s)	Frequency (Hz)	Total fluence (mJ.cm <sup>-2</sup> )
1	0	0	0	0	0	0
2	1500	22	20	4	5	440
3	1500	22	20	10	2	440
4	1500	22	20	20	1	440
5	2100	42	2	1	2	84
6	2100	42	5	2,5	2	210
7	2100	42	10	2	5	420
8	2100	42	10	5	2	420
9	2100	42	10	10	1	420
10	2100	42	15	7,5	2	630
11	2100	42	20	10	2	840
12	2100	42	25	12,5	2	1050
13	3000	87	5	1	5	435
14	3000	87	5	2,5	2	435
15	3000	87	5	5	1	435

Yeasts growth monitoring after PL treatments on YPD solid medium.



residence time of the wine in the device.

To estimate the theoretical fluence applied to the wine during one pass in the reactor at  $9 \text{ L h}^{-1}$ , the following calculation was made:

$$F_{Total} = n_{flash} * F_{flash}$$

$$n_{flash} = Freq * RTD$$

$$RTD = \frac{Q}{V_{tube}}$$

$$V_{tube} = L_{tube} * 2 * \pi * R^2$$

Considering the reactor's geometry, wine was exposed to PL only half of its residence time in the reactor. Postulating this, the following approximation was made:

$$F_{Total} = \frac{n_{flash} * F_{flash}}{2}$$

Flash frequency was set at 4.5 Hz, the reactor was placed at 15 cm distance from the lamp and the input voltage was set at 4000 V, which corresponded to a fluence per flash of  $169 \text{ mJ cm}^{-2}$  at the surface of the reactor. The measured RTD was 20 s, so the wine theoretically received up to 45 flashes resulting in a total fluence of  $7.6 \text{ J cm}^{-2}$  per pass in the reactor. Each 500 mL of inoculated wine batches was then treated with the following modalities:  $0 \text{ J cm}^{-2}$  (OFF: circulated in the reactor without PL treatment),  $7.6 \text{ J cm}^{-2}$  (T1: circulated once with PL),  $15.2 \text{ J cm}^{-2}$  (T2: circulated twice with PL) and finally  $22.8 \text{ J cm}^{-2}$  (T3: circulated thrice with PL). Samples (5 mL) were collected under sterile conditions right after inoculation (T0) and after each treatment (OFF, T1, T2 and T3).

### 2.3. Yeasts enumeration before and after red wine PL treatments

Microbial counts were determined in triplicates by plating serial 10-fold dilutions of the samples and 10 or 100  $\mu\text{L}$  were plated in 9 cm diameter petri dishes. Yeasts were enumerated on YPD plates after 7 days of incubation at  $25 \text{ }^\circ\text{C}$ . The number of colonies detected was expressed in  $\text{CFU mL}^{-1}$  and the limit of detection was  $10 \text{ CFU mL}^{-1}$ . The OFF modality was used as reference for  $\log_{10}$  reduction determination. Analyses of variance (ANOVA) were performed followed by post-hoc Tukey tests (*HSD.test* function from *agricolae* package, R software).

## 3. Results

### 3.1. Sensitivity to PL treatment: large screening on petri dishes

In this first part, PL sensitivity was assessed for 198 yeast strains belonging to 14 different species (Table 1). These species are known to be associated with grape and/or wine. Between 2 and 3 strains per species were chosen to represent the genetic diversity of the species. In addition, a special attention was given to two species, namely *B. bruxellensis* and *S. cerevisiae*, for their negative and positive importance in winemaking respectively, using different strains from various origins.

### 3.2. Monitoring the impact of pulsed light treatment on yeast growth

Different modalities were studied with variation of fluence per flash, total fluence and frequencies, resulting in 15 combinations (Table 2), in order to estimate their impact on yeast growth. The growth was monitored daily, up to 10 days. For each strain, each initial density (4 densities) and each modality, the growth area was measured automatically

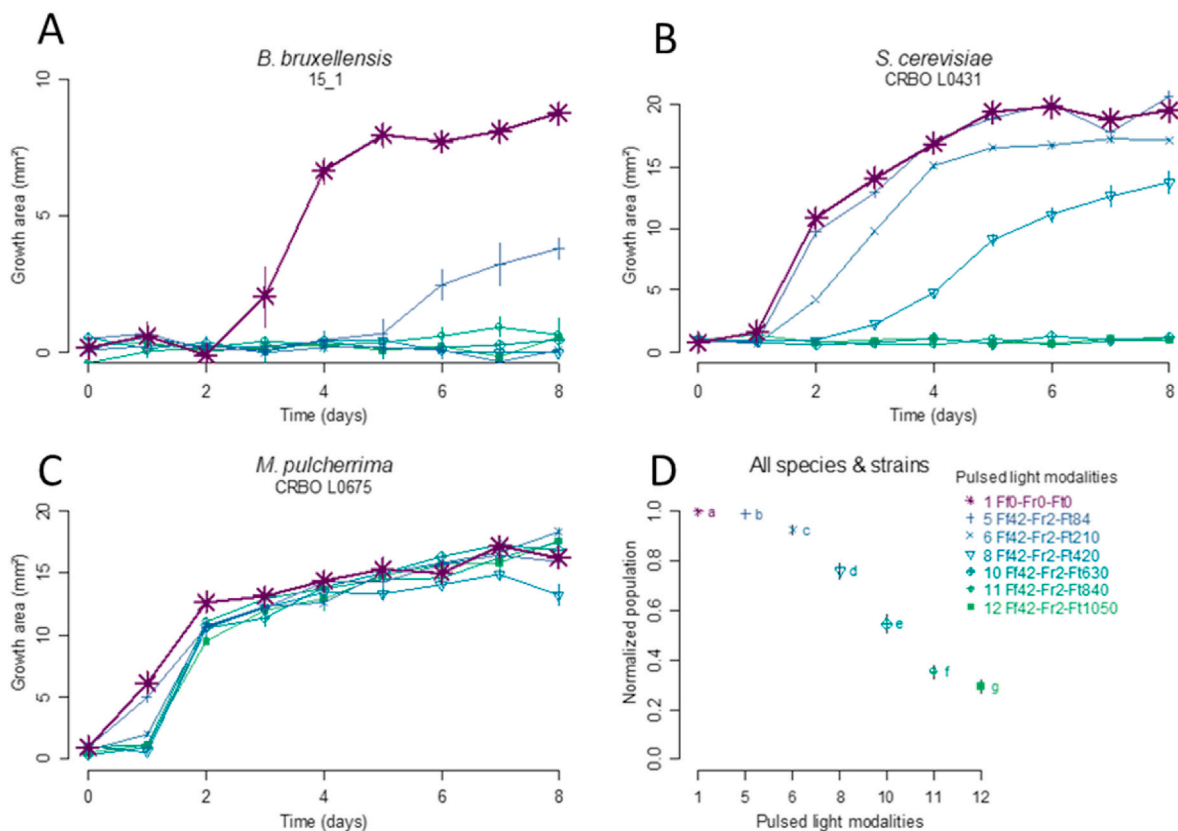


Fig. 2. Growth kinetics of strains depending on the total fluence applied during the pulsed light treatment ( $0, 84, 210, 420, 630, 840$  and  $1050 \text{ mJ cm}^{-2}$ ). (A) *B. bruxellensis* 15\_1, (B) *S. cerevisiae* CRBO L0431, (C) *M. pulcherrima* CRBO L0675, (D) Mean of the Normalized population (Area Under the Curve AUC normalized using the control -not-treated- modality 1) for all the strains used in this study (198).

using home-made R scripts. Triplicates were performed so that more than 350.000 data points were included in the dataset.

First, the total fluence impact was assessed (Fig. 2). Modalities 1, 5, 6, 8, 10, 11 and 12 were applied at 2 Hz with a fluence per flash of 42 mJ cm<sup>-2</sup>, the only difference being the number of flashes applied (respectively 0, 2, 5, 10, 15, 20 or 25) which corresponded to different total fluences (respectively 0, 84, 210, 420, 630, 840 and 1050 mJ cm<sup>-2</sup>). Fig. 2 shows three distinct behaviours associated with total fluence increase, as well as the sensitivity to PL treatments for all the tested strains.

Seven modalities were used (1, 5, 6, 8, 10, 11 and 12). Ff stand for fluence per flash (mJ.cm<sup>-2</sup>), Fr for frequency (Hz) and Ft for total fluence (mJ.cm<sup>-2</sup>). The density of 100 cells/drop was used for comparison. Growth area was measured in mm<sup>2</sup>. For D, the letters correspond to significant differences between each modality (Kruskal-Wallis test).

*B. bruxellensis* strains showed a highly sensitive behaviour to PL. For example, for strain 15\_1 (Figs. 2A), 84 mJ cm<sup>-2</sup> (modality 5: 2 flashes at 42 mJ cm<sup>-2</sup>, 2 Hz) were sufficient to halve its growth and all other treatments were sufficient to prevent its growth. For strain *S. cerevisiae* CRBO L0431, PL treatment with 84 mJ cm<sup>-2</sup> (modality 5) did not impact its growth, while treatments with 210 and 420 mJ cm<sup>-2</sup> (modalities 6 and 8) reduced yeast growth by 20% and 35% respectively. Only total fluences higher than 630 mJ cm<sup>-2</sup> (modalities 10, 11 and 12) fully prevented CRBO L0431 growth (Fig. 2B). PL poorly affected *M. pulcherrima* CRBO L0675 strain growth, with none of the applied treatments being sufficient to reduce significantly the final cell growth (Fig. 2C). However, treatments with 210 mJ cm<sup>-2</sup> and higher total fluences increased the lag-phase (about 24 h more than the control). Considering all the strains, all modalities were significantly different from each other, indicating that the higher the fluence, the greater the reduction in growth, thus confirming that the total fluence is the main process parameter affecting yeast growth (Fig. 2D).

In Fig. 3, the PL modalities 1, 3, 8 and 14 were compared to study the influence of the fluence per flash on the yeast growth profiles. Four fluences per flash were applied (0, 22, 42 and 87 mJ cm<sup>-2</sup>) with approximate total fluencies (about 430 mJ cm<sup>-2</sup>) and frequency (2 Hz).

Four modalities were used (1, 3, 8 and 14). Ff stand for fluence per flash (mJ.cm<sup>-2</sup>), Fr for frequency (Hz) and Ft for total fluence (mJ.cm<sup>-2</sup>). The density of 100 cells/drop was used for comparison. Growth area was measured in mm<sup>2</sup>. D: the letters correspond to significant differences between each modality (Kruskal-Wallis test).

Results showed that the impact of fluence per flash varied depending on the yeast considered (Fig. 3). For example, *B. bruxellensis* strain 15\_1 was highly sensitive to PL for the three fluences per flash applied (Fig. 3A). On the contrary, *M. pulcherrima* strain CRBO L0675 appeared poorly sensitive to all fluences per flash treatments tested; only a growth delay was observed again (Fig. 3C). *S. cerevisiae* CRBO L0431 showed an intermediate trend with a wide variation of response: the greater the fluence per flash, the greater the growth was impacted, 80%, 60% and 25% of the maximal growth corresponding to 22, 42 and 87 mJ cm<sup>-2</sup> respectively (Fig. 3B). The lag phase was also impacted. In general, yeast species were more affected by higher fluence per flash (Fig. 3D). The fluence per flash of 22 mJ cm<sup>-2</sup> corresponded to 90% of the maximal growth, 42 mJ cm<sup>-2</sup> to 75% and the 87 mJ cm<sup>-2</sup> to 40%. Thus, for similar total fluence, the impact of fluence per flash had an important impact on yeast growth.

Three groups of three modalities (2, 3 and 4, then 7, 8 and 9, and finally 13, 14 and 15) were used to study the impact of the frequency of flashes (at 1, 2 and 5 Hz) on yeast growth (Fig. 4). Within the three-modality groups, the effects of the fluence per flash and the total fluence were uniform.

Nine modalities were used (2, 3, 4, 7, 8, 9, 13, 14 and 15). Ff stand for fluence per flash (mJ.cm<sup>-2</sup>), Fr for frequency (Hz) and Ft for total

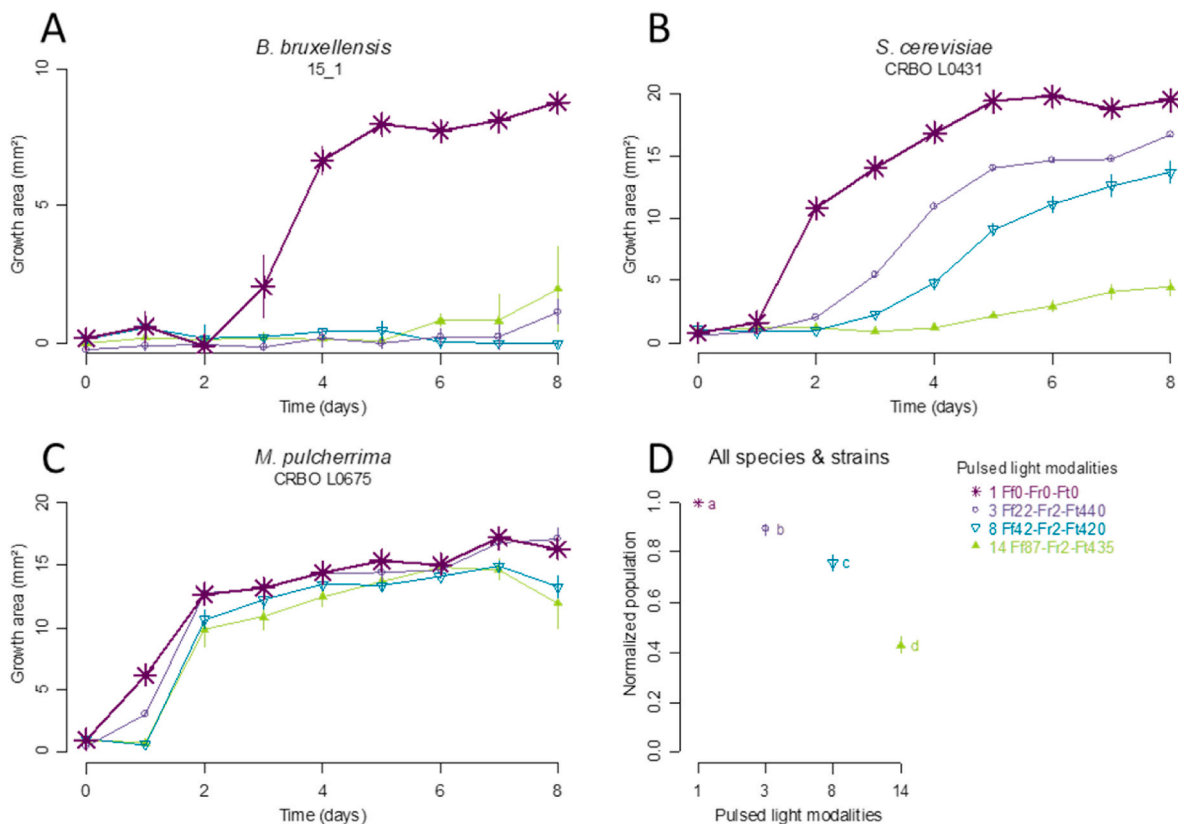
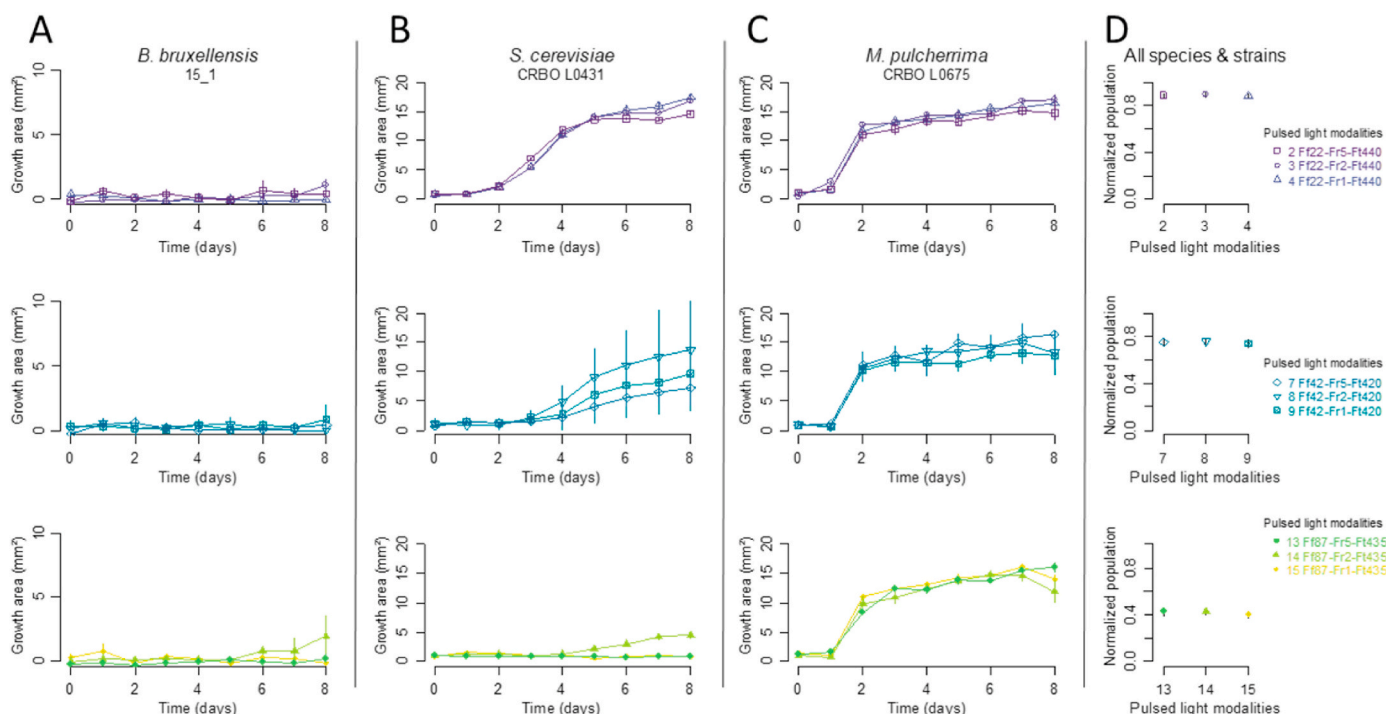


Fig. 3. Growth kinetics depending on four different fluence per flash applied during the pulsed light treatment (0, 22, 42 and 87 mJ cm<sup>-2</sup>). (A) *B. bruxellensis* 15\_1, (B) *S. cerevisiae* CRBO L0431, (C) *M. pulcherrima* CRBO L0675, (D) Mean of the Normalized population (Area Under the Curve AUC normalized with the control modality) for all the strains used in this study (198).



**Fig. 4.** Growth kinetics depending on the frequency applied on the pulsed light treatment (1, 2 and 5 Hz). (A) *B. bruxellensis* 15\_1, (B) *S. cerevisiae* CRBO L0431, (C) *M. pulcherrima* CRBO L0675, (D) Mean of the Normalized population (Area Under the Curve AUC normalized with the control modality) for all the strains used in this study (198).

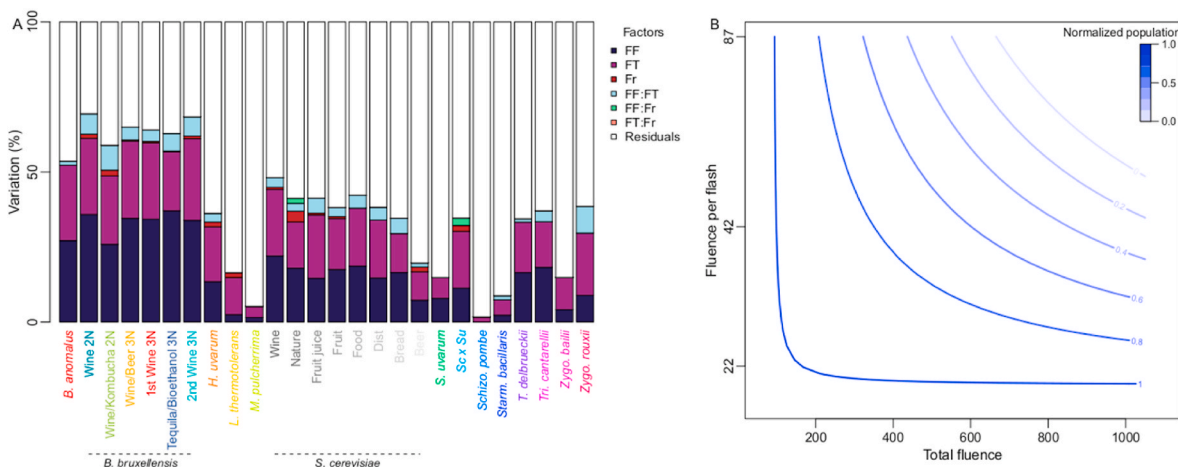
fluence ( $\text{mJ}\cdot\text{cm}^{-2}$ ). The density of 100 cells/drop was used for comparison. Growth area was measured in  $\text{mm}^2$ .

The growth of the three strains was very similar. The three yeasts were not sensitive to the different frequencies used, whatever were the total fluence or the fluence per flash. No significant differences were observed for the three groups of modalities for all strains (198) (Fig. 4D). These results indicate that the frequency does not affect the efficiency of the PL treatment in the tested conditions, when considering same fluence per flash and same total fluence.

### 3.3. Impact of PL treatment parameters

To precise the most important factors (fluence per flash, total fluence, frequency as well as their potential interactions) affecting the

maximal population size, an ANOVA was performed for each strain. The percent of variation explained by each factor was represented on the bar plot and summarized by species or subpopulations (Fig. 5 A). The fluence per flash was the most impacting factor, explaining around 17% of the total variation of population size for all species, with important variation depending on the strain/species (ranging from 0% to 37%). The total fluence also had strong implication and variation regarding the strain/species, with 16.8% of the total variation (ranging from 1.6% to 27%). In accordance with the results above (Fig. 4), the frequency impact was small (0.6%, range of 0–3%). Moreover, interaction between fluence per flash and total fluence was low (3.2% range of 0–8%). The interaction between both factors can be seen on the isocurve response (Fig. 5 B): at  $22 \text{ mJ cm}^{-2}$ , a poor effect of pulsed light treatments on yeast growth was globally achieved, no matter the total fluence,



**Fig. 5.** Factors impacting pulsed light treatment efficiency. (A) Percentage of variation explained by the different factors (fluence per flash, total fluence and frequency) and their interactions for all tested species and strains aggregated by species or subgroups. (B) Isocurve representation of the normalized population size depending on the two main factors, fluence per flash and total fluence ( $\text{mJ}\cdot\text{cm}^{-2}$ ).

compared to 87 mJ cm<sup>-2</sup>, where the impact of the total fluence was stronger. Indeed, the total fluence is an important factor, but only when the fluence per flash is already sufficient to impact yeast growth.

Overall, these analyses suggested that the fluence per flash is the most important factor that conditioned the efficiency of pulsed light treatment. The total fluence is the other factor that affected PL efficiency on yeast growth, but its effectiveness can be detected if a threshold value of the fluence per flash is exceeded.

These analyses also showed that the impact of these factors varied greatly depending on the species and strain. Both *Brettanomyces* species (*B. anomalus*, 3 strains and *B. bruxellensis*, 111 strains) were significantly impacted by fluence per flash and total fluence, while the three tested strains of *Metschnikowia pulcherrima* or *Starmerella bacillaris* were poorly or not impacted. *S. cerevisiae* and many other species displayed an intermediate behaviour.

### 3.4. Interspecific and intraspecific variability according to PL sensitivity

To analyse the impact of pulsed light treatments on the investigated yeast species, a K-means clustering analysis was performed. The maximal population (area in mm<sup>2</sup>) was normalized using the maximal population recorded in absence of PL treatment (modality 1). Four growth trends were identified (Fig. 6), group 1 encompassing very sensitive strains (i.e. sensitive at low fluence per flash/low total fluence), group 2 and 3 containing strains sensitive to higher values of fluence per flash and/or total fluence, and group 4 being sensitive only to the most efficient modalities. Interestingly, a strong variation depending on the

species was observed: all tested strains of *L. thermotolerans*, *M. pulcherrima*, *Starmerella bacillaris*, *Zygo. bailii*, *T. delbrueckii* clustered in the less sensitive group (G4). Conversely, strains of *B. anomalus* and *B. bruxellensis* were exclusively distributed in the most sensitive groups (G1-G3).

K-means clustering identified an optimum of 4 groups (G1-G4), G1 containing the more sensitive strains and G4 the less sensitive ones. Sc x Su stands for *S. cerevisiae* x *S. uvarum*. (A) Distribution of the strains in the different sensitivity groups per species and/or substrates. (B) Heatmap showing the normalized population size (one line per strain) depending on the 15 modalities of pulsed light treatment. Modalities were ordered from the less to the most impacting on yeast growth: 1, 5, 6, 3, 2, 4, 6, 7, 9, 10, 14, 13, 15, 11 and 12. (C) Bidimensional plot showing the normalized population depending on the 15 modalities of pulsed light treatment for each group.

A focus was made on *S. cerevisiae* and *B. bruxellensis* strains to explore the intra-specific diversity to PL response. To compare the different strains, the maximal growth means of all applied treatments was used as proxy and presented in Fig. 7.

AUC means 'Area Under the Curve' and represents the normalized mean growth for the PL modalities. For each subgroup, a Kruskal-Wallis test was performed on AUC to identify significant differences between groups. Different letters (in brackets) denote different significance groups. 2N and 3N stand for diploid and triploid respectively.

For *S. cerevisiae*, strains associated with brewery, bakery and distillery processes were significantly less sensitive to PL treatments compared to wine and fruit juice strains. Wild isolates and strains

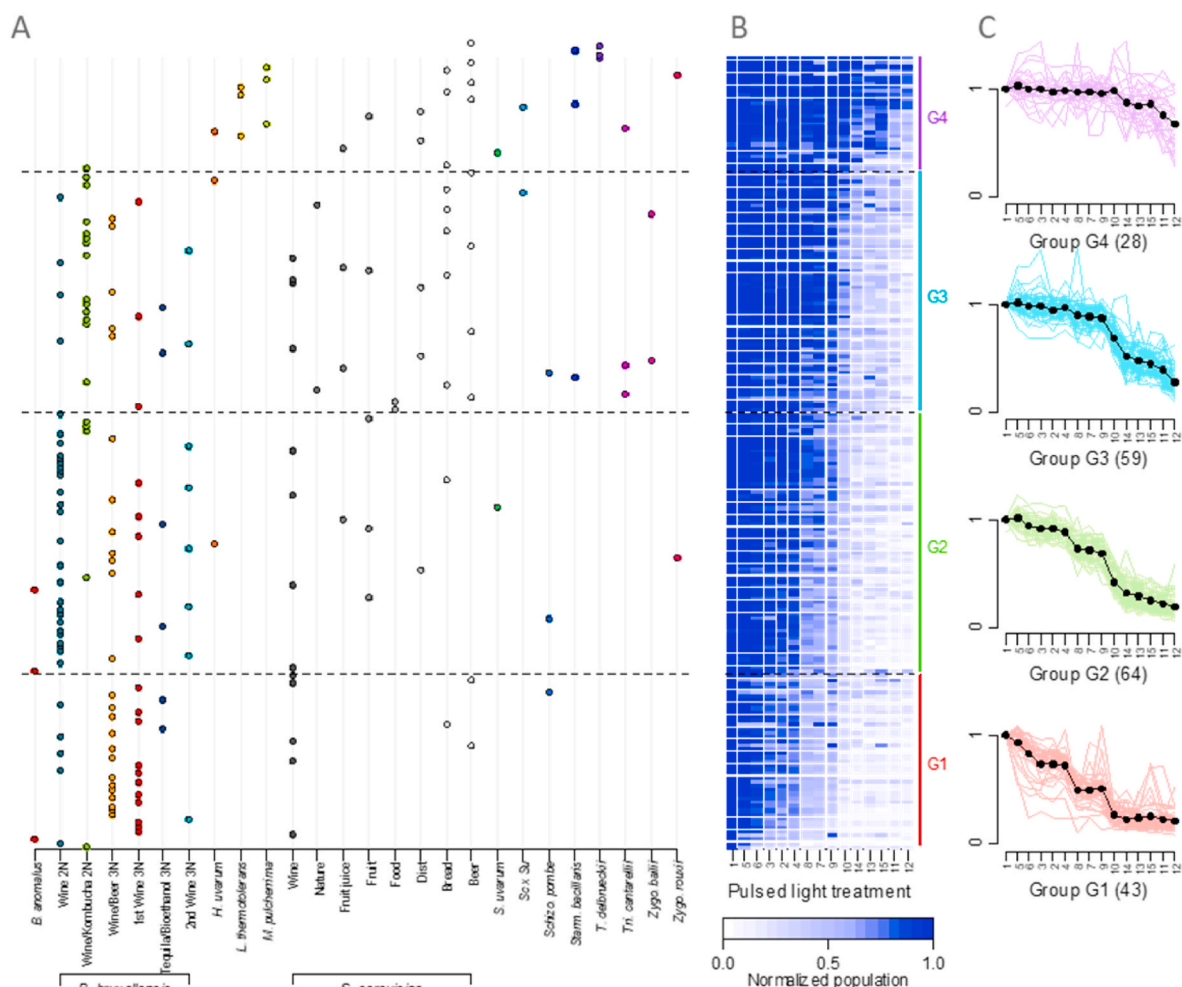


Fig. 6. Clustering of 198 yeast strains depending on their sensitivity to pulsed light treatment.

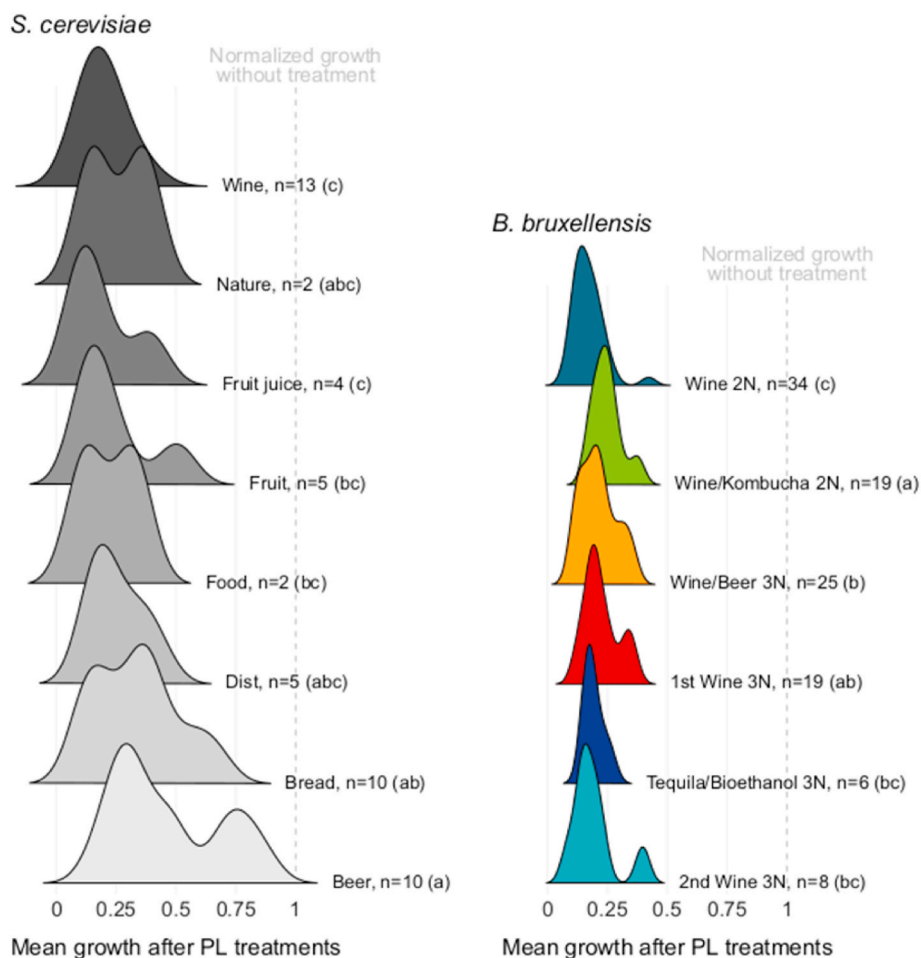


Fig. 7. Influence of pulsed light treatments on the growth of *S. cerevisiae* and *B. bruxellensis* subgroups.

associated with food spoilage (Nature, Food, Fruit, Fruit juice groups) showed intermediate behaviour. By contrast, all six subpopulations of *B. bruxellensis* showed lower maximal growth than *S. cerevisiae*, confirming that *B. bruxellensis* was more sensitive to PL treatments. In the case of *B. bruxellensis*, PL impact varied depending also on the genetic group, with Wine/Kombucha 2N group less sensitive to PL treatment. Wine 2N, Tequila/Bioethanol 3N and 2nd Wine 3N were the most

sensitive. Wine/Beer 3N and 1st Wine 3N had an intermediate behaviour. Altogether, these results showed that the sensitivity to PL treatments varied depending on the yeast species, but also depending on the origin of the strains.

Sensitivity to PL treatment of various yeasts species and strains in red wine.

In the second part of this study, the PL potential was evaluated for

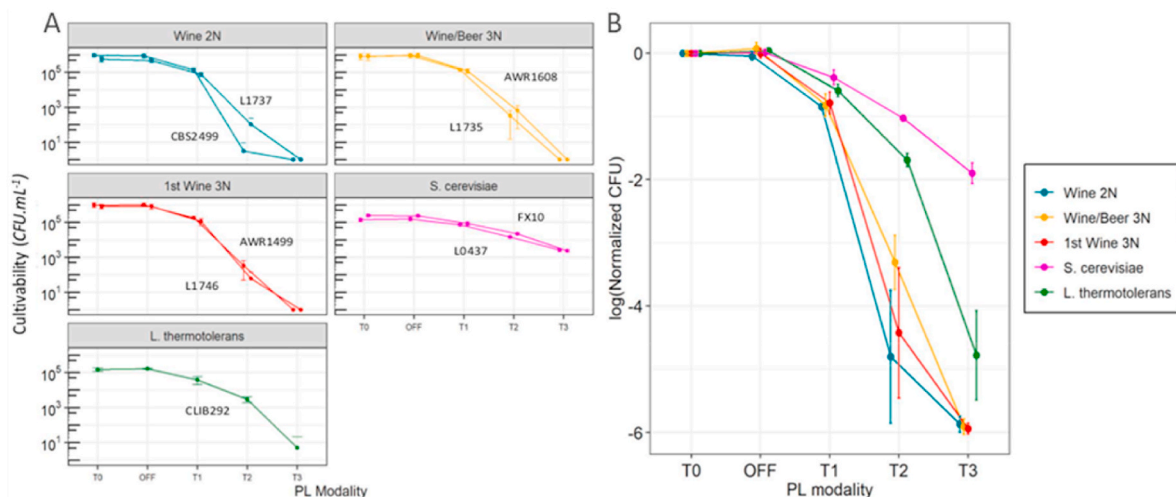


Fig. 8. Cultivability of 9 strains after different PL treatments. (A) Cultivability is expressed in CFU. mL<sup>-1</sup>. (B) The log reduction was normalized using the T0 cell cultivability.

the microbial stabilization of various inoculated yeasts species and strains in red wine (Fig. 8). We selected six strains from *B. bruxellensis* belonging to the three main wine-related genetic groups (Avramova et al., 2018a) already used in another related study (Pilard et al., 2021). To compare the relative sensitivity of these species, two *S. cerevisiae* and one *L. thermotolerans* strains were also selected. Thus, nine yeast strains were submitted to four different PL treatments (Fig. 8). The  $\log_{10}$  reduction from the T0 population was used for the different statistical analyses, to consider variations in initial populations. ANOVA analysis was made to determine the impact of the PL and the strain groups on yeast growth (used in Fig. 8). The PL treatments explained 81% of the variation, while 7% were explained by the strain groups.

Concerning the impact of each modality, no significant differences in viability were observed between the T0 and OFF modalities, evidencing that pumping through the apparatus had no impact on cell viability. The 3 PL modalities, T1 (7.6 J cm<sup>-2</sup>), T2 (15.2 J cm<sup>-2</sup>), T3 (22.8 J cm<sup>-2</sup>) were significantly different from T0 and OFF modalities and from each other, the higher the fluence, the higher the logarithmic reduction detected.

Regarding the sensitivity to PL for the three species, *B. bruxellensis* strains were all highly sensitive compared to other species, resulting approximately in 1  $\log_{10}$  CFU mL<sup>-1</sup> reduction with T1 treatment, 3 to 5  $\log_{10}$  CFU mL<sup>-1</sup> reduction for T2 treatment, and finally more than 6  $\log_{10}$  CFU mL<sup>-1</sup> reduction (below our detection threshold for each strain) with T3 treatment (Fig. 8). *S. cerevisiae* strains expressed the lowest sensitivity, with 0.5, 1 and 2  $\log_{10}$  CFU mL<sup>-1</sup> reduction respectively for treatments T1, T2 and T3. *L. thermotolerans* strain showed an intermediate response to PL compared to the other two species, with 0.5, 2 and 5  $\log_{10}$  CFU mL<sup>-1</sup> reduction respectively for treatments T1, T2 and T3.

Considering the variation within *B. bruxellensis* genetic groups, the Wine 2N and the 1st Wine 3N strains were significantly more sensitive than the Wine/Beer 3N strains. *L. thermotolerans* CLIB292 expressed an intermediate sensitivity, between *B. bruxellensis* and *S. cerevisiae* strains. The two *S. cerevisiae* strains had similar response of growth reduction following PL treatments.

Normalized survival curves are grouped by genetic groups or by species, normalized using the mean at T0. The means of triplicates  $\pm$  standard errors were represented for each modality.

#### 4. Discussion

The present study aimed to explore the PL sensitivity of yeasts related to winemaking and vineyard. A large collection of yeasts was used, encompassing 14 species and 198 strains, and a rapid plate screening method using 15 PL treatments was applied. Furthermore, experiments on nine strains from 3 species were carried out in a continuous flow PL treatments chamber to evaluate the necessary dose for microbial inactivation and validate the species variability observed on plate screening.

##### 4.1. Total fluence and fluence per flash governing the PL efficiency on solid media

The impact of three different treatment parameters governing the PL treatment (e.g., fluence per flash, total fluence and frequency) was investigated. fluence per flash and total fluence strongly impacted the growth reduction (17% and 16.8% respectively). An increasing dose for both parameters was correlated with a higher growth reduction. Although the two parameters were dependent and interlinked for the efficiency of the treatment, similar total fluencies did not induce the same germicidal effect depending on the way the energy was distributed. Indeed, the minimum fluence per flash tested (22  $\mu$ J cm<sup>-2</sup>) weakly impacted cells growth whatever the total fluence applied (Fig. 5 B). Two non-mutually exclusive hypotheses can be made: (i) the content of wavelengths emitted changed depending on the lamp voltage, (ii) a

minimal fluence per flash threshold is needed, indicating a possible “peak effect” (Gómez-López et al., 2007). Concerning the variation of wavelength content depending on the lamp voltage, it has been showed that UV content could decrease when lamp voltage decreased (Wekhof, 2000). Considering that PL efficiency relies (at least partially) on UV-C content, it could explain the lack of low fluence per flash germicidal effect observed here. In addition, some studies point up a possible additional “peak effect” with high energy treatments which could contribute to the germicidal effect of pulsed light (Levy et al., 2012). The existence of this fluence threshold is in contradiction with Bunsen-Roscoe law, which enounces that only the total fluence governs the PL efficiency (Gómez-López and Bolton, 2016; Kramer et al., 2017a). Violation of this law has already been observed on bacteria and fungi in surface treatments with PL, on *Aspergillus niger*, as well as in continuous flow treatments of *Listeria innocua* (Artíguez et al., 2011; Kramer et al., 2017b; Levy et al., 2012; Luksiene et al., 2007). Demonstrating a specific effect of high fluence per flash treatments (not due to the variations of UV content in the emitted spectrum) remains an issue, unresolved by our study whose aim was to determine the impact of three PL parameters (fluence per flash, total fluence and flash frequency) on 14 wine yeast species. Here, the frequency modulation (between 1 and 5 Hz) had almost no effect (0.06%) on PL treatment efficiency. This indicates that for PL applications, the flash frequency can be increased (or reduced) maintaining the same total fluence without affecting the germicidal efficiency, which can be interesting to reduce the treatment time needed at the industrial scale. However, subsequent work will have to measure the possible impact of flash frequency on the wine temperature (not measured here), and its subsequent impact on wine organoleptic quality.

##### 4.2. Cellar resident yeasts expressed higher sensitivity to PL than vineyard yeasts

Concerning the sensitivity between the different yeast species, a great variability was observed (Fig. 6). *Brettanomyces* species were highly sensitive to PL, while on the contrary *L. thermotolerans*, *M. pulcherrima*, *Starm. bacillaris*, *Zygo. bailii*, *T. delbrueckii* were scarcely impacted. A third group, composed of *H. uvarum*, *Saccharomyces* sp., *Schizo. pombe*, *Tri. cantarelli*, *Zygo. Rouxii*, showed an intermediate behaviour. This ranking is globally in accordance with a previous plate screening study led on continuous UV-C treatments, where *Brettanomyces* species were highly impacted when other species such as *M. pulcherrima* or *Starm. bacillaris* were less affected (Pilard et al., 2021). These results are in adequation with the well-described overriding role of UV-C into PL efficiency (Keener and Krishnamurthy, 2014). Secondary metabolites (photoprotective pigments, mycosporine-like amino acids) or coat proteins (Bisquert et al., 2018; Clair et al., 2020; Gao and Garcia-Pichel, 2011; Singaravelan et al., 2008), DNA repair mechanisms (photoreactivation and dark repairs mechanisms) (Friedberg et al., 2005; Zhang et al., 2017), wall thickness and composition (Beauchamp and Lacroix, 2012) and, possibly, clustering ability (shielding effect) are factors involved in the protection of microorganisms from light damages, whose amount or efficiency may vary from one species to another. The less sensitive species to PL are particularly associated with vineyards and grape berries, therefore frequently exposed to sun light (Sipiczki, 2016; Varela and Borneman, 2017; Zott et al., 2008). On the contrary, *B. bruxellensis* or *B. anomalus* are barely isolated from grape berries but mainly from anthropized environments (cellar, equipment, barrels), thus less exposed to UV radiations (Loureiro and Malfeito-Ferreira, 2003; Wang et al., 2015). UV radiations were already shown to influence and modulate yeast community at ecological scale (Libkind et al., 2009; Longan et al., 2017). *T. delbrueckii*, *L. thermotolerans* and *M. pulcherrima*, which were less affected by PL treatments, can be used by the winemakers as technological auxiliaries for acidification, biocontrol agent or to improve aromas in grape musts (Roudil et al., 2019; Windholtz et al., 2021). Thus, PL treatments might be used to eliminate the unwanted yeasts, without impairing the

establishment of positive ones.

Regarding the results on red wine PL treatments, the sensitivity of *B. bruxellensis* strains was also highly marked, with 6 log<sub>10</sub> CFU mL<sup>-1</sup> reduction (below the detection limit) compared to *S. cerevisiae* strains (2 log<sub>10</sub> CFU mL<sup>-1</sup> reduction) for a total fluence of 22.8 J cm<sup>-2</sup> (Fig. 8). This result is in agreement with the plate screening results. The viability reduction of *B. bruxellensis* was strong, which underlines the interest of using PL to prevent wine spoilage caused by this yeast. Surprisingly, *L. thermotolerans* strain, which was among the less sensitive strain in plate screening, was more impacted than *S. cerevisiae* in the wine. This could be explained by the physiological state of this species in red dry wine: indeed, the adaptation of this species to wine was long and tedious compared to the others (data not shown).

#### 4.3. Intraspecific sensitivity within *B. bruxellensis* and *S. cerevisiae*

A focus on *B. bruxellensis* (111 strains) and *S. cerevisiae* (49 strains) yeast species was made, for their relevance in beverages (Hirst and Richter, 2016; Tubia et al., 2018). Although *S. cerevisiae* species was less sensitive than *B. bruxellensis*, important variability was observed between groups in both species (Fig. 7). Within *B. bruxellensis* genetic subpopulations as recently defined by Eberlein (Eberlein et al., 2021), the Wine 2N isolates were more affected than the 1st Wine 3N and Wine/Beer 3N isolates on plate screening. This trend was also observed in wine treatment, with a small subset of strains. Surprisingly, the Wine 2N group was found to be much affected by PL than the 1st Wine 3N and Wine/Beer 3N groups, while this group expressed lower sensitivity to continuous UV-C treatments (Pilard et al., 2021). This might be attributable to the additional effects of photothermal and photophysical mechanisms due to PL high intensity and large emission spectrum. Regarding *S. cerevisiae*, different behaviours were observed within the subpopulations described, but the Wine and Fruit juice isolates showed an increased sensitivity compared to isolates from Beer and Bread environments. Although being responsible of the necessary fermentation of sugar into ethanol, *S. cerevisiae* can act as spoiling microorganism causing an unwanted ‘re-fermentation’ of sweat wine due to its ethanol and SO<sub>2</sub> resistance (Divol et al., 2006; Malfeito-Ferreira, 2019). Hence, PL utilization for *S. cerevisiae* managing in winemaking might be a promising track.

## 5. Conclusion

This study brings information on the impact of three factors governing PL treatments on yeast sensitivity (fluence per flash, total fluence, and frequency). In accordance with previous studies, total fluence and fluence per flash affected the PL efficiency. In addition, our results suggest that the fluence per flash must be set at or above a threshold value to guarantee the effectiveness of PL treatment (due to UV decrease and/or peak effect). Once this threshold value is reached, higher fluencies per flash and higher total fluencies increased the treatment efficiency. By contrast, the frequency does not affect the treatment efficiency, although its impact on wine temperature remains to be assessed. Thus, a possible reduction in processing times can be envisioned and optimization for the continuous processing of liquids can be developed using efficient criteria. In addition, a strong diversity regarding the PL sensitivity was observed at inter specific level. By chance, spoilage yeasts showed higher sensitivity to PL treatments than “beneficial” yeasts. These results were confirmed in continuous flow PL treatments of red wine, *B. bruxellensis* strains being highly sensitive (6 log<sub>10</sub> CFU mL<sup>-1</sup> reduction, below the detection limit, for a total fluence of 22.8 J cm<sup>-2</sup>). However, considering PL application for winemaking, the impact of the treatment on wine’s organoleptic properties should be evaluated, which has not been done in this study and will be the subject of further work. The right combination of fluence per flash and total fluence should be used to efficiently stabilize wines with no or minimal impact of PL on wine’s properties. Chemical and sensorial analyses of PL

treated wines must be carried out to address these questions.

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## Declaration of competing interest

The co-author Philippe Marullo is affiliated with BIOLAFFORT. This does not alter the authors’ adherence to all the journal’s policies on sharing data and materials.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.fm.2022.104121>.

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## Conclusion et perspectives

Ces deux études ont mis en lumière plusieurs résultats qui pourront être utiles pour l'application de ces technologies dans la filière viti-vinicole, mais aussi dans la compréhension de leur impact sur la croissance des microorganismes.

Tout d'abord, concernant la technologie de lumière pulsée (PL), grâce au jeu de données acquis, l'impact de trois paramètres clés sur la viabilité cellulaire a été évalué, la fluence par flash, la fluence totale et la fréquence des flashes. La fluence par flash est le paramètre influençant le plus l'efficacité du traitement, suivi de la fluence totale. La fréquence n'est que peu impactant dans la gamme appliquée (1-5 Hz). L'effet seuil observé concernant la fluence par flash semble être en contradiction avec certains résultats de la littérature, notamment la loi de réciprocité de Bunsen-Roscoe (l'efficacité du rayonnement est la même peu importe que la fluence soit obtenue avec un taux de fluence élevé et un temps d'exposition court ou avec un taux de fluence faible et un temps d'exposition long) (Gómez-López and Bolton, 2016). Des études précédentes ont fait état de violation de cette loi à plusieurs reprises (Artíguez et al., 2011; Kramer et al., 2017; Levy et al., 2012). Cependant, l'homogénéité du spectre d'émission n'a pas pu être testée en fonction de la fluence appliquée par flash et il a été montré que la part des UV-C dans l'émission d'un flash peut dépendre de la fluence appliquée. Ces dernières variations observées étaient mineures (Levy, 2010). Afin de mieux comprendre les paramètres clés impactant l'efficacité de la technologie PL, c'est un paramètre qu'il faudra correctement caractériser.

Au niveau microbiologique, les deux études ont montré des similarités importantes dans la sensibilité des espèces. De manière intéressante, les espèces de levures que l'on retrouve plus volontiers dans les chais sont plus sensibles que les espèces associées au vignoble (associées aux baies et à la vigne). Ces dernières ne sont généralement qu'une source mineure de déviation dans l'élaboration du vin car peu résistantes aux conditions qui y règnent (peu de nutriments, forte teneur en éthanol, peu d'oxygène), contrairement aux espèces du chai (*e.g. B. bruxellensis*). Ces résultats démontrent l'intérêt certain de ces technologies dans le contrôle des populations de levures néfastes en œnologie.

Les traitements de vins rouges très contaminés (entre  $10^5$  et  $10^6$  cellule/mL) ont montré l'efficacité de ces deux méthodes, permettant des réductions de la viabilité en dessous du seuil de détection des souches inoculées dans la plupart des cas (réduction 5-6 log). Il est important de souligner que les vins rouges utilisés étaient très absorbants, opaques, limitant la pénétration des rayons. Bien que cela n'ait pas été le cœur de ces études, l'utilisation d'un pilote approprié est très certainement déterminant dans l'efficacité des traitements et sera un levier d'amélioration important pour l'industrialisation de ces méthodes.

Concernant les variations de sensibilité des souches de *B. bruxellensis*, des différences significatives ont été observées au niveau des populations génétiques, bien qu'elles soient plus faibles que les variations interspécifiques. Cependant, les variations de sensibilité des populations ne sont pas parfaitement conservées entre les deux technologies. De manière générale, la population *kombucha* (Wine/Kombucha 2N) présente la moins grande sensibilité aux deux traitements. La population *wine 1* (1st Wine 3N) présente une sensibilité relative importante aux UV-C, qui n'a pas été observée avec le traitement PL. Finalement, la population diploïde *wine 3* (Wine 2N) est relativement peu sensible aux UV-C, mais présente une plus grande sensibilité aux traitements PL (Tableau 3). Ces deux dernières

observations ont été confirmées en traitement sur vin, et mettent en évidence des modes d’actions qui pourraient être légèrement différents entre les deux méthodes.

La variabilité des réponses des populations de *B. bruxellensis* aux méthodes de contrôle et de stabilisation microbiologique (sulfites, chitosane, UV-C et PL) commence à être mise en évidence au fil de la littérature. Ces données mettent en lumière la nécessité d’une utilisation raisonnée, adaptée et non systématiques de ces technologies (Tableau 3). La détection et l’identification de la (les) population(s) impliquée(s) dans une contamination sont donc nécessaires pour adapter le traitement. Plusieurs outils d’identification sont à la disposition des laboratoires et des professionnels ou sont en train d’être développés. L’utilisation d’outils moléculaires (« typBrett » commercialisé par Laboratoire Excell), d’intelligence artificielle couplée à de la microscopie optique peuvent permettre l’identification de populations, aidant le vinificateur dans le choix de la méthode à utiliser (Albertin et al., 2018; Avramova et al., 2018b; Lebleux et al., 2021). Ces variations de sensibilité rendent d’autant plus important l’utilisation de plusieurs isolats représentatifs de la diversité génétique de *B. bruxellensis* dans l’étude de nouvelles méthodes de contrôle de cette levure, ce qui a rarement été fait jusqu’à présent.

Cependant, un aspect majeur n’a pas été approché lors de ces deux études, l’impact sur la qualité du vin de ces deux traitements. Dans la littérature, l’influence des UV-C sur les propriétés organoleptiques d’un vin semble minime ou obtenue pour des doses bien supérieures à celles conseillées pour la stabilisation microbiologique (Diesler et al., 2019; Durner et al., 2017). Lors de travaux réalisés par l’équipe des procédés du laboratoire, l’influence visuelle et organoleptique du traitement de 10 vins a été étudiée. De manière générale, les impacts sur la couleur du vin étaient imperceptibles par l’œil, et une légère réduction des concentrations en esters ainsi qu’une augmentation de la concentration de certains thiols a été observée (Pilard, 2022). Les influences organoleptiques de la PL sur le vin n’ont pour l’instant fait l’étude d’aucune publication scientifique. La caractérisation de ces impacts devrait être plus ardue que pour les UV-C du fait des nombreux paramètres impliqués (fréquences, fluence par flash, fluence totale, ajout de filtres). Cette variabilité sur les paramètres opérationnels de cette technologie peut aussi être une source de modulation de l’impact de la qualité des vins et nécessitera une attention particulière pour le développement de cette technologie pour la filière viti-vinicole.

Tableau 3: Evaluation de l’efficacité de 4 méthodes de contrôle sur les différentes populations de *B. bruxellensis*.

	<u>populations</u>						Sources
<u>Méthode de stabilisation</u>	Beer	kombucha	Teq/EtOH	wine 1	wine 2	wine 3	
Sulfites	Sensible	sensible	sensible	tolérant	résistant	sensible	(Avramova et al., 2018b)
Chitosan	plutôt résistante	intermédiaire	intermédiaire	sensible	intermédiaire	intermédiaire	(Paulin et al., 2020)
UV-C	intermédiaire	plutôt tolérante	intermédiaire	sensible	intermédiaire	plutôt tolérante	(Pilard et al., 2021)
PL	intermédiaire	plutôt tolérante	sensible	intermédiaire	sensible	sensible	(Pilard et al., 2021)
Présence dans le vin (%)	18%	4%	<1%	43%	3%	32%	(Cibrario et al., 2019)

## Discussion générale

*Brettanomyces bruxellensis* est une levure retrouvée dans de nombreux procédés fermentaires, pouvant être considérée comme une cause importante d'altération microbiologique (vin, bière, bioéthanol) ou au contraire comme auxiliaire technologique (kombucha, bière de spécialité). L'étude de la génétique des populations sur un nombre important d'individus (>1400) a permis de mettre en évidence une structure influencée par la ploïdie, la niche d'isolation et la géographie, validée par des études génomiques (Avramova et al., 2018a; Eberlein et al., 2021; Gounot et al., 2020; Serra Colomer et al., 2020a). La variabilité phénotypique de *B. bruxellensis* a été régulièrement soulignée et discutée, cependant sans prendre en compte la structure génétique de la population. La présence de caractéristiques phénotypiques propres à certaines populations, associés à l'augmentation de la valeur adaptative dans certains environnements (résistance aux sulfites par exemple (Avramova et al., 2018b; Varela et al., 2019)) permet de poser la question de l'adaptation aux niches anthropisées de cette espèce. Pour y répondre, l'étude phénotypique de nombreuses souches (151) représentatives de la diversité génétique de *B. bruxellensis* a été réalisée. Une première approche a été de mesurer dans cinq environnements (kombucha, vin, moût de raisin, bière et wort) des paramètres de croissance, de fermentation, de la production/consommation de métabolites d'intérêts, des traits d'histoire de vie ainsi que de composés volatils. Une seconde étude a été conduite (151 souches) sur la capacité à métaboliser les nitrates, avec une étude des relations génotype-phénotype. Les résultats obtenus ont permis d'évaluer et de démontrer que la variabilité phénotypique est en partie structurée par les populations identifiées. Des variations phénotypiques entre les populations qui permettent un changement de la valeur adaptative ont été mises en évidence, et indiquent une plausible adaptation de certaines populations à des environnements anthropisés.

### Caractères spécifiques de l'espèce

Aux travers de ces études, les principales caractéristiques de l'espèce *B. bruxellensis* ont été affinées. La capacité à réaliser la fermentation alcoolique, la survie dans des milieux riches en éthanol, à consommer les malto-oligosaccharides des céréales, l'assimilation des nitrates, la faible production de glycérol, la forte production d'acide acétique ainsi que la production de phénols volatils semblent être des caractères propres à l'ensemble de l'espèce (Chapitre 2 et Chapitre 3). De plus, la capacité de consommation des sucres du malt et l'assimilation des nitrates semblent être sous relâchement sélectif avec la perte (possiblement répétée) de ces phénotypes pour les souches de certaines populations. La dégénérescence de fonctions non utilisées et/ou la réduction des caractères métaboliques est un phénomène observé dans l'évolution du phylum des levures bourgeonnantes (Shen et al., 2018). Cette réduction a été observée sur des données génomiques comparatives entre une souche isolée de bioéthanol (fermentation en continue) et de vin (Tiukova et al., 2019). La spécialisation des populations de *B. bruxellensis* est donc possiblement associée à une réduction des caractères phénotypiques non essentiels dans les nouvelles niches colonisées ainsi que le gain possible ou la sélection de fonctions nécessaires à la colonisation des niches (Figure 26 et Figure 27). Cette spécialisation de populations de micro-organismes en réponse à des pressions anthropiques a été soulignée chez plusieurs espèces (Legras et al., 2018; Ropars et al., 2020). Les mécanismes moléculaires menant à la perte de fonction ont été étudiés pour l'assimilation des nitrates. Une modification du nombre de copies des gènes, avec des évènements de pertes d'hétérozygotie a été souligné. Les évènements de perte d'hétérozygotie, qui ont été mis en lumière précédemment, façonnent les génomes de *B. bruxellensis* (Eberlein et al., 2021). Nos résultats suggèrent que ces évènements peuvent influencer la valeur adaptative de l'espèce.

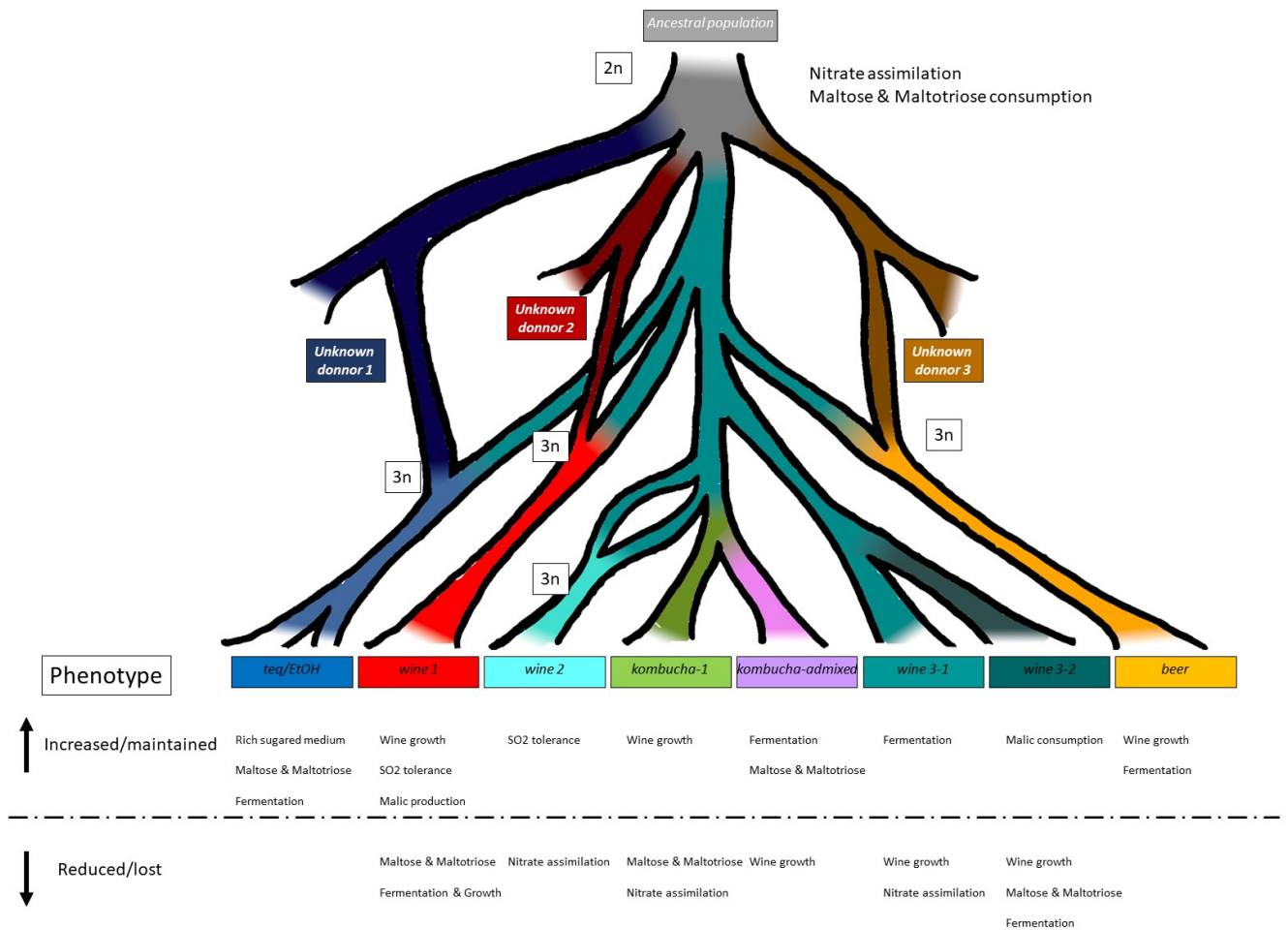


Figure 26: Scénario schématique d'évolution de l'espèce *B. bruxellensis*. « 2n » équivaut à diploïde, « 3n » à triploïde.

### Caractères impliqués dans la divergence phénotypique des populations

D'autres caractères phénotypiques présentent des disparités importantes suivant les différents populations. La capacité de croissance importante dans le vin des populations *wine 1* et *beer*, les capacités fermentaires importantes (à l'échelle de l'espèce) des souches *beer*, l'influence différente sur la couleur finale du milieu des groupes génétiques, la production d'acide malique du groupe *wine 1* et sa consommation par le groupe *wine 3-2*, ainsi que la possible production de SO<sub>2</sub> pour le groupe *wine 1* sont des caractères phénotypiques très marqués entre les populations (Figure 27). Notre hypothèse est que ces caractères sont le résultat d'une évolution divergente des populations en réponse à des pressions de sélection différentes. Les causes génétiques de ces changements phénotypiques restent à étudier. Pour comprendre les mécanismes moléculaires et valider des hypothèses, des mutants fonctionnels seront nécessaires. Les outils de transformation génétique aléatoire chez *B. bruxellensis* sont disponibles (Miklenic, 2013; Miklenić et al., 2015; Schifferdecker et al., 2016; Varela et al., 2018; Varela and Borneman, 2022). Cependant ces outils sont non-ciblés, et ne permettent pas l'inactivation de gènes spécifiques chez *B. bruxellensis*. L'utilisation de *S. cerevisiae* pour une expression hétérologue est une possibilité déjà expérimentée dans la littérature (Godoy et al., 2014; González et al., 2017; Varela et al., 2019). Récemment, l'utilisation de l'outil CRISPR-Cas9 a

permis de cibler certains gènes avec plus ou moins d'efficacité dû à l'état polyploïde (Varela et al., 2020), dévoilant un outil prometteur pour mieux comprendre la biologie et le métabolisme de cette levure.

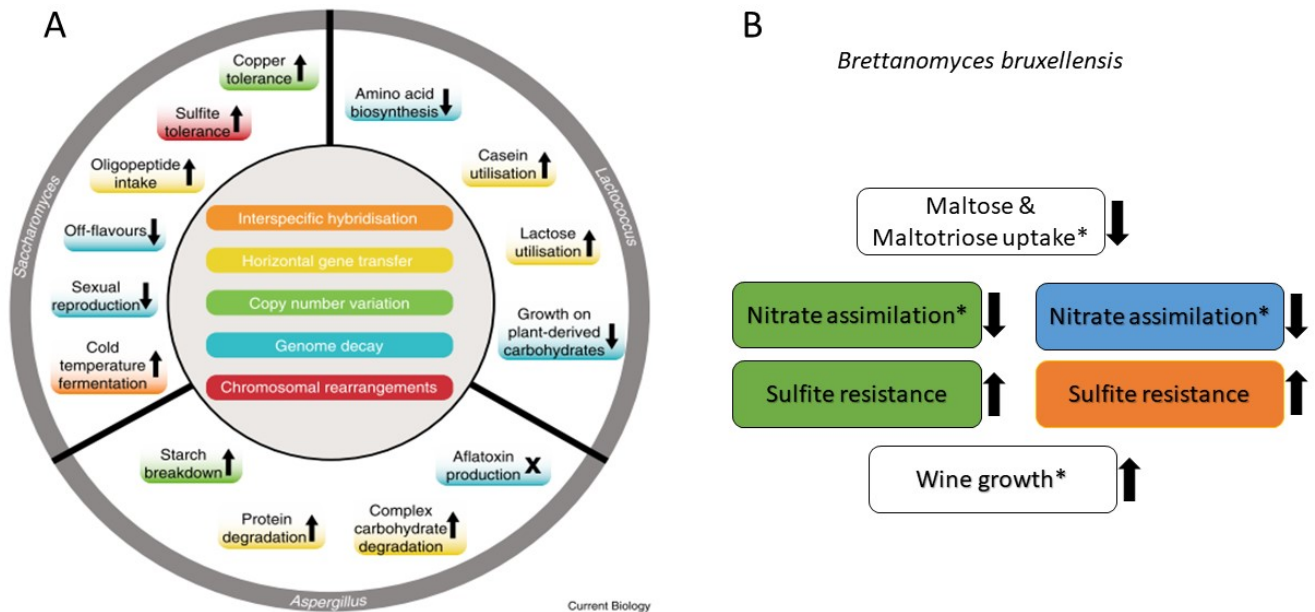


Figure 27: Principaux phénotypes en réponse à l'adaptation à des niches anthropisées. Adapté de Motthalamme et al., (2022 et de Steensels et al., (2019). Les étoiles indiquent les signes mis en évidence lors de ces travaux de thèse. Les mécanismes moléculaires n'ont pas été élucidés pour la dégradation de l'amidon ainsi que pour la croissance dans le vin.

### Quels indices sur la niche d'origine de *B. bruxellensis* ?

Dans cette partie, qui est très spéculative, nous chercherons à retracer l'habitat d'origine de *B. bruxellensis* à l'aide d'indices phénotypiques. En effet, jusqu'à présent, *B. bruxellensis* a toujours été isolée à partir d'environnements associés à l'être humain. L'obtention et l'étude d'isolats « naturels » seraient une ressource biologique importante pour étudier l'adaptation évolutive chez cette espèce. En utilisant les données phénotypiques obtenues au travers de cette thèse et les caractères décrits comme traits d'espèce, il est possible d'obtenir de potentiels indices sur l'environnement ancestral type de *B. bruxellensis*. La capacité de consommation de malto-oligosaccharides (issus de céréales) a été précédemment mise en avant (Chapitre 2). La présence de nombreux gènes codant des  $\beta$ -glucosidases avec une activité enzymatique qui semble forte (très variables d'une étude à l'autre et suivant les isolats), la capacité d'utiliser des sucres tels que la cellobiose, etc., pourraient indiquer la présence de légumes, fruits et feuilles (Crauwels et al., 2015; Kuo et al., 2018; Mansfield et al., 2002; Serra Colomer et al., 2020b; Vervoort et al., 2016). La présence de nitrate peut être conséquente dans ces différents éléments et servir de source d'azote à *B. bruxellensis* (céréales, légumes, fruits) (Colla et al., 2018). La capacité de croissance de la majeure partie des souches de *B. bruxellensis* dans le vin (Chapitre 2) confirme son adaptation aux milieux riches en éthanol. Il est donc très probable que sa niche originelle soit relativement riche en éthanol, produit par elle-même ou d'autres espèces (telles que *S. cerevisiae*). L'oxygène semble être un facteur clé dans le développement de *B. bruxellensis* induisant une forte production d'acide acétique (Aguilar Uscanga et al., 2003; Capusoni et al., 2016). La production d'acide acétique permet l'acidification importante du milieu, l'inhibition de la croissance des autres micro-organismes, puis la consommation de cet acide lorsque les sources de carbones préférentielles seront épuisées (« Make Accumulate and Consume ») (Dzialo et al., 2017; Rozpędowska et al., 2011). La production d'acide acétique attire aussi des insectes (comme

*Drosophila melanogaster*) qui sont des vecteurs de dissémination (Becher et al., 2018). La présence du complexe I de la chaîne respiratoire (seule espèce Crabtree-positive à l'avoir conservé dans son génome mitochondrial) permet le recyclage du NAD(P)H en présence d'oxygène (Procházka et al., 2010). L'effet 'Custers' a été décrit chez cette espèce (arrêt important de la croissance et de la fermentation dû au non recyclage du NAD(P)H) lors du passage d'un milieu en aérobie à un milieu en anaérobie strict (Pereira et al., 2012; Teles et al., 2022; Tiukova et al., 2013). Cependant, cet effet semble être supprimé en présence de nitrates (Galafassi et al., 2013a). Pour finir, la présence en grande quantité d'oxygène induit un ralentissement de la croissance et semble contre-productif (Capusoni et al., 2016). La production d'acide acétique semble donc être favorable à *B. bruxellensis* jusqu'à un certain point, ce qui pourrait indiquer une préférence pour un apport limité en oxygène. La faible vitesse de croissance de cette levure et la capacité de formation de VNC et de biofilms pourrait indiquer des périodes d'absence et de privation de nutriments importantes (saisonnalité). Une autre information est la sensibilité aux rayons lumineux de cette espèce (UV-C ou PL, Chapitre 4) par rapport aux autres espèces de levures testées (Chapitre 4). Cela pourrait indiquer un mode de vie dans la pénombre, à l'abri des rayonnements UV. L'ensemble de ces indices (e.g. la présence de graines et de fruits de manière saisonnière, peu ventilé, et à l'abri du soleil dans une cavité par exemple) pourrait concorder avec des réserves ou caches d'animaux (oiseaux, petits mammifères par exemple). Ces environnements sont encore mal connus mais ressemblent, sur bien des aspects, aux milieux anthropisés colonisés par *B. bruxellensis* et expliqueraient ses grandes capacités de persistance (Cibrario et al., 2019; Ruxton et al., 2014; Wiens et al., 2008). Ces hypothèses ont été rapidement discutées dans la revue publiée dans *Molecular Ecology* (Chapitre 1). Identifier l'habitat d'origine de cette espèce et isoler de nombreux individus 'sauvages' permettraient de comprendre si les changements génétiques (hybridations, LOH, CNVs) et les conséquences phénotypiques mis en évidence dans la littérature et au travers de ces travaux de thèse sont intervenus lors de l'anthropisation de *B. bruxellensis* ou sont antérieurs/ultérieurs.

### Hybridation & polyploïdie

L'une des particularités de *B. bruxellensis* est la présence d'évènements d'hybridations (intra- et interspécifiques) amenant à de nombreux individus triploïdes. L'hybridation interspécifique a parfois été vue comme une fin, une impasse évolutive, due à la perte de fertilité des hybrides. Cependant, les études de génomique comparative démontrent que plutôt d'être une impasse, l'hybridation joue un rôle majeur dans l'adaptation, la diversification et l'évolution des espèces (Stelkens and Bendixsen, 2022). L'augmentation de la ploïdie semble être un élément clé dans l'adaptation et l'évolution, car elle permet une diminution de la contre-sélection des mutations grâce à la redondance des gènes et permet donc l'acquisition potentielle de nouveaux phénotypes (Gladieux et al., 2014; Gorkovskiy and Verstrepen, 2021; Ohno, 1970; Todd et al., 2017; Wertheimer et al., 2016). L'important nombre d'hybrides dans les milieux anthropiques pose la question de savoir si ces évènements d'hybridation sont fréquents mais éliminés au cours du temps, ou rares mais sélectionnés dans les différentes niches. La prévalence des hybrides chez *B. bruxellensis* (>50% des isolats) pour le nombre d'hybridations indépendantes identifiées (4 à 5 pour le moment) semble indiquer que la deuxième hypothèse est privilégiée chez cette espèce (Avramova et al., 2018a; Eberlein et al., 2021). La datation des évènements de polyploïdisation permettrait une avancée importante dans la compréhension de ces mécanismes dans l'adaptation évolutive aux niches anthropisées. La formation de ces hybrides triploïdes est une question aussi importante tant sur les mécanismes d'hybridation que sur l'apparition de génomes haploïdes au sein de cette espèce. Les phénomènes d'hybridation chez *Saccharomyces*

spp. font intervenir principalement la reproduction sexuée. La reproduction sexuée semble peu probable chez *B. bruxellensis*, d'une part, car l'analyse des génomes indique qu'il n'y a pas d'échange génomique entre les populations et, d'autre part, car aucune forme sexuée n'a encore pu être observée à ce jour (Eberlein et al., 2021; Tiukova et al., 2019). La fusion de protoplastes (hybridation dite 'somatique') peut être une voie alternative à la formation de tels hybrides (Steensels et al., 2014). A notre connaissance, il n'existe qu'un seul isolat haploïde décrit dans cette espèce, ce qui semble être un état rare (Roach and Borneman, 2020). Enfin, la polyploïdie est généralement décrite comme un phénomène coûteux sur le plan des ressources et de l'énergie, et donc rapidement contre-sélectionné en l'absence de pression de sélection particulière (Comai, 2005). Chez *B. bruxellensis*, le maintien de nombreux isolats triploïdes suggère que la triploïdie et/ou l'hybridation conférerait à cette levure une valeur adaptative forte. Une exploration plus fine des haplotypes ciblés par les mécanismes évolutifs (en cours chez nos collaborateurs strasbourgeois) devrait permettre une meilleure compréhension de l'apport respectif des génomes supplémentaires et de l'augmentation du niveau de ploïdie dans la valeur adaptative de l'espèce.

### **Adaptation de *B. bruxellensis* : domestication, synanthropisation, pestification ?**

Des signatures d'adaptation divergente ont été identifiées au cours de ce travail de thèse. Différentes formes d'adaptation ont été décrites dans la littérature scientifique, parmi lesquelles la domestication, la synanthropisation ou encore la pestification. Quel est le substantif qui correspond le mieux à *B. bruxellensis* ?

Dans la définition du terme *domestication*, l'espèce doit être bénéfique, apporter un avantage technologique à l'être humain, et parfois dépendre exclusivement de l'être humain pour sa survie et développer des relations de dépendances importantes par accumulation de mutations délétères (Chapitre 1) (Moyers et al., 2018). Ce cas-là pourrait correspondre en partie aux isolats rencontrés dans la fabrication de bière de spécialités (type Lambic, craft-beer) ou de kombucha dans lesquels la présence de *B. bruxellensis* est recherchée et nécessaire (Antolak et al., 2021; Serra Colomer et al., 2019). Cependant, aucune relation de dépendance entre la levure et son environnement n'a été mise en évidence par nos travaux. L'utilisation de souches sélectionnées ('starter') chez cette espèce ne semble pas être autant développée par l'industrie d'après nos connaissances que pour d'autres espèces comme *S. cerevisiae*. Les pressions de sélection rencontrées ne semblent pas être assez fortes et/ou anciennes en comparaison de *S. cerevisiae* (Gallone et al., 2018). Les modes de propagations sont aussi différents ('backslopping' vs inoculation spontanée) (De Roos and De Vuyst, 2019; Whittington et al., 2019). De plus, la présence de *B. bruxellensis* dans les autres environnements (e.g. œnologique, bioéthanol) est vue comme problématique. Il semble donc prématuré d'associer le terme de *domestication* à cette espèce, même dans le milieu brassicole et de la fabrication de kombucha au vu des éléments scientifiques actuels.

Dans la majorité des environnements, la présence de *B. bruxellensis* est vue comme une contamination. Plusieurs termes ont été utilisés pour décrire des organismes causant des altérations. Le terme de 'synanthropisation' a été utilisé pour des espèces exposées à des niches anthropiques dont la présence ne profite pas à l'être humain ("*those which are regularly isolated from the ecological niches formed or transformed by humans in the course of habitation and activity*" (Glushakova et al., 2016)). Cette terminologie a été utilisée pour les rats, les pigeons ainsi que des espèces de plantes invasives (Lebedev et al., 2021; Sotek et al., 2015; Stępień, 2009). Cependant, les récentes données génomiques et phénotypiques (notamment obtenues lors de cette thèse) indiquent que *B. bruxellensis*



a été particulièrement influencée par les environnements anthropiques, ce qui est plus que le simple fait de « vivre proche » de ces procédés. Le terme de synanthropisation semble donc limité, et pas du tout adapté pour les souches utilisées volontairement (bières de spécialité et kombucha).

La *pestification* est un autre terme parfois employé, associé à des micro-organismes pathogènes (De Gracia et al., 2015; Saleh et al., 2014). Les auteurs définissent ce terme comme le processus par lequel un agent pathogène vivant à l'origine dans la nature s'adapte aux conditions biotiques et abiotiques d'environnements anthropiques (Gladieux et al., 2018). Ce terme semble plus indiqué par rapport à synanthropisation concernant l'impact et les modifications liées au changement de niche écologique. Cependant, *B. bruxellensis* n'est pas un micro-organisme pathogène, et sa présence ne provoque pas de risque particulier pour la santé humaine quels que soient les procédés considérés. Les vinificateurs trouveraient sans aucun doute le terme de *peste* particulièrement adapté pour décrire les déviations organoleptiques importantes provoquées par *Brett*.

Sur le plan rhétorique, il est donc encore difficile d'identifier le terme scientifique correspondant le mieux à l'adaptation de l'espèce *B. bruxellensis* aux procédés fermentaires auxquels cette espèce se trouve associée. Les termes *domestication* et *synanthropisation* pourraient correspondre à certaines populations (Figure 26). Sur le plan évolutif en revanche, *B. bruxellensis* s'impose comme un micro-organisme de premier choix pour étudier les phénomènes d'adaptation divergente en lien avec des environnements anthropisés et son étude devrait permettre une meilleure compréhension des mécanismes évolutifs sous-jacents.

## Conclusion

Ces travaux de thèse ont mené aux conclusions suivantes :

- i) La variabilité phénotypique de *B. bruxellensis* est liée en partie à la structure génétique de l'espèce.
- ii) Plusieurs caractères phénotypiques suggèrent une évolution divergente des populations de *B. bruxellensis* dans les différentes niches écologiques anthropisées que l'espèce colonise.
- iii) L'assimilation des nitrates et la consommation des sucres du malte sont des traits propres à l'espèce *B. bruxellensis*, sous relâchement sélectif dans certains milieux.
- iv) La perte d'hétérozygotie et la variation du nombre de copie de gènes impactent la capacité de l'espèces à consommer les nitrates et semblent être des mécanismes d'adaptation majeurs chez *B. bruxellensis*.
- v) L'utilisation de méthodes de stabilisation microbiologique physiques par rayonnement sont des approches efficaces dans le contrôle des populations de levures d'altérations en œnologie et particulièrement pour *B. bruxellensis*.

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## Annexes :

Annexes relatives à la revue : *Brettanomyces bruxellensis*: Overview of the genetic and phenotypic diversity of an anthropized yeast

Supplemental Table 1. Correspondence between the different subpopulations described in populational studies of *B. bruxellensis*.

Genetic groups as described in Gounot et al. (2020)	Group name from Avramova et al. (2018)	Genetic groups as described in Colomer et al. (2020)	Genetic groups as described in Eberlein et al. (2021)	Ploidy	Main niches of isolation
G2N1	L14165-like	Farmhouse	kombucha	Diploid	Beer, wine
G2N2				Diploid	Kombucha, wine
G2N3	CBS2499-like	Wine 2n	wine 3	Diploid	Wine
G3N1	AWRI1608-like	Lambic	beer	Allotriploid	Beer, wine
G3N2	AWRI1499-like	Wine 3n	wine 1	Allotriploid	Wine
-	L0308-like	-	wine 2	Autotriploid	Wine
-	CBS5212-like	Tequila	teq/EtOH	AlloTriploid	Bioethanol, tequila

Supplemental Table 2. Physical, chemical and biotechnological treatments used against *B. bruxellensis*. Adapted from Pinto et al. (2020) (Pinto et al., 2020).

Treatment	References	Number of isolates	
	Avramova et al. (2018)	145	
SO <sub>2</sub>	Vigentini et al. (2013)	108	
	Dimopoulou et al. (2019)	22	
	Gaseous O <sub>3</sub>	Englezos et al. (2019)	3
Electrolysed water	Cravero et al. (2018)	1	
Chemical methods	Petrova et al. (2016)	3	
	Chitosan	Bağder Elmacı et al. (2015)	1
		Paulin et al. (2020)	53
	Dimethyldicarbonate	Vincent Renouf et al. (2008)	10
	Colloidal silver	Gil-Sánchez et al. (2019)	1
Essential Oils	García-Ríos et al. (2018)	1	

Physical methods	High Hydrostatic Pressure	van Wyk & Silva (2017)	1
	Pulsed Electric Fields	van Wyk et al. (2019)	3
	High Power Ultrasounds	Breniaux et al. (2019)	1
	Filtration	Umiker et al. (2013)	2
		Duarte et al. (2017)	1
	UV-light	Fredericks et al. (2011)	1
		Pilard et al. (2021)	111
Micro-waves	Zeng et al. (2014)	1	
Biotechnological approaches	Starter cultures of <i>S. cerevisiae</i> / <i>O. oeni</i>	Berbegal et al. (2017)	1
	Killer toxin Saccharomycin	Branco et al. (2019)	1
	Killer toxin SeKT killer toxin	Mazzucco et al. (2019)	1
	Avidin	von Cosmos & Edwards (2016)	6
	killer toxin <i>Ustilago maydis</i>	Santos et al. (2011)	17

Supplemental Table 3: Non-exhaustive list of references studying *B. bruxellensis* isolates. This bibliographic list was used to produce Figure 3.

Study	Number of strains	Geographical origins	Substrates	Processes
(Abbott et al., 2005)	3	NA	Bio-ethanol	Bio-ethanol
(Agnolucci et al., 2009)	5	Africa; Europe	Wine; Beer	Various processes
(Agnolucci et al., 2010)	7	Europe	Wine	Wine
(Agnolucci, Scarano, Rea, Toffanin, & Nuti, 2007)	NA	Europe	Wine	Wine
(Uscanga et al., 2000)	1	America	Bio-ethanol	Bio-ethanol
(Uscanga et al., 2003)	1	America	Bio-ethanol	Bio-ethanol

(Albertin et al., 2018)	33	Europe; America; Oceania	Wine; Beer	Various processes
(Angela et al., 2020)	1	Asia	Kombucha	Kombucha
(Arroyo-López et al., 2006)	1	Europe	Olives	Other
(Avramova et al., 2018a)	1488	Europe; America; Africa; Asia; Oceania	Beer; Bio- ethanol; Kombucha; Tequila; Wine; Soft drink; Cider	Various processes
(Avramova et al., 2019)	3	Europe; Oceania	Wine	Wine
(Avramova et al., 2018b)	145	Europe; Oceania; Africa; America; Asia	Beer; Bio- ethanol; Kombucha; Tequila; Wine	Various processes
(Bağder Elmacı et al., 2015)	1	Europe	Beer	Beer
(Barata et al., 2008)	35	Europe; America	Wine; Beer	Various processes
(Basílio et al., 2008)	1	America	Bio-ethanol	Bio-ethanol
(Bassi et al., 2018)	1	America	Bio-ethanol	Bio-ethanol
(Benito et al., 2009)	9	Europe	Wine	Wine
(Berbegal et al., 2017)	1	Europe	Wine	Wine
(Blomqvist et al., 2010)	3	Europe	Bio-ethanol	Bio-ethanol
(Blomqvist et al., 2012)	1	Europe	Bio-ethanol	Bio-ethanol
(Bokulich et al., 2012)	NA	NA	Beer	Beer
(Borneman et al., 2014)	4	Europe; Oceania	Wine	Wine



(Branco et al., 2019)	1	Europe	Wine	Wine
(Cajueiro et al., 2017)	1	America	Bio-ethanol	Bio-ethanol
(Capozzi et al., 2016)	7	Europe	Wine	Wine
(Cartwright et al., 2018)	2	America	Wine	Wine
(Chandra et al., 2016)	2	Europe	Wine	Wine
(Chatonnet et al., 1992)	10	Europe	Wine	Wine
(Cheng et al., 2017)	1	Europe	Wine	Wine
(Chescheir et al., 2015)	1	Oceania	Wine	Wine
(Cibrario et al., 2019)	1411	Europe; America; Africa; Asia; Oceania	Bio-ethanol; Wine; Beer; Kombucha	Various processes
(Cibrario et al., 2020a)	49	Europe; America; Africa; Oceania	Bio-ethanol; Wine; Beer; Kombucha	Various processes
(Cibrario et al., 2020b)	8	Europe; America; Oceania	Wine	Wine
(Claussen, 1904)	NA	NA	Beer	Beer
(Codato et al., 2018)	1	America	Bio-ethanol	Bio-ethanol
(Conterno et al., 2013)	1	Europe	Wine	Wine
(Conterno et al., 2006)	48	Europe; America; Asia; Oceania	Beer; Wine	Various processes
(Coton et al., 2017)	NA	NA	Kombucha	Kombucha
(Coulon et al., 2010)	NA	Europe	Wine	Wine

(Crauwels et al., 2015)	7	Europe; Oceania; America	Wine; Beer	Various processes
(Crauwels et al., 2017)	8	Europe; Oceania; America	Wine; Beer; Soft drink	Various processes
(Crauwels et al., 2014)	26	Europe; America; Africa; Oceania	Beer; Wine	Various processes
(Cravero et al., 2018)	1	Europe	Beer	Beer
(Curtin et al., 2007)	244	Oceania	Wine	Wine
(Curtin et al., 2012)	1	Oceania	Wine	Wine
(Curtin et al., 2012)	41	Oceania	Wine	Wine
(da Silva et al., 2019)	30	America	Bio-ethanol	Bio-ethanol
(Da Silva, Leite, & De Morais, 2016)	NA	America	Bio-ethanol	Bio-ethanol
(Daenen et al., 2007)	16	Europe	Beer	Beer
(de Barros Pita and Tiukova, 2013)	1	America	Bio-ethanol	Bio-ethanol
(de Souza Liberal, Carazzolle, Pereira, Simões, & de Morais, 2012)	2	America; Europe	Bio-ethanol; Beer	Various processes
(de Souza Liberal et al., 2007)	NA	America	Bio-ethanol	Bio-ethanol
(Di Toro et al., 2015)	48	Europe	Wine	Wine
(Dias et al., 2003a)	21	NA	Wine; Beer; Soft drink	Various processes
(Dias et al., 2003b)	1	Europe	Wine	Wine
(Dimopoulou et al., 2019a)	22	Europe	Wine	Wine
(Dimopoulou et al., 2019b)	8	Europe; America	Wine; Beer	Various processes

(Duarte et al., 2017)	1	Europe	Wine	Wine
(DuBois, 2017)	6	Europe; America; Oceania	Wine	Wine
(Englezos et al., 2019)	3	Europe	Wine	Wine
(Fabrizio et al., 2015)	4	Europe; Africa	Wine	Wine
(Fadda et al., 2001)	1	Europe	Feta	Other
(Fournier et al., 2017)	9	Europe; Oceania; Africa	Wine; Bio- ethanol; Beer	Various processes
(Fredericks et al., 2011)	1	Europe	Beer	Beer
(Freer, 2002)	12	NA	Beer; Wine	Various processes
(Freer et al., 2003)	6	NA	Beer; Wine	Various processes
(Galafassi et al., 2013a)	1	Europe	Wine	Wine
(Galafassi et al., 2011)	28	Europe; America; Oceania	Wine; Beer; Bio-ethanol	Various processes
(Gerbaux et al., 2000)	NA	Europe	Wine	Wine
(Gil-Sánchez et al., 2019)	1	NA	Wine	Wine
(Godoy, García, Peña, Martínez, & Ganga, 2014)	1	America	Wine	Wine
(Godoy, Martinez, Carrasco, & Ganga, 2008)	1	America	Wine	Wine
(Godoy et al., 2017)	3	America; Europe; Oceania	Wine	Wine

(Godoy, Vera-Wolf, Martinez, Ugalde, & Ganga, 2016)	1	America	Wine	Wine
(González-Arenzana et al., 2019)	NA	Europe	Wine	Wine
(González et al., 2017)	1	America	NA	Not available
(Gounot et al., 2020)	53	Europe; Africa; Oceania; America	Wine; Beer; Bio-ethanol; Soft drink	Various processes
(Gracin et al., 2016)	NA	Europe	Wine	Wine
(Granato et al., 2015)	19	Europe; America; Oceania	Wine; Beer; Bio-ethanol	Various processes
(Guo et al., 2016)	1	Europe	Wine	Wine
(Hellborg and Piškur, 2009)	30	Europe; America; Oceania	Wine; Beer; Soft drink	Various processes
(Heresztyn, 1986)	3	Europe	Wine	Wine
(Hixson et al., 2012)	3	Europe	Wine	Wine
(Joseph et al., 2013)	5	Europe; America; Oceania	Beer; Wine	Various processes
(Joseph et al., 2007)	40	Europe; Asia; America; Oceania; Africa	Wine	Wine
(Kregiel et al., 2018)	1	NA	Soft drink	Other
(Križanović et al., 2020)	1	Europe	Wine	Wine
(Kumara et al., 1993)	1	Europe	Beer	Beer
(Kuo et al., 2018)	1	Asia	NA	Not available

(Lebleux et al., 2020)	65	NA	Wine	Wine
(Leite et al., 2012)	1	America	Bio-ethanol	Bio-ethanol
(Lentz and Harris, 2015)	5	America; Europe	Beer	Beer
(Longin et al., 2016)	2	Europe	Wine	Wine
(Louw et al., 2016)	3	Europe; Africa; Oceania	Wine	Wine
(Madsen et al., 2017)	2	Europe	Wine	Wine
(Martyniak et al., 2017)	2	Europe	Beer	Beer
(Mazzucco et al., 2019)	1	NA	Wine	Wine
(Meroth et al., 2003)	3	Europe; Africa	Wine; Beer	Various processes
(Millet and Lonvaud-Funel, 2000)	NA	NA	Wine	Wine
(Miot-Sertier and Lonvaud-Funel, 2007)	9	Europe; Africa;	Wine; Beer	Various processes
(Moktaduzzaman et al., 2015)	1	Europe	Wine	Wine
(Moktaduzzaman et al., 2016)	30	NA	Wine; Beer	Various processes
(Morata et al., 2013)	1	Europe	Wine	Wine
(Morrissey et al., 2004)	NA	NA	Cider	Other
(Nardi et al., 2010)	1	Europe	Wine	Wine
(Neto et al., 2014)	1	America	Bio-ethanol	Bio-ethanol
(Olsen et al., 2015)	1	America	Bio-ethanol	Bio-ethanol
(Oro et al., 2019)	24	Europe	Wine	Wine
(Parente et al., 2018)	1	America	Bio-ethanol	Bio-ethanol
(Parente et al., 2014)	1	America	Bio-ethanol	Bio-ethanol

(Passoth et al., 2007)	1	Europe	Bio-ethanol	Bio-ethanol
(Peña-Moreno et al., 2019)	1	America	Bio-ethanol	Bio-ethanol
(Pereira et al., 2012)	9	America	Bio-ethanol	Bio-ethanol
(Petrova et al., 2016)	2	America	Wine	Wine
(Peynaud and Domercq, 1956)	6	Europe	Wine; Beer	Various processes
(Phowchinda et al., 1995)	1	Europe	Beer	Beer
(Piškur et al., 2012)	1	Europe	Wine	Wine
(Procházka et al., 2010)	1	Europe	Wine	Wine
(Reis et al., 2014)	1	America	Bio-ethanol	Bio-ethanol
(Renouf and Lonvaud-Funel, 2007)	4	NA	NA	Not available
(Renouf et al., 2008)	10	NA	NA	Not available
(Roach and Borneman, 2020)	1	America	Wine	Wine
(Romano et al., 2008)	15	NA	Wine	Wine
(Romano et al., 2017)	1	NA	Wine	Wine
(Rozpędowska et al., 2011)	1	Europe	Wine	Wine
(Santos et al., 2011)	17	Europe	Wine	Wine
(Schifferdecker et al., 2016)	30	Europe; America; Oceania	Wine; Beer	Various processes
(Schopp et al., 2013)	15	America	Wine	Wine
(Serpaggi et al., 2012)	1	Europe	Wine	Wine
(Serra Colomer et al., 2020a)	64	Europe; America; Africa; Oceania	Beer; Bio-ethanol; Kombucha; Tequila; Wine	Various processes

(Serra Colomer et al., 2020b)	5	Europe; America	Beer	Beer
(Smith and Divol, 2018)	6	Africa; Europe; Oceania	Wine; Beer	Various processes
(Spaepen and Verachtert, 1982)	12	NA	Beer	Beer
(Spitaels et al., 2014)	NA	Europe	Beer	Beer
(Tchobanov et al., 2008)	1	Europe	Wine	Wine
(Teles et al., 2018)	1	America	Bio-ethanol	Bio-ethanol
(Teoh et al., 2004)	1	NA	Kombucha	Kombucha
(Tiukova et al., 2014)	1	America	Bio-ethanol	Bio-ethanol
(Tiukova et al., 2013)	1	America	Bio-ethanol	Bio-ethanol
(Tiukova et al., 2019)	1	America	Bio-ethanol	Bio-ethanol
(Tran et al., 2020)	1	Europe	Kombucha	Kombucha
(Tyrawa et al., 2019)	9	Europe; America	Beer; Wine	Various processes
(Umiker et al., 2013)	2	America	Wine	Wine
(Valdes et al., 2014)	1	America	Wine	Wine
(Valdetara et al., 2020)	2	Europe; Oceania	Wine	Wine
(Van Oevelen et al., 1977)	15	Europe	Beer	Beer
(van Wyk and Silva, 2017)	3	Oceania; Europe	Wine	Wine
(van Wyk et al., 2019)	3	Oceania; Europe	Wine	Wine
(Vanbeneden et al., 2008)	NA	NA	Beer	Beer
(C. Varela et al., 2019)	2	Oceania	Wine	Wine

(C. Varela et al., 2020)	2	Oceania	Wine	Wine
(Vigentini et al., 2013)	108	Europe; America; Asia; Oceania; Africa	Beer; Wine; Cider	Various processes
(Vigentini et al., 2008)	17	Europe; Africa	Wine	Wine
(von Cosmos and Edwards, 2016)	6	America; Europe; Oceania	Wine	Wine
(Wang et al., 2019)	1	NA	NA	Not available
(Woolfit et al., 2007)	1	Europe	Wine	Wine
(Yarrow and Ahearn, 1971)	1	America	Beer	Beer
(Zeng et al., 2014)	1	Asia	Fermented rice	Other
(Zepeda-Mendoza et al., 2018)	3	Europe	Wine	Wine
(Zuehlke and Edwards, 2013)	3	America	Wine	Wine

*Annexes relatives à l'article: Wine yeast species show strong inter- and intra-specific variability in their sensitivity to ultraviolet radiation*

Supplemental Table 1: Yeast species and strains used in this work

Species	Strain	Genetic group	Collection	Reference
<i>B. anomalus</i>	BR 23-4	NA	UBO	
<i>B. anomalus</i>	CLIB 304	NA	CLIB	
<i>B. anomalus</i>	NRRL Y-17522 T	NA	NRRL	
<i>B. bruxellensis</i>	12_LT_VGC3_c_10	1st Wine 3N	ISVV	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	15_1	Wine/Kombucha 2N	Thessaloniki	Dimopoulou, Food microbiol, 2019
<i>B. bruxellensis</i>	1961_MX_M1_E2	Wine 2N	ISVV	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	20T13_02	1st Wine 3N	ISVV	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	20T13_05	Wine/Beer 3N	ISVV	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7



<i>B. bruxellensis</i>	2OT13_07	Wine/Beer 3N	ISVV	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	2OT14_01	Wine/Beer 3N	ISVV	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	2OT14_03	Wine/Beer 3N	ISVV	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	33_2	1st Wine 3N	Thessaloniki	Dimopoulou, Food microbiol, 2019
<i>B. bruxellensis</i>	AWRI1499	1st Wine 3N	AWRI	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	AWRI1608	Wine/Beer 3N	AWRI	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	CBS 2499	Wine 2N	CBS	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	CBS 5512	Tequila/Bioethanol 3N	CBS	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	CBS 6055	Tequila/Bioethanol 3N	CBS	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	CDR217	1st Wine 3N	InterRhone	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	DK24A-V	Wine/Beer 3N	UNINA	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	DK4C	1st Wine 3N	UNINA	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	ISA1601	Wine/Kombucha 2N	ISA	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	ISA2211	2nd Wine 3N	ISA	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	ISA2397	Wine/Beer 3N	ISA	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L0308	2nd Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L0417	1st Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7

<i>B. bruxellensis</i>	L0469	Wine 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L0614	Wine 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L14155	1st Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L14163	Wine 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L14169	Tequila/Bioethanol 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L14174	1st Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L14175	1st Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L14190	1st Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L14194	1st Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1703	1st Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L17108	Tequila/Bioethanol 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L17111	1st Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L17112	Wine/Beer 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L17114	Wine/Beer 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1714	Wine 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1715	Tequila/Bioethanol 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1727	1st Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1729	Wine/Beer 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7

<i>B. bruxellensis</i>	L1733	2nd Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1735	Wine/Beer 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1737	Wine 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1741	Wine/Beer 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1746	1st Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1749	Wine/Beer 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1751	Wine 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1753	Wine/Kombucha 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1754	Wine/Kombucha 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1755	Wine/Kombucha 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1756	Wine/Kombucha 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1757	2nd Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1758	Wine/Beer 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1759	Wine/Kombucha 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1760	Wine/Kombucha 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1761	Wine 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1762	Wine 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1763	Wine 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7

<i>B. bruxellensis</i>	L1764	Wine/Beer 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1765	Wine 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1766	Wine 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1771	Wine/Beer 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1782	2nd Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	LB15107g	Wine/Beer 3N	ISVV	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	LB15110g	Wine/Beer 3N	ISVV	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	NL045	Wine/Beer 3N	IFV	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	NL050	1st Wine 3N	IFV	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	NL059	Wine/Beer 3N	IFV	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	SJ12_4	Tequila/Bioethanol 3N	ISVV	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	UWOPS_92_298_4	Tequila/Bioethanol 3N	UWOPS	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	VP1519	Wine/Beer 3N	Foggia	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	VP1539	2nd Wine 3N	Foggia	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	VP1544	2nd Wine 3N	Foggia	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	YJS5301	Wine/Kombucha 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5302	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5319	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5320	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5340	Wine/Kombucha 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077

<i>B. bruxellensis</i>	YJS5345	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5347	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5349	Wine/Kombucha 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5357	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5363	Wine/Kombucha 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5373	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5384	Wine/Kombucha 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5385	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5392	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5397	Wine/Beer 3N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5398	Wine/Kombucha 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5402	Wine/Kombucha 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5406	Wine/Kombucha 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5408	1st Wine 3N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5416	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5417	Wine/Kombucha 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5420	Wine/Kombucha 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5422	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5426	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5431	Wine/Kombucha 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5434	1st Wine 3N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5445	1st Wine 3N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5449	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5456	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5459	1st Wine 3N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5463	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5469	1st Wine 3N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077

<i>B. bruxellensis</i>	YJS5473	1st Wine 3N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5476	1st Wine 3N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5478	1st Wine 3N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5479	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5485	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5487	1st Wine 3N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>H. uvarum</i>	L1433	Group B	CRBO	Albertin et Al, Frontiers microbiol, 2016, 10.3389/fmicb.2015.01569
<i>H. uvarum</i>	NZ15	Group A	CRPR	Albertin et Al, Frontiers microbiol, 2016, 10.3389/fmicb.2015.01569
<i>H. uvarum</i>	Y-1614	Group C	NRRL	Albertin et Al, Frontiers microbiol, 2016, 10.3389/fmicb.2015.01569
<i>L. thermotolerans</i>		18 Domestic 2	Foggia	Hranilovic et al, 2017, plos one, 10.1371/journal.pone.0184652
<i>L. thermotolerans</i>	AEB	Mix Europe/ North America	Commercial strain (AEB)	Hranilovic et al, 2017, plos one, 10.1371/journal.pone.0184652
<i>L. thermotolerans</i>	CLIB292	Domestic 1	CLIB	Hranilovic et al, 2017, plos one, 10.1371/journal.pone.0184652
<i>M. pulcherrima</i>	L0675	NA	CRBO	
<i>M. pulcherrima</i>	NZ268	NA	CRPR	
<i>M. pulcherrima</i>	Y-7111	NA	NRRL	
<i>S. cerevisiae</i>	A24	NA	ISVV	Albertin et al 2011, AEM, 10.1128/AEM.02547-10
<i>S. cerevisiae</i>	Fx10	NA	Commercial starter (Laffort company, France)	
<i>S. cerevisiae</i>	GN	NA	ISVV	Albertin et al 2011, AEM, 10.1128/AEM.02547-10
<i>S. cerevisiae</i>	L0431	NA	CRBO	
<i>S. cerevisiae</i>	L0432	NA	CRBO	
<i>S. cerevisiae</i>	L0433	NA	CRBO	
<i>S. cerevisiae</i>	L0437	NA	CRBO	
<i>S. cerevisiae</i>	SB	NA	ISVV	Albertin et al 2011, AEM, 10.1128/AEM.02547-10
<i>S. cerevisiae</i>	X5	NA	Commercial starter (Laffort company, France)	
<i>S. cerevisiae</i>	Y7327	NA	NRRL	
<i>S. uvarum</i>	U1	NA	ISVV	Da silva et al, 2015, plos one, 10.1371/journal.pone.0123834
<i>S. uvarum</i>	U3	NA	ISVV	Da silva et al, 2015, plos one, 10.1371/journal.pone.0123834

<i>Sc x Su</i>	DU23	NA	ISVV	Da silva et al, 2015, plos one, 10.1371/journal.pone.0123834
<i>Sc x Su</i>	EU23	NA	ISVV	Da silva et al, 2015, plos one, 10.1371/journal.pone.0123834
<i>Schizo. pombe</i>	L0442	NA	CRBO	
<i>Schizo. pombe</i>	Y-11791	NA	NRRL	
<i>Schizo. pombe</i>	Y-12796	NA	NRRL	
<i>Starm. bacillaris</i>	10_372	Blue (italy/greece)	Debrecen	Masneuf-Pomarede et al, 2015, Fems Yeast Res, 10.1093/femsyr/fov045
<i>Starm. bacillaris</i>	L0473	Grey (miscellaneous)	CRBO	Masneuf-Pomarede et al, 2015, Fems Yeast Res, 10.1093/femsyr/fov045
<i>Starm. bacillaris</i>	NZ12	Purple (Spain/France)	CRPR	Masneuf-Pomarede et al, 2015, Fems Yeast Res, 10.1093/femsyr/fov045
<i>T. delbrueckii</i>	B172	Grape/Wine	UOA	Albertin et Al, Plos One, 2014, 10.1371/journal.pone.0094246
<i>T. delbrueckii</i>	CLIB 230	Nature Americas	CLIB	Albertin et Al, Plos One, 2014, 10.1371/journal.pone.0094246
<i>T. delbrueckii</i>	L0705	Bioprocess	CRBO	Albertin et Al, Plos One, 2014, 10.1371/journal.pone.0094246
<i>Tri. cantarellii</i>	L0412	NA	CRBO	
<i>Tri. cantarellii</i>	L0416	NA	CRBO	
<i>Tri. cantarellii</i>	L0419	NA	CRBO	
<i>Zygo. bailii</i>	CLIB 213	NA	CLIB	
<i>Zygo. bailii</i>	L0446	NA	CRBO	
<i>Zygo. bailii</i>	L0536	NA	CRBO	
<i>Zygo. rouxii</i>	CLIB 233	NA	CLIB	
<i>Zygo. rouxii</i>	L0314	NA	CRBO	

AWRI: Wine Microorganism Culture Collection, Australian Wine Research Institute, Adelaide, Australia

Debrecen: University of Debrecen, Hungary

CBS: CBS-KNAW, Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre, institute of the Royal Netherlands Academy of Arts and Sciences (Koninklijke Nederlandse Akademie van Wetenschappen), Utrecht, Netherlands

Foggia: University of Foggia, Italy  
IFV: Institut Français de la Vigne et du Vin, Pole Val de Loire, France

InterRhône: Inter-Rhône, Avignon, France

ISVV: Institut des Sciences de la Vigne et du Vin, Villenave d'Ornon, France

CLIB: Collection de Levures d'Interet Biotechnologique, now CIRM-Levures, INRA, France

NRRL: Northern Regional Research Laboratory, ARS Culture Collection, Peoria, USA

CRBO: Centre de Ressources Biologiques (Enologie, Villenave d'Ornon, France

Thessaloniki: Aristotle University of Thessaloniki, Greece

CRPR: Centre de Recherche Pernod-Ricard, Creteil, France

Annexes relatives à l'article: Evaluating the influence of operational parameters of pulsed light on wine related yeasts: focus on inter- and intra-specific variability sensitivity

Supplemental Table 1: Yeast species and strains used in this work

Species	Strain	Genetic group or Ecological niche	Collection	Reference
<i>B. anomalus</i>	BR 23-4	NA	UBO	
<i>B. anomalus</i>	CLIB 304	NA	CLIB	
<i>B. anomalus</i>	NRRL Y-17522 T	NA	NRRL	
<i>B. bruxellensis</i>	12_LT_VGC3_c_10	1st Wine 3N	ISVV	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	15_1	Wine/Kombucha 2N	Thessaloniki	Dimopoulou, Food microbiol, 2019
<i>B. bruxellensis</i>	1961_MX_M1_E2	Wine 2N	ISVV	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	20T13_02	1st Wine 3N	ISVV	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	20T13_05	Wine/Beer 3N	ISVV	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	20T13_07	Wine/Beer 3N	ISVV	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	20T14_01	Wine/Beer 3N	ISVV	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	20T14_03	Wine/Beer 3N	ISVV	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	33_2	1st Wine 3N	Thessaloniki	Dimopoulou, Food microbiol, 2019
<i>B. bruxellensis</i>	AWRI1499	1st Wine 3N	AWRI	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	AWRI1608	Wine/Beer 3N	AWRI	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	CBS 2499	Wine 2N	CBS	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	CBS 5512	Tequila/Bioethanol 3N	CBS	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	CBS 6055	Tequila/Bioethanol 3N	CBS	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7



<i>B. bruxellensis</i>	CDR217	1st Wine 3N	InterRhone	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	DK24A-V	Wine/Beer 3N	UNINA	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	DK4C	1st Wine 3N	UNINA	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	ISA1601	Wine/Kombucha 2N	ISA	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	ISA2211	2nd Wine 3N	ISA	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	ISA2397	Wine/Beer 3N	ISA	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L0308	2nd Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L0417	1st Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L0469	Wine 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L0614	Wine 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L14155	1st Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L14163	Wine 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L14169	Tequila/Bioethanol 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L14174	1st Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L14175	1st Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L14190	1st Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L14194	1st Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1703	1st Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7

<i>B. bruxellensis</i>	L17108	Tequila/Bioethanol 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L17111	1st Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L17112	Wine/Beer 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L17114	Wine/Beer 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1714	Wine 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1715	Tequila/Bioethanol 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1727	1st Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1729	Wine/Beer 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1733	2nd Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1735	Wine/Beer 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1737	Wine 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1741	Wine/Beer 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1746	1st Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1749	Wine/Beer 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1751	Wine 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1753	Wine/Kombucha 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1754	Wine/Kombucha 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1755	Wine/Kombucha 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7

<i>B. bruxellensis</i>	L1756	Wine/Kombucha 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1757	2nd Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1758	Wine/Beer 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1759	Wine/Kombucha 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1760	Wine/Kombucha 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1761	Wine 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1762	Wine 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1763	Wine 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1764	Wine/Beer 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1765	Wine 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1766	Wine 2N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1771	Wine/Beer 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	L1782	2nd Wine 3N	CRBO	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	LB15107g	Wine/Beer 3N	ISVV	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	LB15110g	Wine/Beer 3N	ISVV	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	NL045	Wine/Beer 3N	IFV	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	NL050	1st Wine 3N	IFV	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	NL059	Wine/Beer 3N	IFV	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7

<i>B. bruxellensis</i>	SJ12_4	Tequila/Bioethanol 3N	ISVV	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	UWOPS_92_298_4	Tequila/Bioethanol 3N	UWOPS	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	VP1519	Wine/Beer 3N	Foggia	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	VP1539	2nd Wine 3N	Foggia	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	VP1544	2nd Wine 3N	Foggia	Avramova et al, 2018, Scientific reports, :10.1038/s41598-018-22580-7
<i>B. bruxellensis</i>	YJS5301	Wine/Kombucha 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5302	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5319	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5320	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5340	Wine/Kombucha 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5345	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5347	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5349	Wine/Kombucha 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5357	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5363	Wine/Kombucha 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5373	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5384	Wine/Kombucha 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5385	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5392	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5397	Wine/Beer 3N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5398	Wine/Kombucha 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5402	Wine/Kombucha 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5406	Wine/Kombucha 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5408	1st Wine 3N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5416	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077

<i>B. bruxellensis</i>	YJS5417	Wine/Kombucha 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5420	Wine/Kombucha 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5422	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5426	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5431	Wine/Kombucha 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5434	1st Wine 3N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5445	1st Wine 3N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5449	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5456	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5459	1st Wine 3N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5463	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5469	1st Wine 3N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5473	1st Wine 3N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5476	1st Wine 3N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5478	1st Wine 3N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5479	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5485	Wine 2N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>B. bruxellensis</i>	YJS5487	1st Wine 3N	YJS	Gounot et al, 2020, GBE, 10.1093/gbe/evaa077
<i>H. uvarum</i>	L1433	Group B	CRBO	Albertin et Al, Frontiers microbiol, 2016, 10.3389/fmicb.2015.01569
<i>H. uvarum</i>	NZ15	Group A	CRPR	Albertin et Al, Frontiers microbiol, 2016, 10.3389/fmicb.2015.01569
<i>H. uvarum</i>	Y-1614	Group C	NRRL	Albertin et Al, Frontiers microbiol, 2016, 10.3389/fmicb.2015.01569
<i>L. thermotolerans</i>	18	Domestic 2	Foggia	Hranilovic et al, 2017, plos one, 10.1371/journal.pone.0184652
<i>L. thermotolerans</i>	AEB	Mix Europe/ North America	Commercial strain (AEB)	Hranilovic et al, 2017, plos one, 10.1371/journal.pone.0184652
<i>L. thermotolerans</i>	CLIB292	Domestic 1	CIRM-Levures	Hranilovic et al, 2017, plos one, 10.1371/journal.pone.0184652
<i>M. pulcherrima</i>	L0675	NA	CRBO	
<i>M. pulcherrima</i>	NZ268	NA	CRPR	
<i>M. pulcherrima</i>	Y-7111	NA	NRRL	
<i>S. cerevisiae</i>	A24	Dist	Hambleton Bard	Albertin et al 2011, AEM, 10.1128/AEM.02547-10

<i>S. cerevisiae</i>	Fx10	Wine	Commercial starter (Laffort company, France)	
<i>S. cerevisiae</i>	GN	Wine	ISVV	Albertin et al 2011, AEM, 10.1128/AEM.02547-10
<i>S. cerevisiae</i>	CRBO L0431	Wine	CRBO	
<i>S. cerevisiae</i>	CRBO L0432	Wine	CRBO	
<i>S. cerevisiae</i>	CRBO L0433	Wine	CRBO	
<i>S. cerevisiae</i>	CRBO L0437	Wine	CRBO	
<i>S. cerevisiae</i>	SB	Wine	ISVV	Albertin et al 2011, AEM, 10.1128/AEM.02547-10
<i>S. cerevisiae</i>	X5	Wine	Commercial starter (Laffort company, France)	
<i>S. cerevisiae</i>	Y7327	Beer	NRRL	
<i>S. uvarum</i>	U1	NA	ISVV	Da silva et al, 2015, plos one, 10.1371/journal.pone.0123834
<i>S. uvarum</i>	U3	NA	ISVV	Da silva et al, 2015, plos one, 10.1371/journal.pone.0123834
<i>Sc x Su</i>	DU23	NA	ISVV	Da silva et al, 2015, plos one, 10.1371/journal.pone.0123834
<i>Sc x Su</i>	EU23	NA	ISVV	Da silva et al, 2015, plos one, 10.1371/journal.pone.0123834
<i>Schizo. pombe</i>	L0442	NA	CRBO	
<i>Schizo. pombe</i>	Y-11791	NA	NRRL	
<i>Schizo. pombe</i>	Y-12796	NA	NRRL	
<i>Starm. bacillaris</i>	10_372	Blue (italy/greece)	Debrecen	Masneuf-Pomarede et al, 2015, Fems Yeast Res, 10.1093/femsyr/fov045
<i>Starm. bacillaris</i>	L0473	Grey (miscellaneous)	CRBO	Masneuf-Pomarede et al, 2015, Fems Yeast Res, 10.1093/femsyr/fov045
<i>Starm. bacillaris</i>	NZ12	Purple (Spain/France)	CRPR	Masneuf-Pomarede et al, 2015, Fems Yeast Res, 10.1093/femsyr/fov045
<i>T. delbrueckii</i>	B172	Grape/Wine	UOA	Albertin et Al, Plos One, 2014, 10.1371/journal.pone.0094246
<i>T. delbrueckii</i>	CLIB 230	Nature Americas	CIRM-Levures	Albertin et Al, Plos One, 2014, 10.1371/journal.pone.0094246
<i>T. delbrueckii</i>	L0705	Bioprocess	CRBO	Albertin et Al, Plos One, 2014, 10.1371/journal.pone.0094246
<i>Tri. cantarellii</i>	L0412	NA	CRBO	
<i>Tri. cantarellii</i>	L0416	NA	CRBO	
<i>Tri. cantarellii</i>	L0419	NA	CRBO	
<i>Zygo. bailii</i>	CLIB 213	NA	CIRM-Levures	
<i>Zygo. bailii</i>	L0446	NA	CRBO	
<i>Zygo. bailii</i>	L0536	NA	CRBO	

<i>Zygo. rouxii</i>	CLIB 233	NA	CIRM- Levures	
<i>Zygo. rouxii</i>	L0314	NA	CRBO	
<i>S. cerevisiae</i>	227	Beer		
<i>S. cerevisiae</i>	CLIB-382	Beer	CIRM- Levures	Albertin et al 2009, J. EVOL. BIOL., 10.1111/j.1420-9101.2009.01828.x
<i>S. cerevisiae</i>	CLIB-650	Beer	CIRM- Levures	Albertin et al 2009, J. EVOL. BIOL., 10.1111/j.1420-9101.2009.01828.x
<i>S. cerevisiae</i>	Nottingham	Beer		
<i>S. cerevisiae</i>	premium gold	Beer		
<i>S. cerevisiae</i>	S-04	Beer		
<i>S. cerevisiae</i>	US-56	Beer		
<i>S. cerevisiae</i>	Windsor	Beer	Danstar	Albertin et al 2009, J. EVOL. BIOL., 10.1111/j.1420-9101.2009.01828.x
<i>S. cerevisiae</i>	Y7328	Beer	NRRL	
<i>S. cerevisiae</i>	CLIB-215	Bread	CIRM- Levures	Albertin et al 2009, J. EVOL. BIOL., 10.1111/j.1420-9101.2009.01828.x
<i>S. cerevisiae</i>	CLIB-319	Bread	CIRM- Levures	Albertin et al 2009, J. EVOL. BIOL., 10.1111/j.1420-9101.2009.01828.x
<i>S. cerevisiae</i>	CLIB-324	Bread	CIRM- Levures	Albertin et al 2009, J. EVOL. BIOL., 10.1111/j.1420-9101.2009.01828.x
<i>S. cerevisiae</i>	CLIB-646	Bread	CIRM- Levures	Albertin et al 2009, J. EVOL. BIOL., 10.1111/j.1420-9101.2009.01828.x
<i>S. cerevisiae</i>	Hirondelle	Bread	Lesaffre	Albertin et al 2009, J. EVOL. BIOL., 10.1111/j.1420-9101.2009.01828.x
<i>S. cerevisiae</i>	SBA	Bread	Lesaffre	Albertin et al 2009, J. EVOL. BIOL., 10.1111/j.1420-9101.2009.01828.x
<i>S. cerevisiae</i>	SBB	Bread	Lesaffre	Albertin et al 2009, J. EVOL. BIOL., 10.1111/j.1420-9101.2009.01828.x
<i>S. cerevisiae</i>	SBC	Bread	Lesaffre	Albertin et al 2009, J. EVOL. BIOL., 10.1111/j.1420-9101.2009.01828.x
<i>S. cerevisiae</i>	SBD	Bread	Lesaffre	Albertin et al 2009, J. EVOL. BIOL., 10.1111/j.1420-9101.2009.01828.x
<i>S. cerevisiae</i>	SBE	Bread	Lesaffre	Albertin et al 2009, J. EVOL. BIOL., 10.1111/j.1420-9101.2009.01828.x
<i>S. cerevisiae</i>	alcotec 48	Dist	Hambleton Bard	Albertin et al 2009, J. EVOL. BIOL., 10.1111/j.1420-9101.2009.01828.x
<i>S. cerevisiae</i>	963	Dist	NA	Albertin et al 2009, J. EVOL. BIOL., 10.1111/j.1420-9101.2009.01828.x
<i>S. cerevisiae</i>	YB-427	Dist		
<i>S. cerevisiae</i>	YB-428	Dist		
<i>S. cerevisiae</i>	Y-767	Food	NRRL	
<i>S. cerevisiae</i>	YB-360	Food		
<i>S. cerevisiae</i>	Y-6678	Fruit	NRRL	
<i>S. cerevisiae</i>	Y-7568	Fruit	NRRL	
<i>S. cerevisiae</i>	Y-965	Fruit	NRRL	
<i>S. cerevisiae</i>	YB-210	Fruit		
<i>S. cerevisiae</i>	YB-4081	Fruit		
<i>S. cerevisiae</i>	Y-129	Fruit juice	NRRL	
<i>S. cerevisiae</i>	Y-2230	Fruit juice	NRRL	
<i>S. cerevisiae</i>	Y-6275	Fruit juice	NRRL	

<i>S. cerevisiae</i>	Y-6278	Fruit juice	NRRL	
<i>S. cerevisiae</i>	Y-35	Nature	NRRL	
<i>S. cerevisiae</i>	Y-7567	Nature	NRRL	
<i>S. cerevisiae</i>	CLIB-154	Wine	CIRM- Levures	Albertin et al 2009, J. EVOL. BIOL., 10.1111/j.1420-9101.2009.01828.x
<i>S. cerevisiae</i>	CLIB-157	Wine	CIRM- Levures	Albertin et al 2009, J. EVOL. BIOL., 10.1111/j.1420-9101.2009.01828.x
<i>S. cerevisiae</i>	CLIB-328	Wine	CIRM- Levures	Albertin et al 2011, AEM, 10.1128/AEM.02547-10
<i>S. cerevisiae</i>	CLIB-479	Wine	CIRM- Levures	Albertin et al 2009, J. EVOL. BIOL., 10.1111/j.1420-9101.2009.01828.x
<i>S. cerevisiae</i>	Y-1301	Wine	NRRL	

AWRI: Wine Microorganism Culture  
Collection, Australian Wine Research  
Institute, Adelaide, Australia

CBS: CBS-KNAW, Centraalbureau voor  
Schimmelcultures (CBS) Fungal  
Biodiversity Centre, institute of the Royal  
Netherlands Academy of Arts and  
Sciences (Koninklijke Nederlandse  
Akademie van Wetenschappen), Utrecht,  
Netherlands

CLIB: Collection de Levures d'Interet  
Biotechnologique, now CIRM-Levures,  
INRA, France

CRBO: Centre de Ressources Biologiques  
(Enologie, Villenave d'Ornon, France

CRPR: Centre de Recherche Pernod-  
Ricard, Creteil, France

Debrecen: University of Debrecen,  
Hungary

Foggia: University of Foggia, Italy

IFV: Institut Français de la Vigne et du  
Vin, Pole Val de Loire, France

InterRhône: Inter-Rhône, Avignon, France

ISVV: Institut des Sciences de la Vigne et  
du Vin, Villenave d'Ornon, France

NRRL: Northern Regional Research  
Laboratory, ARS Culture Collection,  
Peoria, USA

Thessaloniki: Aristotle University of  
Thessaloniki, Greece

UNINA: University of Napoli, Italy

UOA: Hellenic Collection of Pathogenic  
Fungi, University of Athens, Greece

UWOPS: Culture collection of the  
University of Western Ontario, Canada

YJS: YJS collection, Schacherer's lab,  
Universite de Strasbourg, France