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Exploration of the phenomena associated with the production of pyrrole and pyridine derivatives responsible for mousy off-flavours in wine

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Par Pierre MOULIS

Exploration of the phenomena associated with the production of pyrrole and pyridine derivatives responsible for mousy off-flavours in wine

Sous la direction de Patricia BALLESTRA
et de Doris RAUHUT

Soutenance le 27 juin 2023

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"*I only hope that we don't lose sight of one thing*
It was all started by a mouse."
Walt Disney, 1954

Declaration of authorship

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Abstract

In recent years, consumer demand for organic food has continued to grow. The actors of the wine sector are attentive to these expectations. Wine production is evolving with decreases in the use of certain inputs that can directly impact the quality of wine. For example, there has been an upsurge in the "mousy off-flavour" defect in wines with reduced sulphite content. Today, three molecules seem to be at the origin of this defect: 2-acetylpyrroline (APY), 2-acetytetrahydropyridine (ATHP), and 2-ethyltetrahydropyridine (ETHP). Our work, combining chemical, microbiological and oenological approaches, provides answers to the mechanisms of appearance of this defect in wine.

In order to provide answers, methods for quantifying *N*-heterocyclics responsible for mousiness have been developed using SBSE-GC-MS techniques. The method of determination in wine made it possible to quantify the compounds in 74 different wines. The second method is adapted to a chemically defined medium, containing all the precursors known to date "mousy off-flavours". It makes it possible to test microorganisms for their ability to produce the mousy off-aroma under standardized conditions. These methods have been used to show that the proportion of the two tautomers of 2-acetyltetrahydropyridine is not impacted by the pH of the wine and buccal cavity.

Microorganisms were isolated from 25 wines with the defect. The most frequently encountered species are *Saccharomyces cerevisiae*, *Oenococcus oeni,* or *Brettanomyces bruxellensis*. Among the 353 microorganisms isolated, only strains of *B. bruxellensis*, *Lentilactobacillus hilgardii*, *Oenococcus oeni* are able to produce mousy compounds in model medium. Strains of collections belonging to these three species were then studied under the same conditions. The results show that the levels and ratios of the three N-heterocycles show significant variation between species but that all the strains evaluated (22 strains of *B. bruxellensis*, 10 strains of *L. hilgardii*, 20 strains of *O. oeni*) have the capacity to produce at least one of the three compounds. Further model media tests have shown that there may be interaction between microorganisms to produce mousy compounds.

The study of these interactions was continued in the wine environment. A white Ugni must was vinified using 2 different strains of *Saccharomyces cerevisiae*. And the production of the mousy compounds was then followed over 24 days. The results showed that, regardless of the microorganisms that produce, one of the two strains of *S. cerevisiae* contributes to the development of conditions for greater production of APY in wines.

Finally, production kinetics were undertaken in a model medium to determine at what physiological stage *B. bruxellensis* could produce these compounds. ATHP production is not linear. The compound appears promptly in significant concentrations, especially at the beginning of the stationary growth phase and then it’s disappearing. Hypotheses have been made about the physiological role of this compound.

Key words: Mousy off-flavours; Wines; Brettanomyces bruxellensis; lactic acid bacteria; Organoleptic deviations

Résumé

Depuis quelques années, la demande des consommateurs pour les aliments biologiques n'a cessé de croître. Les acteurs de la filière vitivinicole sont à l'écoute de ces attentes. La production de vins évolue avec des diminutions d'utilisations de certains intrants pouvant impacter directement la qualité du vin. C'est ainsi que l'on note une recrudescence du défaut « des goûts de souris » dans les vins à teneur réduite en sulfites. A l'heure actuelle, trois molécules sembleraient être à l'origine de ce défaut : le 2-acétylpyrroline (APY), le 2-acétyltétrahydropyridine (ATHP) et le 2-éthyltétrahydropyridine (ETHP). Notre travail, combinant des approches chimiques, microbiologiques et œnologiques permet d'apporter des éléments de réponse quant aux mécanismes d'apparition de ce défaut dans le vin.

Afin d'apporter des éléments de réponses, des méthodes de quantifications des N-hétérocycliques responsables du goût de souris ont été développées en utilisant des techniques de SBSE-GC-MS. La méthode de dosage dans le vin a permis de quantifier les composés dans 74 vins différents. La deuxième méthode est adaptée à un milieu chimiquement défini, contenant tous les précurseurs connus à ce jour « des goûts de souris ». Elle permet de tester les microorganismes pour leur capacité à produire le goût de souris en conditions standardisées. Ces méthodes ont permis de montrer que la proportion des deux tautomères du 2-acétyltétrahydropyridine n'est pas impactée par les différences de pH entre celui du vin et celui buccal.

Des microorganismes ont été isolés à partir de 25 vins présentant le défaut. Les espèces les plus fréquemment rencontrées sont des *Saccharomyces cerevisiae*, des *Oenococcus oeni* ou bien des *Brettanomyces bruxellensis*. Parmi les 353 microorganismes isolés, seules les souches de *B. bruxellensis*, *Lentilactobacillus hilgardii*, *Oenococcus oeni* sont capables de produire les composés sourissés en milieu modèle. Des souches de collections appartenant à ces trois espèces ont ensuite été étudiées dans les mêmes conditions. Les résultats montrent que les niveaux et les rapports des trois *N*-hétérocycles présentent d'importantes variations selon les espèces mais que toutes les souches testées (22 souches de *B. bruxellensis*, 10 souches de *L. hilgardii*, 20 souches de *O. oeni*) ont la capacité de produire au minimum un des trois composés. D'autres essais en milieu modèle ont montré qu'il pouvait y avoir une interaction entre microorganismes pour la production des composés du goût de souris.

L'étude de ces interactions a été poursuive en milieu vin. Un moût d'Ugni blanc a été vinifié à l'aide de 2 souches de *Saccharomyces cerevisiae* différentes. Et la production des composés sourissés a ensuite été suivie sur 24 jours. Les résultats ont montré que, peu importe les microorganismes producteurs, une des deux souches de *S. cerevisiae* contribue au développement de conditions permettant une production de l'APY plus importante dans les vins.

Enfin des cinétiques de productions ont été entrepris en milieu modèle afin de déterminer à quel stade physiologique *B. bruxellensis* pouvait produire ces composés. La production d'ATHP n'est pas linéaire. Le composé apparait de façon ponctuelle dans des concentrations importantes, notamment en début de phase stationnaire de croissance. Des hypothèses ont été formulées sur le rôle physiologique de ce composé.

Mots clefs: Goût de souris; Vins; *Brettanomyces bruxellensis*; Bactéries Lactiques; Déviations organoleptiques

Zusammenfassung

In den letzten Jahren ist die Nachfrage der Verbraucher nach Bio-Lebensmitteln weiter gestiegen. Die Akteure des Weinsektors achten auf diese Erwartungen. In der Weinproduktion ist ein Rückgang in der Anwendung bestimmter Maßnahmen, die sich direkt auf die Weinqualität auswirken können, zu verzeichnen. So hat beispielsweise der „Mäuselton“ bei Weinen mit reduziertem Sulfitgehalt zugenommen. Derzeit scheinen drei Moleküle Verursacher dieses Defekts zu sein: 2-Acetylpyrrolin (APY), 2-Acetyltetrahydropyridin (ATHP) und 2-Ethyltetrahydropyridin (ETHP). Die durchgeführte Forschungsarbeit, die chemische, mikrobiologische und önologische Ansätze kombiniert, liefert Antworten auf die Mechanismen des Auftretens dieses Fehltons im Wein.

Um Antworten zu finden, wurden Methoden zur Quantifizierung der N-Heterozyklen, die für den „Mäuselton“ verantwortlich sind, mithilfe von SBSE-GC-MS-Techniken entwickelt. Die Bestimmungsmethode für Wein ermöglichte es, diese Substanzen in 74 verschiedenen Weinen zu quantifizieren. Die zweite Analysenmethode ist auf ein chemisch definiertes Medium abgestimmt, das alle bisher bekannten Vorstufen des „Mäuseltons“ enthält. Sie ermöglicht es, Mikroorganismen unter standardisierten Bedingungen auf ihre Fähigkeit zu testen, einen „Mäuselton“ zu erzeugen. Mit diesen Methoden konnte gezeigt werden, dass das Verhältnis der beiden Tautomere von 2-Acetyltetrahydropyridin nicht von den Unterschieden zwischen dem pH-Wert des Weines und der Mundhöhle beeinflusst wird.

Mikroorganismen wurden aus 25 Weinen mit „Mäuselton“ isoliert. Die am häufigsten anzutreffenden Arten sind *Saccharomyces cerevisiae*, *Oenococcus oeni* oder *Brettanomyces bruxellensis*. Von den 353 isolierten Mikroorganismen sind nur Stämme der Arten *B. bruxellensis*, *Lentilactobacillus hilgardii* und *Oenococcus oeni* in der Lage, Substanzen, die einen „Mäuselton“ verursachen, im Modellmedium zu produzieren. Anschließend wurden Stämme aus der Sammlung, die zu diesen drei Arten gehörten, unter denselben Bedingungen untersucht. Die Ergebnisse zeigen, dass die Konzentrationen und Verhältnisse der drei N-Heterozyklen signifikante Unterschiede zwischen den Arten aufweisen, dass jedoch alle untersuchten Stämme (22 Stämme von *B. bruxellensis*, 10 Stämme von *L. hilgardii*, 20 Stämme von *O. oeni*) die Fähigkeit haben, mindestens eine der drei Komponenten zu produzieren. Weitere Versuche in Modellmedien haben gezeigt, dass es bei der Produktion mäuselnder Substanzen zu einer Interaktion zwischen Mikroorganismen kommen kann.

Die Untersuchung dieser Wechselwirkungen wurde im Weinmilieu fortgesetzt. Ein Ugni blanc Most wurde mit zwei verschiedenen *Saccharomyces cerevisiae* Stämmen vinifiziert. Anschließend wurde die Produktion der mäuselnden Komponenten über 24 Tage verfolgt. Die Ergebnisse zeigten, dass unabhängig von den produzierenden Mikroorganismen einer der beiden *S. cerevisiae* Stämme zur Entwicklung von Bedingungen beiträgt, die eine höhere APY-Produktion im Wein ermöglichen.

Schließlich wurden Produktionskinetiken im Modellmedium durchgeführt, um festzustellen, in welchem physiologischen Stadium *B. bruxellensis* diese Verbindungen produzieren kann. Die Produktion von ATHP verläuft nicht linear. Die Verbindung tritt punktuell in hohen Konzentrationen auf, insbesondere zu Beginn der stationären Wachstumsphase, und verschwindet dann wieder. Es wurden Hypothesen über die physiologische Rolle dieser Verbindung aufgestellt.

Schlüsselwörter: Mäuselton; Weine; *Brettanomyces bruxellensis*; Milchsäurebakterien; Organoleptische Abweichungen

Valorisation of research work

Scientific publications

Kiyomichi, D.; Franc, C.; **Moulis, P.**; Riquier, L.; Ballestra, P.; Marchand, S.; Tempère, S.; de Revel, G. Investigation into mousy off-flavor in wine using gas chromatography-mass spectrometry with stir bar sorptive extraction. Food Chem. **2023 Jun** 15;411:135454. doi: 10.1016/j.foodchem.2023.135454. Epub 2023 Jan 9. PMID: 36681024.

Moulis, P.; Miot-Sertier C.; Cordazzo, L.; Claisse, O.; Franc, C.; Riquier, L; Albertin, W.; Marchand, S.; de Revel, G.; Rauhut, D.; Ballestra P. Which microorganisms contribute to off-flavour in our wines? Subbmitted to OENO One, **April 2023**.

Displayed presentation in a national congress

Moulis, P.; Ballestra P.; Rauhut, D. "How *Brettanomyces bruxellensis* is bringing the mousiness in our wine?". Summer school Sustainability, of the Hochschule Geisenheim University, Geisenheim, (Germany), **September 2021**.

Oral communication

Moulis, P.; Rauhut, D.; Ballestra P. "Exploration of the occurrence of the mousy compounds in several matrices." Lecture seminar of the Hochschule Geisenheim University, Geisenheim, (Germany), **17 December 2020**.

Moulis, P.; Miot-Sertier C.; Cordazzo, L.; Claisse, O.; Franc, C.; Riquier, L; Albertin, W.; Marchand, S.; de Revel, G.; Rauhut, D.; Ballestra P. "Enquête sur le "goût de souris": on vous dit tout!". Journées techniques Vigne & Vin BIO, Prigonrieux, (France), **3 March 2023**.

Moulis, P.; Rauhut, D.; Ballestra P. "Which microorganisms contribute to off-flavour in our wines?". Lecture seminar of the Hochschule Geisenheim University, Geisenheim, (Germany), **6 April 2023**.

Thesis environment

This project involves a cotutelle between Université de Bordeaux and Hochschule Geisenheim University. The doctoral supervisors are Dr. Patricia Ballestra (Unité Mixte de Recherche Œnologie, Institut des Sciences de la Vigne et du Vin, Université de Bordeaux, France) and Prof. Doris Rauhut (Department of Microbiology and Biochemistry, Hochschule Geisenheim University (HGU), Germany). In addition to exchanges between Bordeaux and Geisenheim, a 6 month-research stay, was planned in Adelaide (AWRI, Australia). Due to the COVID-19 pandemic, the Australian research stay has never been realized.

This research was funded within the framework of Bordeaux-Adelaide-Geisenheim (BAG) international project alliance, through the French-German Doctoral College CDFA-03-18, the Région Nouvelle-Aquitaine, and the Land of Hessen.

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List of abbreviations

4EG : 4-ethylguaiacol
4EP : 4-ethylphenol
AAB : acetic acid bacteria
ADI : arginine deiminase
AF : alcoholic fermentation
ANOVA : analyse of variance
AP : 2-acetylpyridine
APY : 2-acetylpyrroline
ADHP : 2-acetyldihydropyridine
ATHP : 2-acetyltetrahydropyridine
AWRI : Australian wine research institute
BADH : 4-aminobutyraldehyde dehydrogenase
CAS : chemical abstracts service
CRBO : centre de ressources biologiques œnologique
DNA : deoxyribonucleic acid
ETHP : 2-ethyltetrahydropyridine
FEVS : Fédération des exportateurs de vins et spiritueux de France
FSC : forward scatter
GABA : gamma-amino butyric acid
GABAld : gamma-amino butyraldehyde
GC : gas chromatography
HPLC : high performance liquid chromatography
HS : headspace
IBMP : 3-*iso*butyl-2-methoxypyrazine
ISO : international organization for Standardization
IUPAC : international union of pure and applied chemistry
LAB : lactic acid bacteria
MALDI TOF : matrix assisted laser desorption ionisation time of flight
MLF : malolactic fermentation
MLVA : multi-locus variable number analysis
MS : mass spectrometry
NaCl : sodium chloride
*N*HAM : *N*-heterocycle assay medium
NMR : Nuclear magnetic resonance
PCR : polymerase chain reaction
PDMS : polydimethylsiloxane
pH : potential Hydrogen
pK_a : acidity constant
PI : propidium iodide
PTFE : polytetrafluoroethylene
RGJ medium: red grape juice medium
SBSE : stir bar sorptive extraction
SSC : side scatter
SO_2 : sulphur dioxide
SNIFF : sniffing
VBNC : viable but non-cultivable
YPD : yeast peptone dextrose

General introduction and objectives

Wine production in France has a significant commercial impact with a +7,9 billion euros export gross revenue in 2016, wine and spirits representing the second export sector after aeronautics (FEVS, 2017). Any loss in quality or microbiological problem in wine production may then cause a significant economic loss for the industry. Sulphur dioxide, or SO_2, is used in the food industry as a preservative because of its antioxidant and antimicrobial properties and is generally referred as sulphites in beverages. It is added in must and wine at different stages of its elaboration, especially at bottling time, to protect it from oxidation and microbial alteration during ageing or storage. Its maximal concentration in the product is fixed under EU law (Regulation (EC) No 606/2009).

The consumer demand for organic food has been growing these last years. In France, the number of organic exploitations for wine elaboration was multiplied by 4.6 in fifteen years and their number represented 9% of the French vineyard in 2015 (SudVinBio, 2016). Consequently, a European regulation for organic wines elaboration was created in 2012 (Commission Implementation Regulation (EC) No. 203/2012). In this regulation, the authorized additives were reduced compared to conventional wines. More particularly, the regulation for organic wines consists in a decrease of 50 mg/L for total sulphur dioxide for dry wines (Glucose + Fructose < 2 g/L) with a maximum authorized level of 100 mg/L for red dry organic wines. Considering the health concerns linked to SO_2 ingestion and the recent growing consumer interest in wines with less additives (like organic wines), the elaboration of quality wines with reduced SO_2 contents is a major issue for winemakers that want to meet the market demand. Their production requires an increased hygiene in the wineries (stainless steel vat, high degree of sanitary requirement, ...), suitable practices (rapid transformation after harvest, use of inert gases, ...) and regular controls of wine quality to adjust SO_2 contents during the process. Minimising SO_2 in red wines under inadequate conditions can result in oxidation, acceleration of colour ageing, increase in microbial populations and associated organoleptic deviations such as mousy off-flavour.

Mousiness has recently reappeared with the production of low SO_2 content wines and becomes a major problem for this sector. It is described as an extremely unpleasant flavour close to the smell of mice cage or according to other descriptors as “roasty” or “popcorn like” flavour. A related group of nitrogen-containing heterocyclic compounds is associated with mousy-off flavour in wines, including 2-acetyltetrahydropyridine (ATHP), 2-ethyltetrahydropyridine (ETHP) and 2-acetylpyrroline (APY). Production of mousy N-heterocycles in wines is attributed to certain *Brettanomyces* yeasts and certain lactic acid bacteria (LAB). Several strains of *Brettanomyces anomalus* and *Brettanomyces bruxellensis*, known to be associated with the spoilage of wine or other fermented beverages, have been shown to produce a mousy taint when fermenting grape juice or when reinoculated into sound wine. Their ability to produce ATHP, ETHP and in a smaller extent APY, has been confirmed using different chemically defined media containing among others some ethanol, lysine, and ornithine. *Lentilactobacillus hilgardii* and *Levilactobacillus brevis* were the first lactic acid bacteria species linked to mousy off-flavour. These bacteria have been shown to produce large amounts of ATHP and smaller quantities of APY and ETHP

when incubating in a synthetic medium. Several *Oenococcus oeni* strains have also been found to be capable of producing strong mousy off-flavour during growth in an ethanolic grape juice medium. They have been shown to produce the three mousy heterocycles, including ETHP at concentrations higher than that of other LAB strains. The biosynthetic pathway by which *Brettanomyces* and LAB produce mousy off-flavour compounds in wine is unknown. However, several conditions necessary for ATHP and APY production have been described.

The compounds mentioned as precursors are all potentially available in wine and reasons as to why mousy off-flavour forms in some wines and not in others are still not fully understood. The occurrence of mousy off-flavour may depend on LAB and *Brettanomyces* yeasts metabolisms in relation to the concentration of *N*-heterocycles precursors, acetaldehyde concentration (and hence SO_2 content), polyphenols and specific antioxidant power of wines. The investigation of these factors' influences on the production of *N*-heterocycles in red and white wines from different wine growing regions was scheduled.

A better understanding of the phenomena related to the production of mousy off-flavour in wines is expected to develop diagnostic tools to assess the risks of wine spoilage such as molecular screening of wine microbial strains producing significant mousy off-flavour and quantification of mousy off-flavour precursors in wine. The project 'MousyWine' supported by the BAG Alliance was designed to meet this objective. Divided into 4 distinct tasks, it formed the framework of my thesis work.

The first task was to characterize the diversity of wine microorganisms producing mousy off-flavour. To answer this, microorganisms from different mousy wines have been isolated, identified by MALDI-TOFF-MS and their ability to produce mousy compounds have been determined in a chemically defined medium. The screening has been then extended to strains from different collections. For this, a quantitative method of the *N*-heterocycle by SBSE-GC-MS has been developed in wine and in chemically defined medium.

The second task was to explore the microbial mechanisms leading to the production of mousy compounds. Co-inoculations have been tested in the chemically defined medium. Unfortunately, we had some technical issues with the analysis in this period. Therefore, only preliminary results are available. In order to study the link between the production of mousy compounds and yeasts growth phase, production kinetics of *Brettanomyces bruxellensis* were conducted.

The determination of key parameters of mousy off-flavour in wine has been explored in a third work package. Three different musts have been collected, one Ugni blanc from Cognac area (France), one Cabernet Sauvignon from Bordeaux (France), and one Merlot from Bergerac (France) and have been fermented by two strains of *Saccharomyces cerevisiae* to explore the impact of the alcoholic fermentation on the production of the mousiness by different microorganisms. Work on Cabernet Sauvignon and Merlot are still ongoing, for that reason, the results won't be discussed here.

The last task was to develop diagnostic tools to assess the risk of mousy off-aromas production wines. Unfortunately, this part could not be treated during the thesis.

The first chapter of this thesis is devoted to a bibliographical synthesis gathering current knowledges on the defect of the mousy off-aromas. In a first part, the recrudescence defects in wines without added sulphites will be addressed. In a second step, the defect of the mousiness will be analysed from a sensory and chemical point of view, the presence of the compounds responsible for the alterations in other matrices will also be discussed. The third part of this chapter will address vinification in its entirety and microorganisms of œnological interest. Finally, the last part will focus on microorganisms related to the production of mousiness in wine.

The second chapter of this work will present the work carried out on the chemical aspect of mousy off-aromas. Two analytical methods have been developed during this thesis in partnership with Prof. Gilles de Revel laboratory of the UMR Œnologie. The first one was worked out in contribution with Daiki Kiyomichi to assay mousy compounds in wines and was published in 2022. The second method was developed to assay mousy compounds in the chemically defined medium used to study the ability of microorganisms to produce mousy taint. A focus on ATHP tautomers has been done. Proportion of the two tautomeric forms has been explored at pH ranging from 1.57 to 7.72. A collaboration with Dr. Alicia Jouin, working at Prof. Pierre-Louis Teissedre laboratory of the UMR Œnologie, permits to explore the impact of oxygen on the presence of *N*-heterocycles in wine.

The third chapter will present the work carried out in connection with the first task of the project and is therefore mainly focused on the diversity of wine micro-organisms producing mousy off-aroma in model solution. The first part has been submitted in the journal OENO One and is currently being reviewed. The rest of this chapter will present exploratory results on microbial interactions that can impact the production of mousy compounds.

The fourth chapter will deal with the second and third tasks of the project. The results related to production kinetics in *B. bruxellensis* and those obtained during the experiments related to mousy compounds production in different Ugni Blanc matrices will be presented and discussed.

This thesis work therefore focuses on the exploration of the phenomena associated with the production of *N*-heterocyclic compounds in a model medium and in a Ugni blanc wine by microorganisms. The understanding of the production mechanisms of mousy compounds will lead to better understand the appearance of this defect in different processes, in order to limit the development of this off-aroma.

Chapter I.

Literature review

I.1. Defects on the rise in “sulphite-free wines”

I.1.1. Definition of defect

According to the Cambridge dictionary (https://dictionary.cambridge.org), a defect is “a fault or problem in something that spoils that thing or causes it not to work correctly” or is “something that is lacking or is not exactly right in something”. This definition highlights an important point of defects, it is that they are dependent on the matrix in which they are found. However, this definition is not the most suitable for the œnology community. Indeed, if we refer to the dictionary, the defect could come from the absence of qualities whereas in œnology, the first quality of a wine lies in the absence of defect.

This is an objective definition of quality that will suit wine professionals and experts in the field. This objective quality is therefore based on pre-established criteria. If the wine meets all these criteria, then it will be said to be of quality. However, consumers too can say that a wine is of quality. This quality perceived by the consumer will be confronted with the objective quality of experts (Zeithaml, 1988). The perceived quality will then depend entirely on the taster and his personal tastes. Despite this, this definition supports the cultural notion of defects. By speaking of rules and conventions it shows that in another culture with different conventions the defect could be seen as a quality.

Imperfections of a wine can come from various origins: oxidative, microbial, exogenous to the wine or related to precipitation and can impact all sensory levels. It can affect the colour, the taste, or the aroma of the wine.

Off-odours related to microbial developments or to exogenous origin to wine, may appear. Before being entirely perceptible and distinguished for tasters, these alterations mask the qualities of the wine and in particular its fruity aromas, bringing it a certain heaviness. So far, the definition given by the Cambridge dictionary would correspond, the defect would mask the qualities of wine (Campo *et al.*, 2005; San-Juan *et al.*, 2011; Tempère *et al.*, 2017) and would cause the "lack of something". At higher concentration, defects are characteristic, and it becomes possible to identify them. Once recognized in the wine, it is difficult to smell anything else and the wine then loses all its characteristics to become only an ordinary wine, so it becomes difficult to recognize the origin of the wine, its grape variety etc..

The main defects of wine, frequent in the past, have been eliminated thanks to the study of these. Current knowledges makes it possible to vinify quality wines in a controlled manner (Ribéreau-Gayon *et al.*, 2006).

However, the policy of reducing oenological inputs favours the return of certain defects.

I.1.2. Consumer expectations

Since the 2000s, organic and natural wines have experienced a real boom. Organic wines are made using only certified organic grapes, grown without synthetic pesticide, genetically modified organisms (GMO), and chemical fertilisers. The term “Organic wine” is certified since 2012, and means that the

whole process, from harvest to bottle can be certified (Regulation (EU) 2012/203, 2012). A natural wine is a wine vinified without any inputs (Association des Vins Naturels). In 20 years, the surface area of organic vineyards in the Bordeaux region has almost been multiplied by 20 (Vigneron bio, Nouvelle Aquitaine, 2021). This is due to a growing consumer demand for products with fewer inputs. Indeed, consumers are increasingly questioning environmental but also health issues (Lecomte, 2021; Pomarici *et al.*, 2016; Pomarici & Vecchio, 2014; Tait *et al.*, 2019). Whether the effects are actually proven or assumed by consumers, whether they are short-term toxic or chronically toxic, consumers will increasingly move towards "natural" products (Peres *et al.*, 2018).

Different wines and œnological products can then be singled out whether it is the ecological impact of pesticides in the vineyards, or the impact on health of potential residues of these. The same is true for œnological products, for example sulphites, their media coverage has led to the emergence of a new attribute of interest: wines "without sulphites" or "without added sulphites" (Lecomte, 2021).

I.1.3. The use of SO_2

The use of sulphites in œnology dates to the 19th century (Ladrey, 1871; Pasteur, 1866). SO_2 can be added at different stages of winemaking: on the harvest, during post-fermentation stages whether alcoholic fermentation or malolactic fermentation, before aging and finally at bottling. SO_2 is then used to improve the conservation of the wine. The use of SO_2 is regulated (Regulation (EU) 2019/934, 2019) and its maximum permitted levels in wines will depend on the type of wine produced but also on the mode of management of the vineyard, whether organic or conventional.

Food laws require the statement "Contains sulphites" on the labels of food, whether these are added as a formulating ingredient, as processing aids or naturally present. In winemaking, sulphites are produced during alcoholic fermentations by yeasts. This indication must be present once the concentration of these reaches 10 ppm, or 10 mg/L in a wine (Timbo *et al.*, 2004).

SO_2, or sulphur dioxide is a colourless gas. It can be added in different forms in wine, sulphurous acid (H_2SO_3), potassium bisulphite ($KHSO_3$) and potassium metabisulfite ($K_2S_2O_5$) (Regulation (EU) 2019/934, 2019). Once dissolved in an aqueous solution, sulphur dioxide exists in equilibrium between molecular SO_2 ($SO_2.H_2O$), bisulphite ion (HSO_3^-) and sulphite ion (SO_3^{2-}), the proportions of these three compounds will depend on the pH of the aqueous solution and are governed by the acidity constants $pK_{a1} = 1.81$ et $pK_{a2} = 6.91$. The pH of the wine (3-4) being less below pK_{a2} the SO_3^{2-} species is not found. The relative abundance of the three compounds as a function of pH is shown in Figure 1.

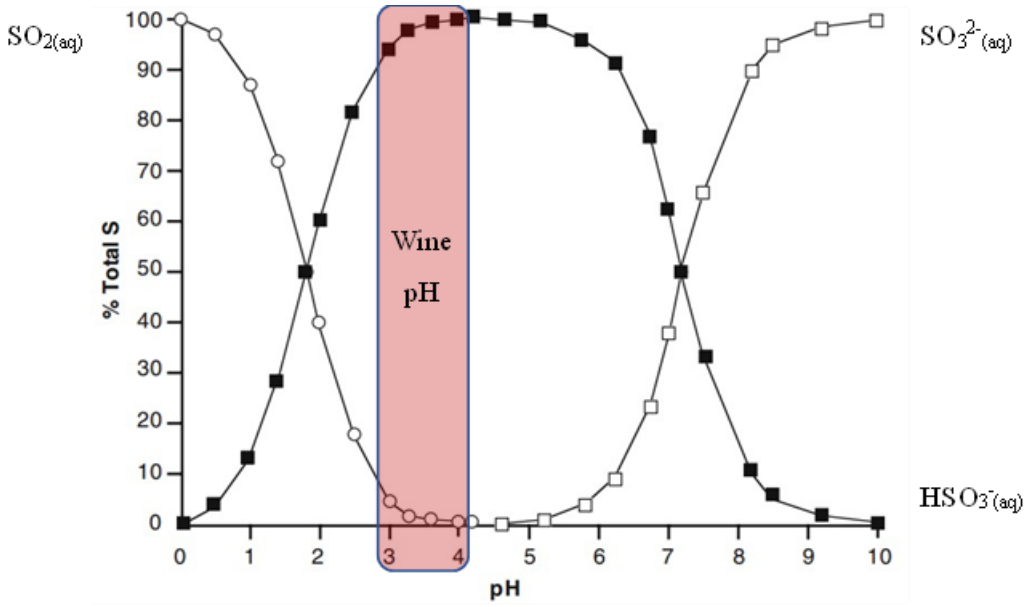


Figure 1 : Relative abundance of molecular SO_2, bisulphite ion and sulphite ion at different pH values (Fugelsang & Edwards, 2007).

The $SO_{2(aq)}$ form, is called molecular SO_2 and is present in a small amount at the wine pH but is the most active form. It is also the only form with antioxidasic capacity, thus inhibiting the enzymatic activity associated with the presence of *Botrytis cinerea*. The most present form at wine pH, is the bisulphite ion form (HSO_3^-) which is also active, but also likely to combine with other compounds of wine. All bisulphite combinations correspond to the "combined SO_2". The bisulphite ion and the molecular SO_2 represent the share of the "free SO_2" (Fugelsang & Edwards, 2007).

Indeed, the "free SO_2" protects the wine with its antioxidant and antimicrobial properties. The fact of drastically reducing its concentration in wines increases the risk of contamination by alteration yeasts or bacteria (Caruso *et al.*, 2002; Divol *et al.*, 2012; Du Toit & Pretorius, 2019; Joyeux *et al.*, 1984; Maier *et al.*, 1986), the risk of oxidation of ethanol to ethanal (Danilewicz, 2016; Elias & Waterhouse, 2010), the risk of oxidation of polyphenol (Danilewicz, 2003; Oliveira *et al.*, 2011) or other compounds related to the volatile matrix of the wine.

Some economists have looked at the issue of these new practices and new "sulphite free wines" labels and have been able to show that consumers are willing to pay more for a wine for a wine with fewer inputs (Raineau, 2018). Romano *et al.* (2020), even showed that the mention of an "organic" label increases the acceptance rate of sensory defects in wines.

I.1.4. Defects described in "sulphite-free wines"

Wine defects are the result of microbial spoilages and/or chemical deviations associated with a failure to control the wine microorganisms on the one hand and the oxidation-reduction balance on the other hand. One of the main challenges of œnology is to have a better knowledge of these defects in order to

avoid all possible organoleptic deviations in wines. Research aimed at defining the origin of these but also their impact, whether on sensory qualities or on health of consumers and/or the environment, is a topical issue with the continuous objective of producing wines without deceptions.

In a study of 72 wines from the Bordeaux appellation of the 2015 and 2016 vintages (including 52 sulphite-free and 20 sulphited wines) (Pelonnier-Magimel *et al.*, 2020), 70.6 % of wines without added SO_2 were described as having a tasting defect (Table 1).

This study also showed that the main defects (more than 50 %) found in these wines were related to was related to oxidation event (Bueno *et al.*, 2010). Furthermore, 35 % of the studied wines had defects related to the development of alteration microorganisms (Figure 2).

Table 1 : Frequency of wines with at least one defect according to use of sulphites and vintage (from Pelonnier-Magimel et al., 2020).

	2015		2016	
	Wine with SO_2	Wine without SO_2	Wine with SO_2	Wine without SO_2
Number of wines with defect	03-déc	20/26	0/8	18/26
Percentage of wines with defect	25%	77%	0%	69%

	2015	2016	Total
Oxidation	37.5 %	33.3 %	35.4 %
Volatile phenols	20.8 %	16.6 %	18.7 %
Mousy off-flavor	12.5 %	0%	6.2 %
Reduction	4.1 %	8.3 %	6.2 %
Vegetable/Herbaceous	0%	8.3 %	4.1 %
Total	74.9 %	66.5 %	70.6 %

Figure 2 : Citation frequencies and word cloud of descriptors of defects in wines without added sulphites with agreement above 60% (from Pelonnier-Magimel et al., 2020).

I.1.4.1. Reduction/Oxidation

The lack of control of the oxidation-reduction balance of wines is the major cause of chemical defects. Too much exposure to oxygen could tip this balance towards an oxidized wine and, on the contrary, the total absence of oxygen could reveal reduction defects associated to sulphur compounds. Excessive protection due to fear of oxygen can lead to alterations in wine reduction, mainly associated with the production of volatile sulphur compounds including hydrogen sulphide (H_2S) and methanethiol (CH_3SH) (Mestres *et al.*, 2000; Park *et al.*, 1994). These compounds are produced during alcoholic fermentation by *Saccharomyces cerevisiae via* the catabolism of methionine and cysteine (Spinnler *et al.*, 2001). An increase in the concentration of these compounds is observed during the storage of bottled wines under reducing conditions (Ugliano, 2013).

The absence of SO_2, which is the main wine antioxidant during vinification, will greatly reduce the protection of the wine against oxygen. Oxidation is a common problem that can affect wines. It occurs when wine is exposed to air for an extended period, which causes excessive oxidation. Oxidation occurs when compounds in wine react with oxygen in the air, which can alter the colour, taste, and aroma of the wine. The key step of the alteration development is the oxidation of ethanol to ethanal (Wildenradt & Singleton, 1974) which is widely associated with green apple smell.

Signs of oxidation in a wine can vary depending on the type of wine and the level of oxidation. White wines can take on a golden or brownish hue, while red wines can turn brown or orange. They will also impact the taste and the colloidal stability of wines (Cruz *et al.*, 2012; Saucier *et al.*, 1997). This oxidation is not direct but the result of a mechanism involving various reaction intermediates, leading to the production of hydrogen peroxides with direct oxidizing power. It will also be catalysed by the presence of cations of iron and/or copper (Danilewicz, 2003). Polyphenols under too much oxidation form quinones, these are unstable and can lead to other reactions resulting on the degradation of the wine colour and taste (Figure 3).

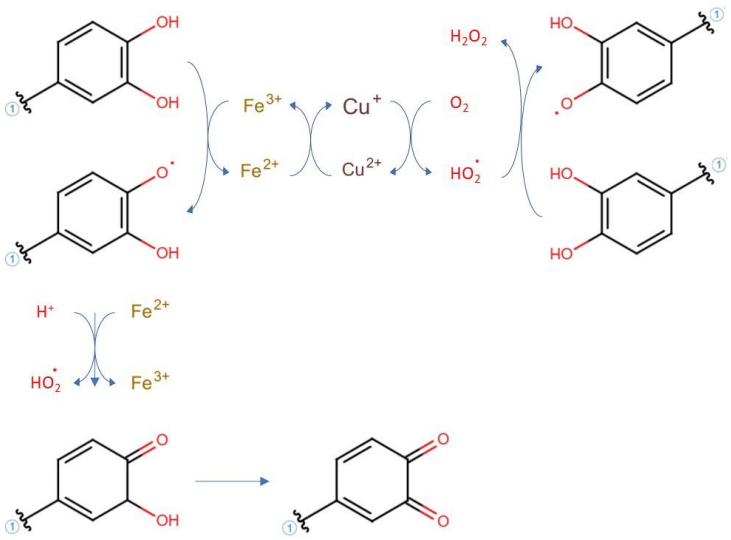


Figure 3 : Scheme of oxidation of phenolic compounds forming quinones and peroxides catalysed by metal cations (adapted from Danilewicz, 2007; Karbowiak et al., 2009).

The taste and aroma of the wine can also change, with notes of nuts, candied fruit, cooked fruit, or oxidized apple.

As mentioned above, the defects depend on the matrix studied. It is important to note that some wines are intentionally oxidized, such as Sherry or Vin Jaune, and this can be considered a deliberate style rather than a defect.

I.1.4.2. Vegetable/Herbaceous

Another defect that emerged in the study done by Pelonnier-Magimel *et al.* (2020) is the vegetal/herbaceous aroma. It is shown that the vegetal character of the "green bell-pepper" type negatively impacts the fruitiness of the wines (Hein *et al.*, 2009). These notes of "green bell-pepper" are directly related to the presence of 2-*iso*butyl-3-methoxypyrazine (IBMP) in wines. This molecule is also found in many other vegetables such as chili, pea, etc. (Hui *et al.*, 2010; Murray & Whitfield, 1975). IBMP is an excellent example to show that the defect is dependent on the matrix in which it is found. In these plants, it is considered qualitative, expected. Similarly, it is also interesting to note that culture will play an important role in its appreciation. Although this alteration is found in unsulphited wines, the origin is quite different. The presence of these odours is often a sign of a lack of maturity of the grapes at harvest (Roujou de Boubée *et al.*, 2000).

I.1.4.3. Volatile phenols

In wines aiming for more natural vinification, with fewer inputs, such as wines without added sulphites, spoilage microorganisms can develop throughout the winemaking process. Volatile phenols, including 4-ethylphenol (4EP) and 4-ethylguaiacol (4EG), are produced by the spoilage yeast *B. bruxellensis* These compounds in wine (ratio 10:1), are responsible for characteristic odours of "horse sweat", "leather" and "stable" (Chatonnet *et al.*, 1992; Tempère *et al.*, 2014) . Volatile phenols, although having a high detection threshold in Bordeaux red wine (440 µg/L for 4EP and 33 µg/L for 4EG) (Heresztyn, 1986), also act below this threshold as a mask of the overall and fruity aroma of the wine (Tempère *et al.*, 2016).

The last defect emerging from the study conducted on wines without added sulphites is the mousy off-flavour.

I.2. Mousiness

I.2.1. Sensory aspect

The mousy off-flavour is an organoleptic deviation that can mask the typicality and quality of a wine. Therefore, it perfectly meets the definition given in the first part. The first description of the mousiness dates from the late nineteenth century (Thudichum, 1894) and refers to an odour perceived retro-nasally of "rodent urine". Currently this defect has been described in many wine-producing countries such as France, the United States, South Africa and Australia (Snowdon *et al.*, 2006), even if, this defect has been on the rise in recent years (Tempere *et al.*, 2019). Massini and Vuchot (2015) attributed this increase, among other things, to the reduction of the use of sulphites. Indeed, although, it has never been scientifically proven that the mousy off-flavour could not appear in a sulphited wine. To our knowledge this alteration has never been described in a conventional wine. Even in sulphite-free wines, mousy wines are not in the majority. 8.8 % of the failing wines had mousy taint as the major defect.(Pelonnier-Magimel *et al.*, 2020).

Once mousy off-flavour is present in a wine, it is impossible to make it disappear and can therefore lead to great losses for winemakers. This defect is described as having "rodent cage and urine", "grilled foods such as popcorn", "crackers", "basmati rice" or "sausage skin" aromas (Tempère *et al.*, 2019). The multiplicity of descriptors may be due to the number of compounds involved and their respective concentrations, the matrix in which they are found but also to the interindividual ability to detect this defect. This ability is due to the behaviour of the compounds responsible for this defect. Most aromatic alterations are felt by ortho-olfaction, the compounds volatilize and are directly noticeable in the glass of wine. On the other hand, the compounds of the mousiness are not volatile at wine pH and only volatilizes once put in the mouth, hence its English name "mousy off-flavour". According to ISO 5492 (2008), “a flavour is a complex combination of the olfactory, gustatory and trigeminal sensations perceived during tasting" and "an off-flavour is an atypical flavour often associated with deterioration or transformation of the product". Although this definition considers the fact that the defect is noticeable only in the mouth, the alteration is known for its aromatic side and no work has been done on its taste or its somesthesic properties. The most appropriate definition of the mousiness would then be that of an aroma "sensory attribute perceptible by the olfactory organ *via* the back of the nose when tasting".

The mousiness is therefore not perceived by all tasters in the same way. According to Grbin *et al.* (1996), sensitivity to mousy off-aromas depends on one's genetic predispositions. There is also a correlation between their individual's saliva, their oral pH, and their ability to sense alteration. The saliva of an individual and the associated oral pH will play a very important role in the volatility of the compounds. Oral pH has significant inter-individual and intra-individual variability. Indeed, it shows fluctuations depending on the food consumed, the time of day and the physiological state of the person (Larsen *et al.*, 1999). Larsen *et al.*, showed that on 11 participants, the oral pH varied between 5.76 and 7.96. This explains why some people are more sensitive to the mousy taint than others, some tasters could even be specific anosmic.

In addition to these difficulties related to tasters, the alteration is often difficult to detect because generally present with other defects such as oxidation or significant volatile acidity or the presence of 4EP. The wine can then be rejected by the professional before putting it in the mouth. The study proposed by Pelonnier-Magimel *et al.* (2020) aimed at discriminating between spoiled wines and sound wines. During the tasting, the olfactory being judged before the taste, mousy wines could be rejected before taste analysis. As consequence, wines having oxidation or volatile phenol defects could have the mousy taint without the taster being able to notice.

The last difficulty related to its tasting also comes from its temporality. As said before, the defect is an aroma and is therefore perceived by retro-olfaction at the time of tasting in the mouth. The difference is that it is felt when tasting but only at the end of the tasting once wine has been spat out or swallowed.

By the way, the odour may then persist beyond 10 minutes (Bartowsky & Henschke, 1995; Grbin *et al.*, 1996).

This difficulty in appreciating the mousy off-aromas during tasting has led professionals to develop detection methods of all kinds. Peynaud & Domercq, (1956) mentioned a method of depositing a drop of wine on the back of the hand before smelling the skin. This method was then reused under the name "Palm & Sniff" (Grbin *et al.*, 1996). The pH of the skin being higher than that of wine, it makes it possible to volatilize the compounds, on the other hand, as does the oral pH, the cutaneous pH shows strong inter- and intra-individual variation (between 4 and 6) (Ali & Yosipovitch, 2013). The use of alkaline paper strips was also proposed to detect the presence of mousiness in a sample. The method consists of dipping the strip in the wine or sample and analysing it sensorially (Grbin *et al.*, 1996; Grbin & Henschke, 2000; Heresztyn, 1986). The paper being basified with sodium hydroxide at a known concentration made it possible to standardize the pH to which the sample was subjected. This method has already proven itself to assess the presence of mousy character on model culture media and some wines (Costello *et al.*, 1993; Grbin & Henschke, 2000; Heresztyn, 1986). Tempère *et al.* (2019) compared this last analysis with a new approach consisting in mimicking the phenomenon in the mouth. For this, they proposed to increase the wine pH approximately to 5 and 7 by the addition of sodium carbonate in order to reduce interindividual variations due to differences in oral pH. The tasters had to judge by ortho-olfaction the intensity of the mousy taint of several samples. The method with the best consensus at that time was the method with wine pH adjustment to 5 using sodium carbonate.

Today, this defect is described as the result of the presence of *N*-heterocyclic compounds in wines. The three currently reported compounds are 2-ethyltetrahydropyridine (ETHP), 2-acetyltetrahydropyridine (ATHP), 2-acetylpyrroline (APY). A mousy wine will generally contain more than one of these compounds (Costello *et al.*, 2001).

I.2.2. Mousy *N*-heterocycles

These compounds (ETHP, ATHP, and APY) have also been found in matrices other than wine in which they are not always portrayed as unpleasant. On the contrary, they often participate favourably in the characteristic aromas of some of these products.

I.2.2.1. 2-Ethyltetrahydropyridine

ETHP is a compound that is rarely described in the literature. Sensorially its perception thresholds are quite high, 140.5 µg/L in water and 150 µg/L by retro-olfaction in wines (Craig & Heresztyn, 1984; Tempère *et al.*, 2019). Despite this, it was the first chemical compound to be associated with the mousy alteration (Tucknott, 1977). Described in cider, it was then sought in wines but it is only much later that the compound had been found (Costello *et al.*, 2001; Grbin *et al.*, 1996). ETHP had been found in two tautomeric forms (Figure 4) even if the enamine form (Figure 4 B) seems to be negligible (Costello & Henschke, 2002).

ETHP [IUPAC names: 2-ethyl-3,4,5,6-tetrahydropyridine and 2-ethyl-1,4,5,6-tetrahydropyridine; CAS numbers: 1462-93-7 and 57634-69-2; molecular weight: 111.18 g/mol] is a tetrahydropyridine in which the hydrogen at position 2 is replaced by an ethyl group, also known as ECTPY. Tetrahydropyridines are six-membered rings composed with one nitrogen atom and five carbon atoms with 2 degrees less unsaturation than pyridine. The tetrahydropyridine ring has two degrees of unsaturation (the ring and a double bond).

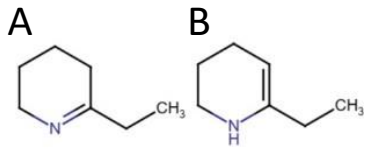


Figure 4 : Tautomers of (A) 2-ethyl-3,4,5,6-tetrahydropyridine and (B) 2-ethyl-1,4,5,6-tetrahydropyridine.

This compound is found in wines at concentrations up to 150 µg/L (Grbin *et al.*, 1996), making it the mousy-compound with the highest concentrations found in wines. Despite these concentrations, it is considered as a compound with little impact on the off-aromas due to its perception threshold more than 100 times higher than ATHP and APY. ETHP is thought to be intimately related to ATHP and appears to be produced only after ATHP (Grbin, 1998).

The presence of ETHP in matrices other than wine or cider is not yet listed.

I.2.2.2. 2-Acetyltetrahydropyridine

ATHP is the second compound associated with mousy taint. Its contribution to the mousy off-aroma in wine has been highlighted and confirmed since 1984 (Costello *et al.*, 2001; Grbin *et al.*, 1996; Heresztyn, 1986). ATHP has been found in wines at concentrations up to 106 µg/L (Costello, 1998).

ATHP [IUPAC names: 1-(3,4,5,6-tetrahydropyridin-2-yl)ethan-1-one and 1-(1,4,5,6-tetrahydropyridin-2-yl)ethan-1-one; CAS numbers: 27300-27-2 and 25343-57-1; molecular weight: 125.17 g/mol] is tetrahydropyridine in which the hydrogen at position 2 is replaced by an acetyl group, also known as ACTPY.

It is certainly found at lower concentrations than ETHP but its perception threshold is of the order of µg/L (Tempère *et al.*, 2019; Teranishi *et al.*, 1975). This compound is unstable and sensitive to oxidation which may explain why it was not identified earlier. Its structure strongly resembles that of ETHP, unlike the acetyl group replacing ethyl. It also exists in two tautomeric forms (Figure 5).

Kuenzler & Pour Nikfardjam (2013) studied 127 wines of German origin from the Württemberg region. These had been qualified as having the defect of the mousy off-aroma. They proposed a way forward chemical production of ATHP in wines from proline and methylglyoxal that would be closely related to

a Maillard reaction (Belitz *et al.*, 2009). On the other hand, in a next study, aiming at showing the production of this chemical compound in a model medium, ATHP was not found. Their samples, however, had mousy character, close to that of ATHP (Pour Nikfardjam & Kunz, 2021).

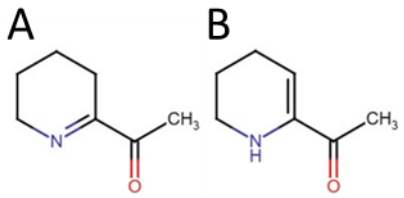


Figure 5 : Tautomers of (A) 2-acetyl-3,4,5,6-tetrahydropyridine and (B) 2-acetyl-1,4,5,6-tetrahydropyridine.

ATHP is an odorous and volatile compound found in many other matrices such as bread, crackers, popcorn, and rice. In these products it is described as having a "roasted popcorn" aroma with an air perception threshold of 0.06 ng/L (Schieberle, 1995). It has been described as a key compound of popcorn aroma. In this matrix, ATHP would have proline and fructose as precursors (Schieberle, 1990). During the thermal transformation of foods such as popcorn, proline reacts with carbohydrates leading to the formation of compounds such as Δ 1-pyrroline, hydroxy-2-propanone or glyceraldehyde. Δ^1-pyrroline and hydroxy-2-propanone are thought to be the key precursors in the formation of ATHP. It has been shown that high pH favours the formation of this compound (Figure 6) (Hofmann & Schieberle, 1998).

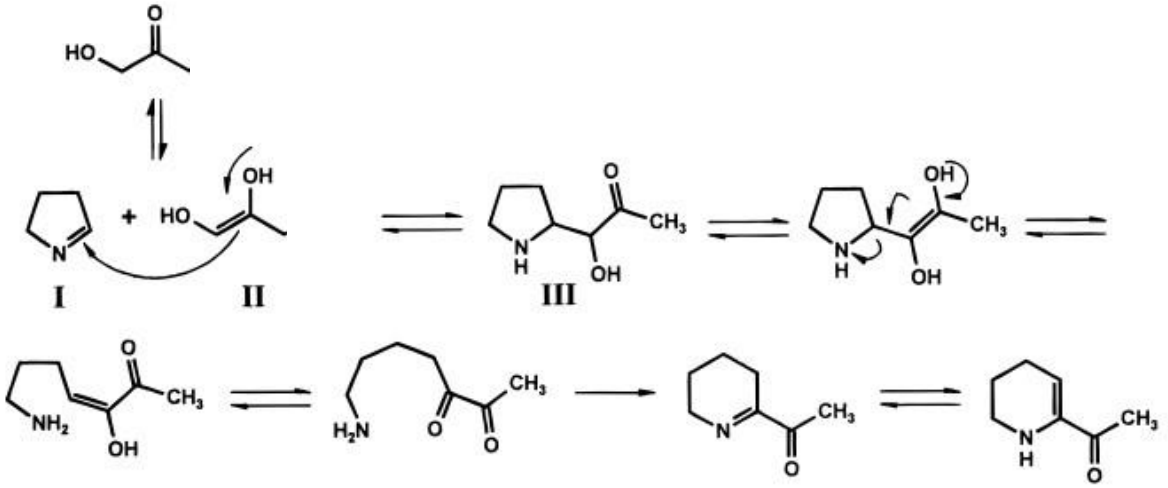


Figure 6 : Hypothetical formation of 2-acetyltetrahydropyridine (ATHP) from the Δ^1-pyrroline (from Hofmann & Schieberle, 1998).

More recently, ATHP has been found in various matrices such as salami but also in cooked food products or roti (peanut, cashew, sesame, cocoa nibs) (Bösl *et al.*, 2021).

I.2.2.3. 2-Acetylpyrroline

APY is the third compound that was identified as responsible for mousy taint. Unlike the previous two compounds, this one was identified by gas chromatography (GC) coupled with olfactometry (Herderich *et al.*, 1995).

APY [IUPAC name: 1-(3,4-dihydro-2H-pyrrol-5-yl)ethanone; CAS number: 85213-22-5; molecular weight 111.14 g/mol] is 1-pyrroline in which the hydrogen at position 2 is replaced by an acetyl. It is also known as 2AP, APR or ACPY. Pyrrolines are five membered rings composed with one nitrogen atom and four carbon atoms with two degrees of unsaturation (the ring and a double bond). The pyrroline ring makes the compound highly unstable. The pyrroline ring is non-reactive and the acetyl group is reactive (Nadaf *et al.*, 2006).

Its perception threshold would be of the order of one-tenth of µg/L (Buttery *et al.*, 1983; Tempère *et al.*, 2019) 10 times lower than that of ATHP. On the other hand, it has so far only been identified at low concentrations in wines (Grbin *et al.*, 1996). Figure 7 B, its enamine form, such as ETHP, does not seem to be present under wine conditions (Costello & Henschke, 2002).

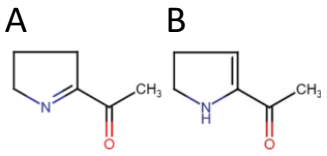


Figure 7 : Tautomers of (A) 2-acetyl-3,4,5-pyrroline and (B) 2-acetyl-1,4,5-pyrroline.

APY is also found in all kinds of foodstuffs (bread, dry ham, green tea, popcorn, etc.), plants (basmati rice, soybeans, pearl millet, spinach, etc.) but also in the animal kingdom (tiger pheromones, mouse urine). In these matrices, it is described as having a "roasted" aroma, "popcorn" (Wakte *et al.*, 2017). It is the key aroma compound of bread crust, popcorn, sweet corn and roasted sesame. Its detection limit in air is 0.02 ng/L (Schieberle, 1995). APY can also be produced by fungi of the genus *Penicillium nalgiovense* (Stahnke, 2000), *Aspergillus spp.* (Shaikh & Nadaf, 2013), etc., and bacteria as *Bacillus cereus* (Adams & De Kimpe, 2007), *Lentilactobacillus hilgardii*, *Pediococcus spp.* (Costello & Henschke, 2002), etc..

The name of the “mousy off-flavour”, “mousiness” in wine is probably due to the presence of APY in the mouse urine (Fujita *et al.*, 2019; Kwak *et al.*, 2008). Kwak *et al.* (2013) showed that APY is a minor compound in the urine of male mice which is not found in female mice urine. On the other hand, it becomes one of the major compounds in the headspace of male mouse urine once dried (Figure 8). This corresponds perfectly to the descriptor, "dirty mouse cage", associated with spoilage in wines.

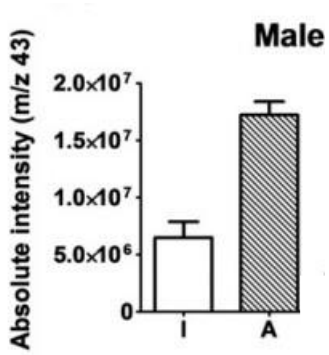


Figure 8 : 2-acetyl-1-pyrroline (APY) headspace urine concentrations. I : Intact (25 µL urine analysed immediately); A : aged and dried for 24 h (adapted from Kwak et al., 2013).

APY is also found in binturong urine, even if the production is not well understood for the mouse and the bearcat and it cannot be explained by their commercial diet. Greene *et al.* (2016) highlighted the relationship between the abundances of APY in binturong urine and the concentration of androstenedione which is a precursor of testosterone and estrone. APY has been also referenced as urine and marking fluid of tiger (*Panthra tigris*) and leopard (*Panthra pardus*) (Brahmachary & Poddar-Sarkar, 2015).

This compound is not found only in the animal kingdom, it is also the main compound that gives aroma to basmati rice and other fragrant rice varieties. This compound then becomes an asset by offering a qualitative aroma sought by consumers. It is not formed when cooking rice but in the aerial parts of the plant in rice fields. Depending on the rice variety, APY is present in different concentrations (Itani *et al.*, 2004). These variations may be due to different genotypes of rice varieties, environmental factors (water potential, temperature), cultural practices (planting density), post-harvest treatment, etc. (Goufo *et al.*, 2010). The addition of nitrogen fertilizer is an example of a cultural practice influencing APY production in rice. Indeed, the presence of nitrogen increases the amount of free proline in rice, thus promoting the formation of APY (Yoshihashi *et al.*, 2002). This is because proline has been identified as a source of nitrogen and a precursor for the formation of this compound. The accumulation of APY in basmati rice is thought to be due to a loss of function of the badh2 gene after deletion of a number of base pairs (Shi *et al.*, 2008). The betaine aldehyde dehydrogenase 2 (BADH2) substrate, γ-amino butyraldehyde, GABald/Δ^1-pyrroline, is the immediate precursor to APY in rice (Bradbury *et al.*, 2008). When BADH2 is functional, it consumes its substrate and oxidizes it to GABA inhibiting APY synthesis. In the absence of BADH2 GABAld/Δ^1-pyrroline activity accumulates and thus induces an increase in APY synthesis. Indeed, GABAld/Δ^1-pyrroline is the precursor of pyrroline (Figure 9).

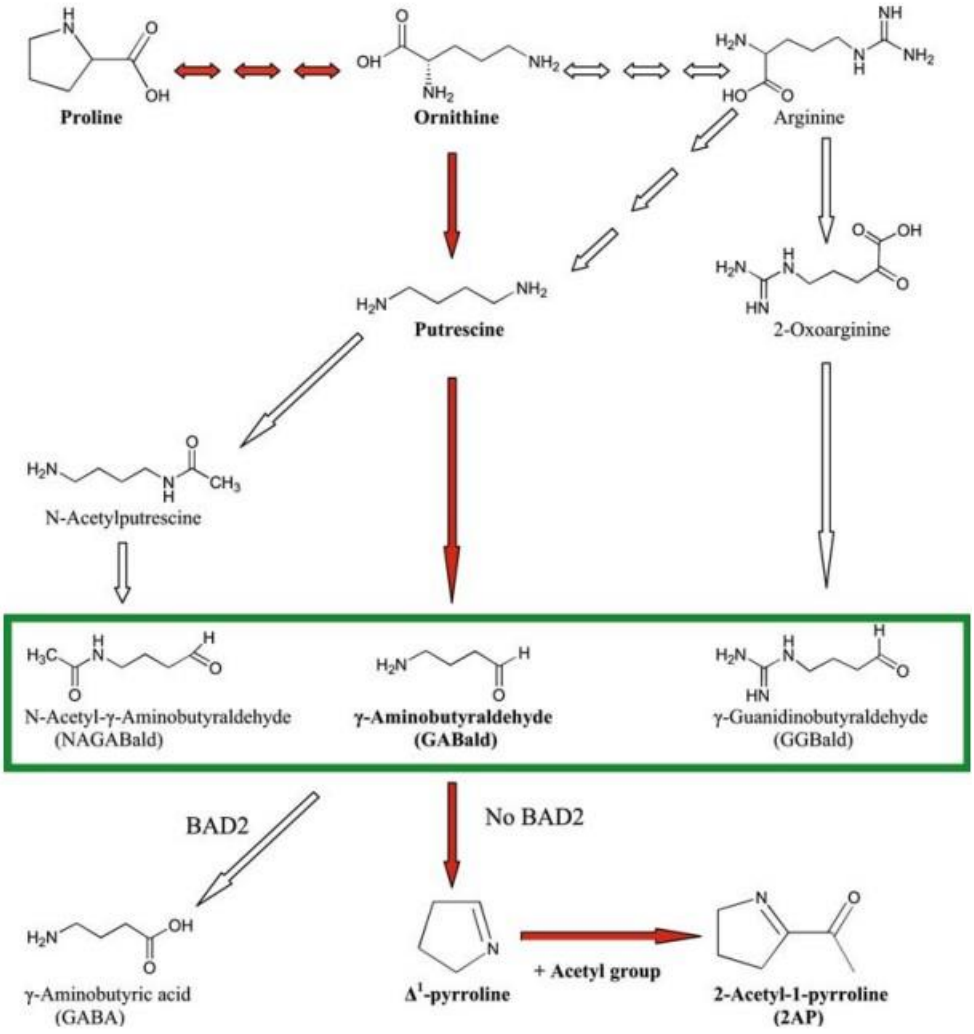


Figure 9 : Pathway from proline to potential substrates (boxed) of BAD1 and BAD2 and to 2-acetyl-1-pyrroline via Δ^1-pyrroline when BAD2 is inactive (filled arrows) or to GABA when BAD2 is active (Bradbury et al., 2008).

The other plants in which APY is found, have a biosynthesis system quite like that present in rice. The same mechanisms have been referenced in coconut (*Cocos nucifera* L.) (Dumhai *et al.*, 2019), *Bassia latifolia* (Wakte *et al.*, 2011), soybean (*Glycine max* L.) (Wu *et al.*, 2009), cucumber (*Cucumis sativus* L.) (Pramnoi *et al.*, 2013). APY has also been identified in different plants, vegetables and fruits even if its origin is not always well known (Hayata *et al.*, 2003; Masanetz *et al.*, 1998; Wong *et al.*, 1998; Wongpornchai *et al.*, 2003).

APY is not only found in plant and animal systems it can also be produced in food products (Corral *et al.*, 2016; Rosso *et al.*, 2018; Straßer & Schieberle, 2014). Most of the time, it is produced by Maillard reactions (Adams & De Kimpe, 2006; Mahatheeranont *et al.*, 2001) and is described as having a roasted-type aromatic quality, popcorn (Söllner & Schieberle, 2009). During the thermal transformation of certain foods such as bread (Schieberle & Grosch, 1985), it seems that the APY reaction scheme is slightly different. Nevertheless, proline remains the source of nitrogen and the precursor of this

compound. But here, Δ^1-pyrroline reacts with 2-oxopropanal to form 2-acetylpyrrolidine which then leads to the formation of APY (Hofmann & Schieberle, 1998, Figure 10).

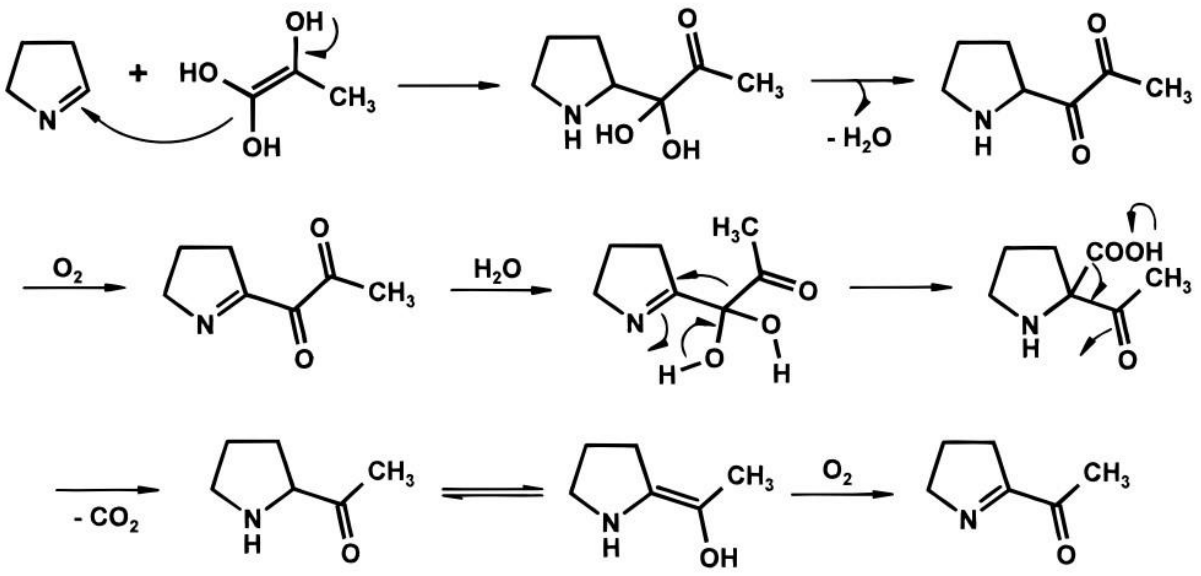


Figure 10 : Formation of 2-acetylpyrroline under aqueous conditions (APY) from the Δ^1-pyrroline (Hofmann & Schieberle, 1998).

Δ^1-pyrroline is the key precursor to the formation of the food flavouring compounds APY and ATHP. This compound is formed from the proline amino acid. ATHP or APY are formed preferentially in a matrix depending on the composition of carbohydrate cleavage products of the matrix. Indeed, if the matrix contains more 2-oxopropanal, APY will preferentially form, as in the bread crust. If on the other hand, the matrix has a greater amount of hydroxy-2-propane, then it will be the ATHP that will be formed preferentially as in popcorn (Hofmann & Schieberle, 1998).

In all these matrices, APY is seen as a sought-after qualitative compound. The only example in the literature associating APY with a mousy taint outside of wine is in wetted ground pearl millet (*Pennisetum americanum*) (Seitz *et al.*, 1993). The concentrations then found in millet is higher than in an aromatic rice, however its origin is not known, proline would still be suspected to be a precursor.

APY also appears in some matrices following microbiological and/or fungal contamination. The liquid culture of several species of *Aspergillus spp.* produced rice odours (Shaikh & Nadaf, 2013). Other studies have shown the production of APY by pathogenic bacterium to humans, *Bacillus cereus*, highlighted the production of the APY. These bacteria are sometimes found in fermented cocoa as spoiled microorganism. Adams & De Kimpe (2007) showed that these bacteria are able to produce APY from ornithine and proline (Figure 11).

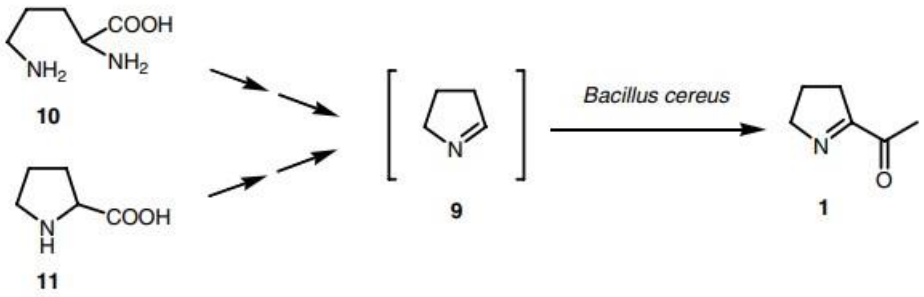


Figure 11 : Proposed pathway for the formation of 2-acetylpyrroline (1) from ornithine (10) and proline (11) via Δ^1-pyrroline (9) by Bacillus cereus *ATCC 27522 and ATCC 14737 (Adams & De Kimpe, 2007).*

In most matrices, amino acids play an important role in the synthesis of mousy compounds. In both plants and processed products, proline is widely recognized as the precursor of these compounds. Ornithine would be implicated as a precursor of APY only when a bacterium is responsible for the production.

I.2.2.4. 2-Acetylpyridine

In recent years, 2-acetylpyridine (AP) has also been suspected to contribute to mousy off-aroma in wines (Hayasaka *et al.*, 2020; Pour Nikfardjam & Kunz, 2021). Kuenzler & Pour Nikfardjam (2013) proposed that AP would result from the oxidation of ATHP. They found AP in 119 out of the 127 wines studied regardless of the style of wine with concentrations up to 530 µg/L for the most concentrated wine. On the other hand, as for ATHP, during their study in 2021, Pour Nikfardjam & Kunz did not find AP in their model solution. They hypothesized that ATHP would have occurred, but not entirely which would lead to the production of an intermediate, 2-acetyldihydropyridine (ADHP) (Figure 12).

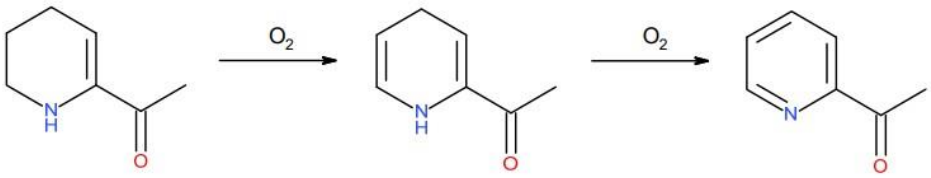


Figure 12 : Proposed pathway of the oxidation of 2-acetyltetrahydropyridine (ATHP) in 2-acetylpyridine (AP) by 2-acetyldihydropyridine (ADHP) (Pour Nikfardjam & Kunz, 2021).

I.2.3. Quantification method in wine

Historically, the three compounds were quantified in wine and model solution by a rather cumbersome method (Costello, 1998; Grbin, 1998). The sample was first filtered and saturated with NaCl. The wine or model solution was then extracted twice at two different pH levels with Freon F11 an organic solvent, its production is internationally banned since 1989 (Canada, 2015). The last organic

phase was finally dried using Na_2SO_4, concentrated and then analysed by gas chromatography coupled with mass spectrometry (GC-MS). All extraction steps are presented in Figure 13.

Pour Nikfardjam & Kunz (2021) followed ATHP and AP by HS-GC-MS and compared the results to HS-GC-SNIFF analysis. By the way, it seems that they were only interested in qualitative analysis without quantifying the compounds.

Recently, a quantification method of the ATHP has been developed in wine by HPLC-MS (Hayasaka, 2019). The method is reliable and robust as well as sufficiently sensitive (Limits of detection: 0.07 µg/L in red wine and 0.08 µg/L in white wine; Limits of quantification: 0.21 µg/L in red wine and 0.23 µg/L in white wine). Although the method is reliable and robust, it can only quantify a single compound, requiring sample preparation which also highlighted the instability of this compound. This instability is depending on the matrix. Pour Nikfardjam & Kunz (2021) proposed that ATHP would oxidize to AP and suggested that the presence of polyphenols could slow down this oxidation. This proposition contradicts the observation of Hayasaka (2019), who has shown that ATHP would have a half-life ($t_{1/2}$) lower in red wines than in white wines. However, this degradation is not explained.

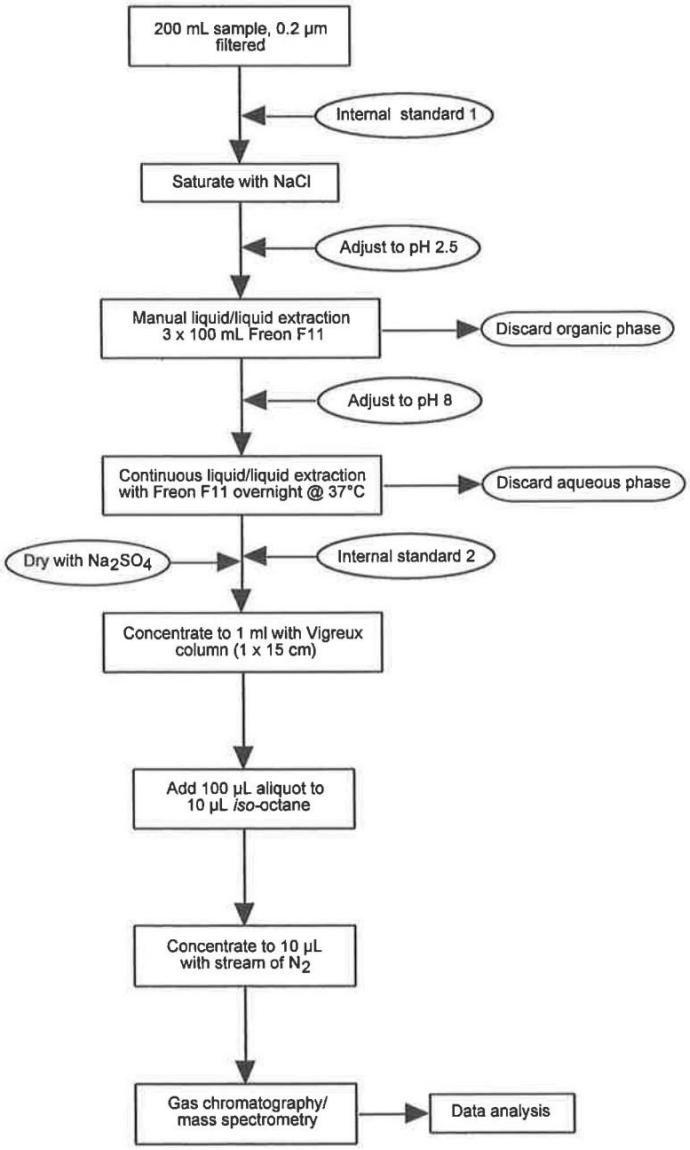


Figure 13 : Detailed protocol of extraction and analysis of the mousy compounds;
Internal standard 1 : 4-acetylpyridine; Internal standard 2 : 3-acetylpyridine (Costello, 1998; Grbin, 1998).

I.3. Microbiology of winemaking

From grapes to wine, microorganisms are numerous and will each play an important role in the production of wine and its quality.

I.3.1. Pre-fermentation steps

Microorganisms are present throughout the winemaking process, from the vine to bottles. Grape berries are colonized by a wide range of microorganisms such as bacteria, yeasts and filamentous fungi (Barata *et al.*, 2012). This endogenous microbial community, consisting mainly of yeasts plays an important role in the grape quality and then in the vinification process. Genera *Aureobasidium*, *Brettanomyces*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Issatchenkia*, *Kluyveromyces*, *Metschnikowia*,

Pichia, *Rhodotorula*, *Saccharomycodes*, *Schizosaccharomyces*, *Sporidiobolus*, *Torulaspora* and *Zygosaccharomyces* are frequently found on the grape skin (Fleet *et al.*, 2002; Martins *et al.*, 2014; Prakitchaiwattana *et al.*, 2004). The leading yeast in œnology, due to its role during alcoholic fermentation (AF), *Saccharomyces cerevisiae*, is rarely isolated in the vineyard (Mortimer & Polsinelli, 1999). It is detected at much lower population levels than other species (Goddard & Greig, 2015). The yeast community found in the vineyard is impacted by various parameters, the maturity of the grapes (Hierro *et al.*, 2006), the geographical area (Gayevskiy & Goddard, 2012), the vintage (Pretorius, 2000) and the method of cultivation. The health status of the harvest will also have a considerable impact. The presence of damaged berries favours the development of certain fungi, *Botrytis cinerea*, but also lactic and acetic bacteria and yeasts, and fermentative such as *Torulaspora spp.* or alteration yeasts such as *B. bruxellensis* (Barata *et al.*, 2012; Mortimer & Polsinelli, 1999).

Once arrived at the desired maturity, the grapes are harvested. The berries are then crushed, the microbial population present on the grape will meet the grape must. The microflora in the vat is then very similar to that of grapes with a wide representation of *non-Saccharomyces* species of genera *Hanseniaspora*, *Torula*, *Pichia, Metschnikowia*, *Candida* (Zott *et al.*, 2010). In order to have better control over the microbial population present, the harvest can be sulphited or non-*Saccharomyces* yeasts can be supplemented to perform biocontrol.

I.3.2. Alcoholic fermentation

The first important microbiological process in winemaking is alcoholic fermentation (AF). During this fermentation, the sugars (glucose and fructose) contained in the must are converted into ethanol and carbon dioxide by the yeasts. Non-*Saccharomyces* yeasts usually start AF but are quickly supplanted by *S. cerevisiae* because they are rapidly inhibited by ethanol production (greater than 5-7 %) and temperature increase (greater than 15°C) (Ciani & Comitini, 2011). On the contrary, *S. cerevisiae* is able to ferment sugars despite the presence of oxygen, grows rapidly and can tolerate higher alcohol contents (up to 16-18 %) (Fleet, 2003; Fugelsang & Edwards, 2007; Thomson *et al.*, 2005).

Unwanted *non-Saccharomyces* yeasts can also grow in the must. To avoid any alterations, some winegrowers will prefer the use of a starter culture or even the use of active yeasts directly in the must. The practice of a starter culture consists of seeding the musts with a starter prepared from a spontaneous fermentation in a small volume of must.

To facilitate fermentation, it is strongly advised to adjust the assimilable nitrogen to avoid any stuck fermentations.

During AF, acetaldehyde is an intermediate compound of sugar metabolism. It can also be produced by oxidation of ethanol by Fenton reaction (Danilewicz, 2003). The use of a sequential fermentation with non-*Saccharomyces*/*Saccharomyces* yeast will tend to produce higher levels of ethanal than those inoculated only with *S. cerevisiae* (Escribano-Viana *et al.*, 2018). The production of ethanal is also

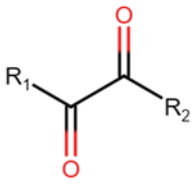
depending on the strain used, the temperature of the AF, the presence of SO_2 etc. (Guittin *et al.*, 2023; Ochando *et al.*, 2020). Among the wine compounds, ethanal is the carbonyl compound with one of the highest affinities with SO_2.

Once AF is complete, when yeasts have consumed almost all carbonaceous sources, significant mortality occurs in yeasts. This autolysis of yeasts has a stimulating effect on the growth of lactic acid bacteria (LAB) in wine (Guilloux-Benatier *et al.*, 1985).

I.3.3.Malolactic fermentation

The wine can be subjected to a second fermentation, malolactic fermentation (MLF). This fermentation is not always desired. It is carried out by LAB and mainly by the species *Oenococcus oeni* (Davis *et al.*, 1985; Dicks *et al.*, 1995). The latter play a crucial role in the vinification of wines: certain strains of *O. oeni* mostly ensures the conduct of the MLF in red wines and part of white wines. They belong to the indigenous microbiota of must and wine and develop spontaneously, during or after AF (Ruiz *et al.*, 2010). Most of the time, MLF proceeds satisfactorily, but adverse conditions or a population of native bacteria that is too low can cause late onset of MLF or languid fermentation. Although called fermentation, it is not one strictly speaking. MLF is the transformation of L-malic acid into L-lactic acid. It is therefore like an enzymatic decarboxylation. The malolactic enzyme that allows this decarboxylation was purified and characterized for the first time in *Lactiplantibacillus plantarum*, in 1975 (Lonvaud, 1975). This decarboxylation leads to a deacidification of the wine, which can lead to a significant improvement in taste: L-malic acid can confer an undesirable acid greenness to the wine; lactic acid is softer and less aggressive on the taste buds of the tongue (de Revel *et al.*, 1999). This will allow red wines to acquire sweet, fleshy, and fat characters. In addition, this results in an increase in the pH of the wine from 0.1 to 0.2 pH unit which results in an organoleptic softening of the wine and a decrease in acidity. (Amerine & Roessler, 1983). The development of *O. oeni* reduces the concentration of substrates available for other species and therefore also leads to microbiological stabilization of the wine. The compounds consumed are not limited to L-malic acid, LAB will also consume vitamins, amino acids, and other substrates.

In addition, MLF also increases the aromatic complexity in wines. Indeed *O. oeni* produces some secondary metabolites such as butan-2,3-dione (diacetyl) by the metabolism of citric acid (de Revel, 1992; Ramos *et al.*, 1995). At low concentrations, diacetyl can contribute positively to the aromatic expression of wines while an excess of diacetyl can nevertheless have a negative impact on tasting (Rankine & Pocock, 1969). LAB can produce other α-dicarbonyl compounds (Figure 14). These molecules, such as glyoxal, methylglyoxal, pentanedione, are extremely reactive, especially with cysteine in wines. The chemical reactions then involved lead to the formation of heterocycles associated with aromatic descriptors "popcorn", "toasted", "hazelnut", "coffee" or "rubber" (Marchand *et al.*, 2000).



	R_1	R_2
glyoxal	H	H
methylglyoxal	CH_3	H
butan-2,3-dione	CH_3	CH_3
pentanedione	CH_3	C_2H_5

Figure 14 : Chemical structure of α-dicarbonyl compounds.

To facilitate the onset of MLF, malolactic starter may be used (Kunkee *et al.*, 1964). These are selected for their technological properties and their adaptation to the environment which can therefore tolerate physicochemical conditions, as acidic pH, relatively low temperatures, the presence of ethanol but also that of sulphur dioxide (Torriani *et al.*, 2011).

As soon as malic acid disappears completely, the wine can be sulphited. This will then be used for its antimicrobial activity to limit microbial developments, which would be undesirable.

The winemakers can therefore use SO_2 at different step of vinification to protect the wine. It can be used at the beginning of fermentation in order to better control microbial populations or at the end of fermentation to reduce the population levels present in the environment and thus prevent any contamination by spoilage microorganisms. In general, the use of sulphites at these different stages responds to risks whether microbiological and/or chemical (Figure 15).

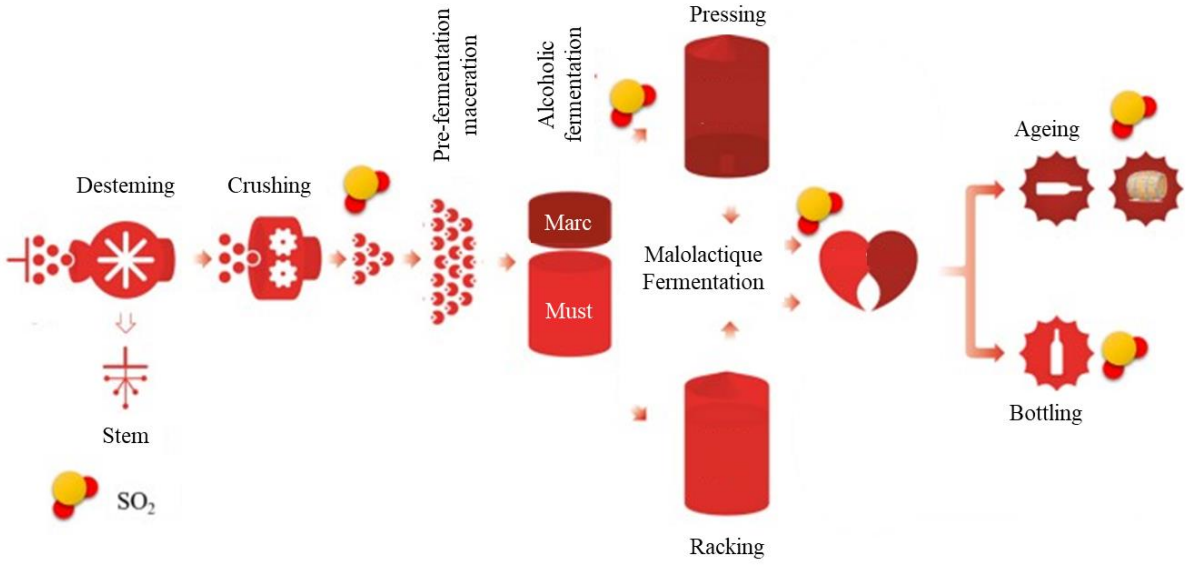


Figure 15 : Modified diagram by © Martin Lavieille representing the use of SO_2 during the winemaking process for red wines (Windholtz, 2020).

I.4. The microbiological risk associated with the mousy off-aromas

The three identified compounds causing mousiness (*Cf*: I.2.2 Mousy *N*-heterocycles; p. 16) can be produced by some LAB or by *Brettanomyces* (Heresztyn, 1986). Studies have demonstrated the ability of *Brettanomyces* yeasts to produce at least two of these compounds while LAB are likely to produce all three. *Brettanomyces* were the first microorganisms to be known to form mousy taint in wines (Peynaud & Domercq, 1956). This spoilage yeast was already monitored for its production of volatile phenols (*Cf:* I.1.4.3 Volatile phenols, p. 14), giving the wine aromatic notes perceived as "leather", "horse sweat" (Chatonnet *et al.*, 1992). LAB are also known to be organisms responsible for mousy off-aroma.

I.4.1. Precursors

Several precursors of the mousy off-aromas have been identified in wine. Ethanol is one of them: in its absence mousy compounds are not produced (Tucknott, 1977), which explains the occurrence of spoilage after FA.

Whether in the form of ethanol, or in the oxidized form, ethanal, it would be essential for the formation of the acetyl group of ATHP and APY. Costello & Henschke, (2002) showed that by replacing ethanol with its deuterated counterpart, d_6-ethanol, the corresponding APY and ATHP are produced in a model medium by lactic acid bacteria. Indeed, in the presence of d_4-ethanal, only the deuterated analogue of APY was found and not ATHP.

The presence of certain amino acids, such as L-lysine and L-ornithine is essential to produce ETHP, ATHP and APY. They are responsible for the formation of heterocyclic structures. When added simultaneously to a model medium, APY and ATHP concentrations increase significantly (Costello & Henschke, 2002). It seems that the heterocyclic structures of the two final compounds are given by the cyclisation of amino acids. These amino acids are naturally present in grape juice and therefore in the must and could thus play the role of precursors of the of mousiness *N*-heterocycles. However, they are not the most common amino acids present in wine (Lehtonen, 1996; Table 2).

Table 2 : Amino acids in seven French red wines (from Lehtonen, 1996).

Amino acid	Abbreviation	Amount in wine (mg/L)
Arginine	Arg	23-64
Hydroxyproline	Hyp	3.3-8.9
Serine	Ser	7.1-14
Aspartic acid	Asp	8.6-19
Glutamic acid	Glu	26-54
Threonine	Thr	5.4-10
Glycine	Gly	11-20
Alanine	Ala	26-64
Proline	Pro	380-790
Methionine	Met	0
Valine	Val	3.6-9.1
Phenylalanine	Phe	7.3-22
Isoleucine	Ile	2.4-5.9
γ-Aminobutyric acid	Gaba	10-47
Leucine	Leu	6.0-13
Histidine	His	3.5-11
Ornithine	Orn	5.3-31
Lysine	Lys	7.5-17
Tyrosine	Tyr	2.0-7.5
Total		393-1050

In wine, the presence of amino acids plays an important role and their composition varies according to the grape varieties and the region of production (Etiévant *et al.*, 1988). L-lysine and L-ornithine are amino acids naturally present in wine at concentrations of about ten mg/L (Lehtonen, 1996). Arginine is usually present in greater amounts than L-lysine and L-ornithine. In addition, many strains of *O. oeni* can use it with the arginine deiminase (ADI) pathway. This pathway involves three enzymes: arginine deiminase, ornithine transcarbamolyase and carbamate kinase (Tonon *et al.*, 2001). The action of arginine deiminase and ornithine transcarbamolyase converts arginine into L-ornithine, a precursor of some mousy *N*-heterocycles produced by lactic acid bacteria.

Although proline is the precursor of APY and ATHP in most plants and various food product (*Cf:* I.2.2.2 2-Acetyltetrahydropyridine; p. 17 ; I.2.2.3 2-Acetylpyrroline; p. 19) and even is present in large quantities in wines, it does not seem to be involved in the production of mousy compounds. Costello (1998) showed that the presence of proline in a model medium containing L-lysine and L-ornithine could completely inhibit the production of APY and could induce a decrease of ATHP production by about 40% with some strains of *L. hilgardii* (Figure 16).

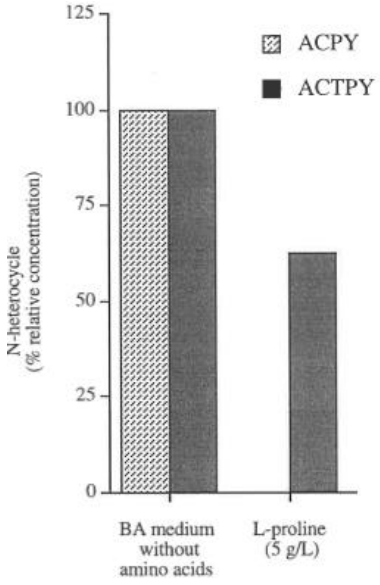


Figure 16 : Effect of the addition of L-proline in a basal assay medium containing L-lysine and L-ornithine on the production of ATHP (ACTPY) and APY (ACPY) by a strain of Lentilactobacillus hilgardii *(Costello, 1998).*

It has been observed that the presence of certain metal ions can impact the production of APY and ATHP. When ferrous ions, manganese, magnesium and calcium are removed in the model medium, the production of APY, ETHP and ATHP is greatly reduced (Snowdon *et al.*, 2006). Although the explanation remains unknown, this information suggests that the amount of metal ions is a physicochemical factor necessary for the development of mousiness in wines. It is possible that it is an enzyme cofactor, or a partner for a redox reaction (Costello & Henschke, 2002).

The oxygen seems to have a real link with the mousy taint, the mechanism involved is unknown. Oxygen and aeration have been shown to stimulate the production of ATHP and ETHP (Grbin, 1998). Of the two, ATHP seems to be preferably produced in a model medium. While in an air limited condition, the ATHP/ETHP equilibrium was reversed.

I.4.2.*N*-heterocycles assay medium

Considering these different precursors, suitable media were developed to study the ability of microorganisms to produce mousy *N*-heterocycles. The 3 main media are presented in Table 3. Grbin, (1998) adapted a medium previously used for monitoring the production of volatile phenols by *B. bruxellensis*. He varied the different constituents to have a better knowledge of the mechanisms involved (Grbin *et al.*, 2007). Costello (1998) compared the ability of different microorganisms to produce mousy off flavour under these conditions (Costello *et al.*, 2001). Subsequently Costello & Henschke (2002) substituted different compounds with labelled one, such as amino acids labelled with carbon 13 or deuterated ethanol. Their objective was to define key substrates for mousy taint. Romano *et al.* (2008),

wanted to characterize *B. bruxellensis* behaviour in terms of growth and volatile compound production in red wine, they adapted Costello's medium to create an *N*-heterocycle *assay* medium (*N*HAM).

Table 3 : Different chemically defined media used to compare the production of microorganisms in model media.

	(Grbin, 1998)	(Costello *et al.*, 2001)	(Romano *et al.*, 2008)
Component	For 1 L		
Ammonium sulphate	2 g	-	-
Biotin	30 mg	-	-
Calcium chloride	310 mg	1.66 g	1 g
Citric acid	200 mg	2 g	2 g
D-Fructose	50 g	50 g	50 g
Ethanal	-	100 mg	100 mg
Ethanol (96%)	-	52 mL	52 mL
Iron sulphate	10 mg	43 mg	43 mg
L-Lysine	100 mg	5 g	5 g
L-Malic acid	-	5 g	5 g
L-Ornithine	-	5 g	5 g
Magnesium sulphate	250 mg	25 g	12.5 mg
Manganese sulphate	-	25 mg	25 mg
myo-Inositol	25 mg	-	-
Nicotinic acid	10 mg	-	-
Potassium chloride	-	4.25 g	4.25 g
Potassium phosphate monobasic	2 g	5.5 g	5.5 g
Pyridoxine	1 mg	-	-
Thiamine	1 mg	-	-
Triammonium citrate	-	-	2 g
Zinc sulphate	2 mg	-	-
Tween 80		-	1 mL
pH	3.8	4.5	4.5

It is notable that the composition of the medium used by Grbin is much poorer than the media used by others. On the other hand, in his study, he varied each parameter to increase knowledge about the production of each compound, varying, for example, L-lysine from 10 mg/L to 1000 mg/L.

I.4.3.Microorganisms involved in the production of the mousy compounds

Microbial contamination can be multiple in a wine produced without the addition of SO_2.

I.4.3.1. Brettanomyces bruxellensis

Brettanomyces bruxellensis is a yeast found in many fermentation processes, which can be considered as an important cause of microbiological alteration (wine, beer) but also as a processing aid (kombucha, specialty beer) (Beech, 1958; Cabranes *et al.*, 1990; Kolfschoten & Yarrow, 1970; Rainbow, 1981; Smith *et al.*, 1981; Tucknott, 1977). Research works in recent years have shown that *B. bruxellensis* has significant genetic and phenotypic diversity (Avramova *et al.*, 2018a; Cibrario *et al.*, 2020; Harrouard *et al.*, 2022; Figure 17).

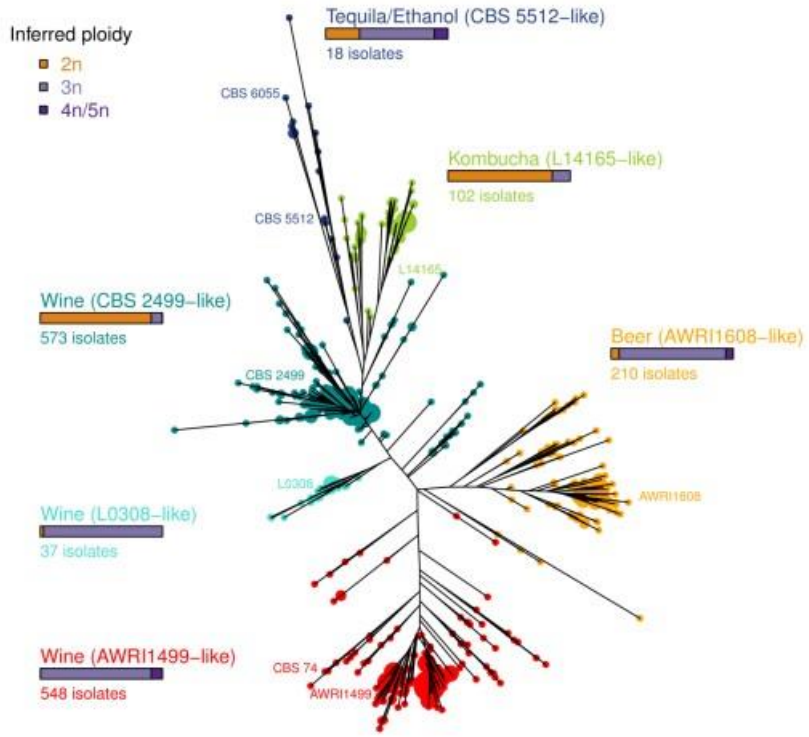


Figure 17 : Dendrogram of 1488 isolates of B. bruxellensis *using 12 microsatellite markers (Avramova et al., 2018a).*

It is also a yeast of interest because of its ability to survive and adapt to the wine conditions (ethanol, pH and SO_2) and its capacity to make some biofilm (Avramova *et al.*, 2018a; Avramova *et al.*, 2018b; Cibrario *et al.*, 2020; Harrouard *et al.*, 2022; Le Montagner *et al.*, 2023). Characterized by a slow growth, it is particularly suitable for nutrient-poor environments, with low pH and high ethanol concentrations.

The genetic groups of *B. bruxellensis* are structured by the environment and the ecological niches in which they are found as well as by their ploidy level. Indeed, it is noted that the Kombucha and Wine 3 groups (CBS 2499-like) are diploid, the Wine 2 group (L0308-like) is autotriploid and the Tequila/Ethanol, Wine 1 (AWRI 1499-like), and Beer groups are allotriploid (Eberlein *et al.*, 2021; Gounot *et al.*, 2020).

The frequency of *B. bruxellensis* contaminations seems to be increasing, this could be linked to changes in vinification practices (significant reduction in the doses of SO_2 used) or to climate change. The origin of these contaminations has been under discussion for several years. At the cellar, *B. bruxellensis* is able to enter the wood of barrels in xylem networks up to 8 mm deep (Fabrizio *et al.*, 2015). Thus, cases of contamination between cellars can occur, notably during the re-use of barrels exchanged between vineyards. Moreover, its ability to survive over long periods in wine, in barrels and its ability to persist on equipment due to various physiological mechanisms (biofilm, invasive growth, ability to enter a viable but non-cultivable (VBNC) during summer), are all elements in favour of the hypothesis of a residual presence in the cellar (Le Montagner *et al.*, 2023; Lebleux *et al.*, 2020; Serpaggi

et al., 2012). Cases of contamination by the same strain for several years in the same cellar indicate a significant survivability in this environment (Cibrario *et al.*, 2020). Its presence has also been reported on bunches of grapes but in much smaller proportions (Oro *et al.*, 2019).

The presence of *B. bruxellensis* has long been associated with adverse effects on the visual and organoleptic qualities of wine (Peynaud & Domercq, 1956; Van der Walt, 1964). Its potential for alteration is particularly related to the loss of colour intensity, the production of volatile acidity, the production of biogenic amines, the production of volatile phenols (Chatonnet *et al.*, 1992; Fugelsang & Edwards, 2007; *Cf*: I.1.4.3 Volatile phenols; p.14). It is responsible for the production of 4EP in wines but also for ATHP and ETHP.

Grbin (1998) started to work on the production of mousiness of different genera of *Brettanomyces*. During his thesis, he highlighted that mousy taint production is a characteristic of the genera. Regarding the different precursors of the mousiness, he proposed a pathway of biosynthesis of ATHP and ETHP few years later (Grbin *et al.*, 2007; Figure 18).

If the production route involving cadaverine is functional in *B. bruxellensis,* one could imagine having interactions between the different microorganisms of the wine. The enzyme lysine decarboxylase is present in distinct species found in wine, whether in yeasts or bacteria (Caruso *et al.*, 2002; Del Prete *et al.*, 2009; Martínez-Pinilla *et al.*, 2013; Tofalo *et al.*, 2016).

Grbin *et al.* (2007), proposed an enzymatic production of ETHP route by yeasts of the genus *Brettanomyces*. The enzyme involved would be a dehydrogenase enzyme and ETHP production would come only after that of ATHP.

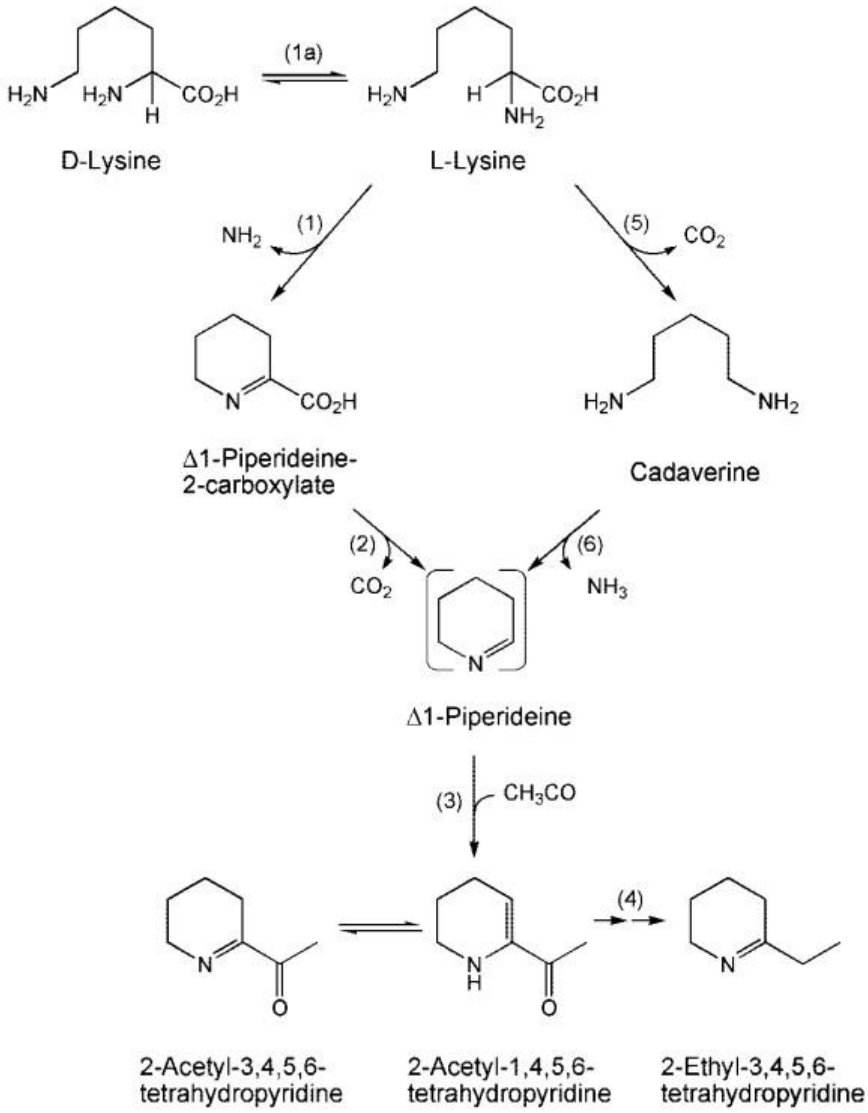


Figure 18 : Proposed pathway(s) of biosynthesis of 2-actyltetrahydropyridine and 2-ethyl-3,4,5,6-tetrahydropyridine; proposed enzyme-mediated reactions: (1) L-lysine R-aminotransferase or R-oxidase (deaminating), (1a) lysine racemase, (2) Δ^1-piperideine-2-carboxylic acid decarboxylase, (3) acylase via acetyl-CoA or other condensation reaction, (4) dehydrogenase, (5) L-lysine decarboxylase, (6) diamine aminotransferase or putrescine oxidase (deaminating) (from Grbin et al., 2007).

I.4.3.2. Lactic acid bacteria

The main characteristic of lactic acid bacteria is the production of lactic acid from carbohydrates. The main genera of lactic acid bacteria present in grape must and in wines are *Liquorilactobacillus*, *Lactiplantibacillus*, *Lentilactobacillus*, *Pediococcus*, *Leuconostoc* and *Oenococcus* (Ribéreau-Gayon *et al.*, 2006).

The bacterial species isolated from wine can be differentiated in particular by their morphology (cockles or bacilli) and their fermentative character regarding glucose (heterofermentative, optional or obligatory heterofermentation and homofermentation).

Beyond their beneficial effect, lactic acid bacteria can also affect the wines quality. There is little knowledge about their action on the components of wine colour, however it is known that they cause colour alteration during MLF, as well as a decrease in the amount of tannins and free anthocyanins (Davis *et al.*, 1986; Ribéreau-Gayon *et al.*, 2006). Some bacteria isolated from wine have also been identified as bacteria that can cause in some cases the fault of ropiness in wines with *Pediococcus parvulus* (Lonvaud-Funel *et al.*, 1993). Nevertheless, the development of LAB in wine can sometimes seriously alter wine sensory qualities and even make it unfit for consumption (Coton *et al.*, 1998; Lonvaud-Funel & Joyeux, 1988; Lucas *et al.*, 2008).

In the same way of *B. bruxellensis,* a great genetic and phenotypic diversity exists in the strains of *O. oeni* (El Khoury, 2014; Lorentzen & Lucas, 2019 (Figure 19) and more broadly the strains of LAB.

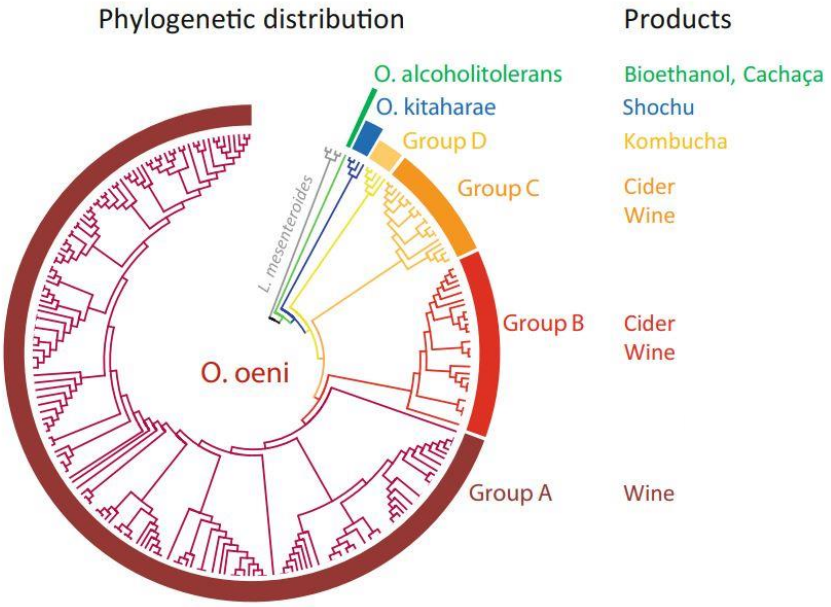


Figure 19 : Phylogenomic tree distribution of 261 Oenococcus spp. *Strains based on genomic distances (from Lorentzen & Lucas, 2019).*

During his thesis, Costello studied the production of mousy compounds of different genera and species of lactic acid bacteria using a high cell density incubation (*Cf*: I.4.2 *N*-heterocycles assay medium; p. 31). He was able to highlight differences in production between the different species. According to this work, *L. hilgardii* is the most productive species of mousy compounds under his conditions. It also appears from these results that *O. oeni*, the predilection strain of MLF can produce the mousy *N*-heterocycles. In addition, heterofermentative bacteria produced more ATHP than homofermentative bacteria.

Based on this information and the results collected following the tracing of the precursors, Costello & Henschke (2002) were able to propose a formation pathway for APY and ATHP by the LAB (Figure 20). The compounds in bold type have been shown as key substrates.

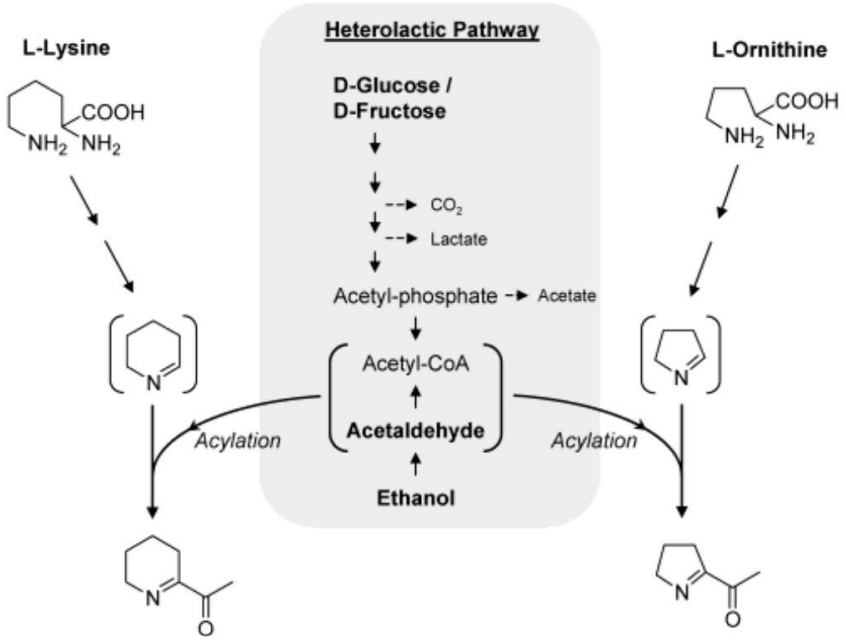


Figure 20 : Proposed pathway for formation of the acetylated mousy N-*heterocycles, 2-acetyl-1-pyrroline and 2-acetyltetrahydropyridine,* L. hilgardii *DSM 20176 (from Costello & Henschke, 2002).*

I.5. Objectives of the thesis

The research objectives of the present work were to explore the phenomena associated with the production of pyrrole and pyridine derivatives responsible for mousy off-aromas in wine.

More specifically, the four objectives of this work were:

- to develop quantification methods in wine and in a standard medium of the mousy compound;
- to characterize the diversity of mousy producers' microorganisms in wine;
- to determine some chemical and/or microbiological parameters which can influence the production of mousiness;
- to develop diagnostic tools to assess the risk of mousy off-aromas production in wines. Unfortunately, this objective could no longer be carried out due to time constraints

Chapter II.

Chemical aspect

This chapter presents the work done at Prof. Gilles de Revel laboratory of the UMR Œnologie. Significant delays were taken during the start of this work mainly due to constraints related to the pandemic. The laboratory was closed for a period of more than 2 months and the planned travel between the different partners had to be postponed requiring regular adaptation. Our first objective was to develop an analytical method using SBSE-GC-MS to quantify the mousy *N*-heterocycles in wines and model culture media in a single run with the minimal time consuming. The first method of quantification has been developed in contribution with Daiki Kiyomichi. Once the quantification methods have been developed in wine and in a model medium, we were interested in the hypothesis of pH dependent ATHP tautomerism proposed by Snowdon *et al.* (2006). Finally, a collaboration with Dr. Alicia Jouin made it possible to look at the impact of oxygen in a "sulphite free wine" on mousy compounds.

II.1. Quantification of the *N*-heterocycles in wines by SBSE-GC-MS

The results of this part were written in the form of a scientific article entitled "Investigation into mousy off-aromas in wine using gas chromatography-mass spectrometry with stir bar sorptive extraction". This article was published in the journal Food Chemistry in January 2023.

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Investigation into mousy off-flavor in wine using gas chromatography-mass spectrometry with stir bar sorptive extraction

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1-(3,4,5,6-Tetrahydro-2-pyridinyl)ethanone (PubChem CID: 520300)
2-Acetyl-1,4,5,6-tetrahydropyridine (PubChem CID: 520194)
2-Ethyl-3,4,5,6-tetrahydropyridine (PubChem CID: 3014008)

ABSTRACT

Three major compounds, 2-acetyl-1-pyrroline (APY), 2-acetyltetrahydropyridine (ATHP) and 2-ethyltetrahydropyridine (ETHP), have been identified as responsible for the mousy off-flavor in wines, although to date quantification data reported in the literature are limited. A simple method for simultaneous quantitation, by SBSE-GC–MS, of these *N*-heterocyclic compounds was developed. Both previously reported tautomers of ATHP, 2-acetyl-1,4,5,6-tetrahydropyridine and 2-acetyl-3,4,5,6-tetrahydropyridine were identified. The limits of detection and quantification of the method were determined in white, rosé and red wines and are lower than previously published concentrations in spoiled wine. ETHP was detected in almost all wines produced with limited use of SO_2. ATHP was detected in almost all wines suspected of mousiness whereas APY was only detected in few cases. This method will provide a support for further studies aimed at understanding the phenomena that influence the occurrence of mousy off-flavor and the oenological parameters that modulate its expression.

1. Introduction

Mousy off-flavor is one of the defects of microbial origin in wine. Mousiness is described as a particularly unpleasant defect (Peynaud & Domercq, 1956) reminiscent of rodent urine (a "dirty mouse cage"), and grilled foods such as popcorn, rice, crackers, and bread crust (Tucknott, 1977; Buttery, Ling, Juliano, & Turnbaugh, 1983; Strauss & Heresztyn, 1984; Herderich, Costello, Grbin, & Henschke, 1995; Bartowsky, 2009). Prior to the 2010 s, such an off-flavor was very uncommon but it has been becoming more frequent in recent years. It is often associated with an increase in pH as well as certain oenological practices, which tend to significantly decrease the use of sulfur dioxide. These practices have become increasingly popular with consumers seeking products from healthier and more environmentally friendly production sources, thus making the addition of SO_2 increasingly controversial (Costanigro, Appleby, & Menke, 2014). For centuries, the wine industry has been using sulfur dioxide for its antimicrobial activity (particularly on the development of undesirable microorganisms such as *Brettanomyces bruxellensis* for example) and antioxidant (chemical or enzymatic) effect (to protect wine compounds from oxidation). In Europe, the regulation No. 2019/934 limits the use of SO_2 in wines and the regulation No. 2019/33 obliges the winemakers to stipulate the mention "contains sulfites" when its concentration is higher than 10 $mg.L^{-1}$. The levels of SO_2 found in wines are mainly due to an addition by the winemakers. However, it is important to note that it can also be produced during alcoholic fermentation by *Saccharomyces cerevisiae* (Wells & Osborne, 2011).

According to Snowdon, Bowyer, Grbin, & Bowyer (2006), the first studies on the subject date from the end of the 19th century. To our knowledge, three *N*-heterocyclic molecules supposedly linked to this defect in wine have been identified: 2-acetyl-1-pyrroline (APY) (Herderich et al., 1995), 2-acetyltetrahydropyridine (ATHP) (Strauss et al., 1984), and 2-ethyltetrahydropyridine (ETHP) (Craig & Heresztyn, 1984).

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Detection thresholds in water by orthonasal evaluation are 1.6 $\mu g.L^{-1}$ for ATHP (Teranishi, Buttery, & Guadagni, 1975) and 0.1 $\mu g.L^{-1}$ for APY (Buttery et al., 1983) whereas it is much higher for ETHP at 140.5 $\mu g.L^{-1}$ (Tempère et al., 2019). Nevertheless, it is difficult to compare these detection threshold values due to the diversity of measurement strategies. Consequently, the data must be handled with care. To our knowledge the comparison between detection threshold and measured values does not enable a direct deduction of the impact of a molecule on the odor of that molecule diluted in a complex matrix such as wine. Even if the measured values suggested that the contribution of ETHP to mousiness may be less significant than that of ATHP and APY, nonetheless this issue should be further explored. Reported concentrations in spoiled wine are between 0.7 and 106 $\mu g.L^{-1}$ for ATHP (Costello, 1998), while for APY a concentration of 7.8 $\mu g.L^{-1}$ was measured (Snowdon et al., 2006). As regards ETHP, concentrations in wine can exceed 150 $\mu g.L^{-1}$ (Grbin, Costello, Herderich, Markides, Henschke, & Lee, 1996). During tasting, a particularity of mousy off-flavor is that it is more strongly perceived in the mouth at the end of tasting by the retronasal route than by the orthonasal route. The presence of a nitrogen atom in the heterocyclic ring enables the existence of a protonated form under specific conditions, i.e. with pH lower than pKa. The protonation of the molecule implies the presence of a positive charge and, as a consequence, an increase of polarity and a loss of volatility. The increase in wine pH during in-mouth tasting leads to a loss of that proton and a gain in the volatility of *N*-heterocycles. The volatile forms can reach the olfactory epithelium and therefore interact with the olfactory receptors. Therefore, it is easier to detect this alteration when the pH of the sample is higher than that of wine, which is the case with oral pH, i.e. between 5.76 and 7.96 (Larsen, Jensen, Madsen, & Pearce, 1999). This observation backs up our remarks regarding the caution with which data concerning detection thresholds should be used.

The origins of mousiness have been explored in the past but have yet to be resolved. The investigations are lacunar mainly because of the absence of easy-to-use quantification methods. A hypothesis as to the occurrence of mousy odors due to a lack of control of the microbial flora of wines and / or musts has been previously advanced (Tucknott, 1977; Grbin, & Henschke, 2000; Costello, & Henschke, 2002). The microorganisms described as able to produce *N*-heterocyclic compounds in wines are lactic acid bacteria (LAB), belonging to the *Lactobacillus* genus or *Oenococcus oeni* species, and yeasts of the *Brettanomyces* genus. Nevertheless, other microorganisms present during winemaking could be implicated. Moreover, nothing is known about the possible link between growth conditions (e.g., nutrients, temperature, pH, various stresses) and the capacity of microbial flora to develop mousy odors. Costello et al. (2002) proposed a biosynthetic pathway for the formation of APY and ATHP by LAB. Both compounds would be produced from L-lysine or L-ornithine, ethanol, and glucose or fructose. In their study of the complex "Brett character" caused by *Brettanomyces* spoilage, Romano, Perello, de Revel & Lonvaud-Funel (2008) provided the first experimental evidence of the production of the mousy off-flavor component ATHP, by *B. bruxellensis* in wine, through GC detection. However, they observed that not all *B. bruxellensis* strains were capable of producing ATHP to the same degree in synthetic media, unlike volatile phenols. Furthermore, they highlighted that ATHP production was favored by the presence of multi-gram amounts of glucose and fructose in wines suggesting that *Brettanomyces* spoilage occurring during a stuck fermentation or in a bottled sweet wine is more likely to result in 'mousiness'. Thus, microorganisms could directly produce the compounds responsible for mousy off-flavor, but microorganisms could also play a role in the formation of precursors such as, for example, methylglyoxal, which is produced during fermentation, and these precursors further chemically transformed into mousy off-flavor compounds. A chemical mechanism proposed by Pour Nikfardjam, & Kunz (2021) initially involves the reaction of methylglyoxal with proline to form in several steps ATHP which could give 2-acetylpyridine (AP) under oxidative conditions.

Recently, a quantification method focusing on ATHP has been reported (Hayasaka, 2019). This method uses liquid chromatography-tandem mass spectrometry interfaced with atmospheric pressure chemical ionization (HPLC-APCI-MS/MS). Limits of detection (LODs) of ATHP for red and white wine with this method were respectively 0.07 $\mu g.L^{-1}$ and 0.08 $\mu g.L^{-1}$, while limits of quantification (LOQs) were 0.21 $\mu g.L^{-1}$ in red wine and 0.23 $\mu g.L^{-1}$ in white wine. However, there are still few quantification data of such *N*-heterocyclic compounds in wine.

To fill the gap and later understand the parameters influencing mousiness, the objective of the current study was to develop a simple and effective method to simultaneously determine trace levels of three mousy *N*-heterocycles in wines. The stir bar sorptive extraction (SBSE) method for the extraction and enrichment of organic compounds from aqueous samples (Baltussen, Sandra, David, & Cramers, 1999) is applied to the trace analysis of various aroma compounds in wines (Fang & Qian, 2006; Franc, David, & de Revel, 2009, Picard, Franc, de Revel, & Marchand, 2018). As a result, SBSE with gas chromatography-mass spectrometry (GC–MS) was chosen and extraction conditions were optimized. In order to provide quantitative data, the method was then applied to analyze mousy *N*-heterocycles in 6 control wines and 68 wines produced without added sulfur dioxide or with limited addition of SO_2. These wines were also analyzed for volatile phenols as it is well known that *B. bruxellensis* produces these compounds (Heresztyn, 1986).

2. Material and methods

2.1. Chemicals

APY (CAS registry No. 85213–22-5, 10 % w/w in toluene) and ATHP (CAS registry No. 27300–27-2, 98 % purity) were purchased from Santa Cruz Biotechnology (California, United States). ETHP (CAS registry No. 1462–93-7, 95 % purity) was provided by eNOVATION chemicals (New Jersey, United States). 4-ethylphenol (EP, CAS registry No. 123–07-9, 99 % purity), 4-ethylguaicol (EG, CAS registry No. 2785–89-9, 98 % purity), 4-vinylphenol (VP, CAS registry No. 2628–17-3, 10 % w/w in propylene glycol) and 4-vinylguaiacol (VG, CAS registry No. 7786–61-0, 98 % purity) were obtained from Sigma-Aldrich Chimie (St Quentin Fallavier, France). [$^2H_3$] 2-*iso*butyl-3-methoxypyrazine (IBMP-d_3, CAS registry No. 588732–63-2, 99.9 % purity) and [$^2H_{10}$] 4-ethylphenol (EP-d_{10}, CAS registry No. 352431–18-6, 99 % purity) which were used as internal standards were supplied by Cluzeau Info Labo (Sainte-Foy-La-Grande, France).

Sodium chloride (NaCl, 98 % purity) and sodium hydroxide pellets (NaOH, 99 % purity) were purchased from VWR chemicals (Fontenay-sous-Bois, France). Milli-Q quality water was obtained from a Milli-Q Reference system (Merck Millipore, Fontenay-sous-Bois, France).

2.2. Wines

The method for the analysis of mousy odorous compounds was optimized and validated using young commercial sauvignon blanc white wine, syrah rosé wine and cabernet sauvignon red wine. The white, rosé and red wine samples were checked prior to their use by means of calibration curves to ensure they contained very low traces of ETHP. APY and ATHP were not detected in any of these 3 wines. Once the analytical parameters were optimized, 15 white wines, 25 rosé wines and 34 red wines produced in France and Japan were analyzed for mousy odorous compounds as well as volatile phenols. These wines were purchased from local stores for "control wines" with customary addition of SO_2, or sponsored by wineries for wines produced without added sulfur dioxide or with limited addition of SO_2. The wines were classified into 'limited' and 'customary' based on indications about SO_2 use on the bottle label or from the producer. They were stored at 15 °C prior to analysis. Each wine was prepared and analyzed in triplicate for both analysis methods. A sensory screening was also performed by the

Table 1
Analytical data for each mousy off-flavor compound.

Compound	CAS No.	Molecular weight ($g.moL^{-1}$)	Log P[a]	Retention time (min)	Specific ions (m/z)
2-acetyl-1-pyrroline (APY)	85213–22-5	111.1	0.86	8.813	68/83/**111**
2-acetyl-2-pyrroline	1314926–27-6	111.1	0.05	–	–
2-acetyl-3,4,5,6-tetrahydropyridine (A-3,4,5,6-THP)	27300–27-2	125.2	1.30	13.667	82/83/97/**125**
2-acetyl-1,4,5,6-tetrahydropyridine (A-1,4,5,6-THP)	25343–57-1	125.2	0.50	17.323	82/83/97/**125**
2-ethyl-3,4,5,6-tetrahydropyridine (ETHP)	1462–93-7	111,2	1.71	10.132	96/**110**/111
2-ethyl-1,4,5,6-tetrahydropyridine	57634–69-2	111.2	–	–	–

[a]Reference: chemicalize.com.
Log P: decimal logarithm of octanol–water partition coefficient.
Quantifying ions are in bold.

experimenter just after the opening of the bottle at the time of preparation of the analytical samples, by means of a retronasal evaluation completed by a test using the "Palm and Sniff" method. In the "Palm & Sniff" technique, one drop of wine is placed on the back of the hand and then the skin is sniffed (Grbin et al., 1996, Peynaud et al., 1956). As skin has a higher pH than wine, it is speculated that this method increases the volatility of mousy off-flavor marker compounds. Forty-eight of the sponsored wines were suspected of mousiness.

2.3. Analysis of mousy odorous compounds

2.3.1. Sample preparation

The SBSE process was carried out using polydimethylsiloxane (PDMS)-coated stir bars (Twisters®) supplied by Gerstel (Mülheim an der Ruhr, Germany). The pH of wine samples was adjusted to 10.5 using an aqueous solution of NaOH (1 M). Ten milliliters of the basified wine sample were then transferred to a wide-base 20 mL-vial previously filled with 2 g of NaCl and vortexed. At that level, NaCl is completely soluble in wine, which prevents damage to the coating and the glass jacket of the stir bar. A volume of 100 µL of the internal standard solution (IBMP-d_3) at 91 $\mu g.L^{-1}$ in ethanol/water: 1/1 was added to each sample after complete dissolution of NaCl and, a 126 µL PDMS-coated stir bar (20 mm × 1.0 mm; length × film thickness) was dropped into the vial, which was subsequently capped with a PTFE-faced rubber stopper. The closed vials were stirred on a 15-position stirring plate for 1 h at 600 rpm and controlled room temperature (20 ± 2 °C). At the end of the extraction time, the stir bars were removed from the samples, quickly rinsed with Milli-Q quality water and dried with lint-free tissue. Each stir bar was then transferred into a glass tube for thermal desorption inside the GC injector.

2.3.2. SBSE-GC–MS instrumentation

Thermal desorption and injection were performed using a Twister thermal desorption unit (TDU) and a Gerstel CIS 4 cooled injection system with a programmable temperature vaporization (PTV) inlet, installed on an Agilent 6890 gas chromatograph combined with an Agilent 5975 Mass Selective Detector (Agilent Technologies, Massy, France), equipped with a Gerstel MPS 2 autosampler (Gerstel, Mülheim an der Ruhr, Germany). An HP-5MS fused-silica capillary column (30 m × 0.25 mm, 0.25 µm, film thickness, SGE, Courtaboeuf, France) was used, with helium as carrier gas (Messer France S.A.S, Suresnes, France) at a constant pressure of 70 kPa, corresponding to an initial flow of 1.3 $mL.min^{-1}$.

2.3.3. SBSE-GC–MS analytical conditions

Thermal desorption of the volatile aroma compounds retained on the PDMS stir bar coating took place in a helium flow at a rate of 50 $mL.min^{-1}$ in the TDU in splitless desorption mode, increasing the temperature from 40 to 280 °C at 60 $°C.min^{-1}$, and holding at the final temperature for 10 min. The TDU transfer line was maintained at 300 °C and the volatile aroma compounds were subjected to cryogenic focusing with CIS at − 100 °C using liquid nitrogen in an empty and straight glass liner, then transferred to the gas chromatograph at 720 $°C.min^{-1}$ from − 100 °C to 290 °C and held for 5 min. Injection was in splitless mode with a 50 $mL.min^{-1}$ helium purge flow at 1.5 min. The GC oven initial temperature was 40 °C, increasing to 80 °C at 3 $°C.min^{-1}$, then to 120 °C at 6 $°C.min^{-1}$, and, finally, to 240 °C at 10 $°C.min^{-1}$ with a final isotherm of 5 min. The solvent delay was of 3 min and the post run time to 40 °C was 8 min. Detection and quantitation were carried out in electron ionization (70 eV) in Selected Ion Monitoring (SIM) mode, based both on the retention time of each compound and the selection of specific ions, with at least three ions for each analyte: two or three for confirmation and one for quantification, on the basis of the best measured signal-to-noise ratio (See Table 1). The internal standard (IBMP-d_3) was quantified with ion m/z 127; qualifier ions were m/z 154 and m/z 95. The MS source was set at 230 °C, the quadrupole at 150 °C, and the transfer line between GC and MS at 280 °C.

Concerning ATHP, two tautomers were observed, 2-acetyl-3,4,5,6-tetrahydropyridine (A-3,4,5,6-THP) and 2-acetyl-1,4,5,6-tetrahydropyridine (A-1,4,5,6-THP). The concentration of each tautomer was calculated individually by arbitrarily considering a 1:1 ratio of both tautomers (Fig. S1, in supplementary material). For better accuracy, ATHP was also calculated by the sum of the peak areas of both tautomers.

2.4. Analysis of volatile phenols

Volatile phenols (4-ethylphenol, EP, 4-ethylguaicol, EG, 4-vinylphenol, VP, and 4-vinylguaiacol, VG) were quantified following a solid-phase microextraction (SPME) and a GC–MS analysis as described by Romano et al. (2008). Briefly, 10 mL of wine were placed in 20 mL vials containing 3.5 g of NaCl and 50 µL of a solution of deuterated 4-ethylphenol (EP-d_{10}) at 56 mg/L in ethanol/water: 1/1 were added as internal standard. Vials were sealed with a PTFE-lined cap (supplied by Chromoptic, Courtaboeuf, France) and vortexed before analysis by HS-SPME-GC–MS. Samples were prepared and analyzed in triplicates.

Analyses were performed with a HP 6890 N gas chromatograph (Agilent Technologies, Massy, France) equipped with an MPS2 autosampler (Gerstel, Mülheim an der Ruhr, Germany) for automated solid-phase microextraction (SPME) and coupled to a HP 5973 quadrupole mass spectrometer (Agilent Technologies, Massy, France). SPME was carried out with 85 µm polyacrylate fibers from Supelco (St Quentin Fallavier, France). Extraction took place with agitation at 35 °C for 50 min and was followed by desorption, in the injection port, at 250 °C for 5 min in splitless mode with a purge flow of 50 $mL.min^{-1}$ after 1 min. The fiber was then inserted for 12 min into the fiber conditioning station at 270 °C before next sample extraction. A HP-5MS fused-silica capillary column (30 m × 0.25 mm, 0.25 µm, film thickness, SGE, Courtaboeuf,

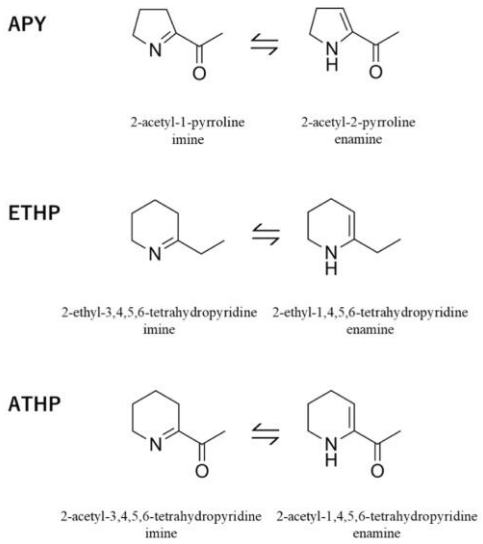

Fig. 1. Chemical structures of the odorous compounds analyzed.

France) was used, with helium as carrier gas (Messer France S.A.S, Suresnes, France) at a constant flow of 1.0 mL.min^{-1}. The initial GC oven temperature of 60 °C was maintained for 1 min, and was then increased to 125 °C at 3 °C.min^{-1}, then to 220 °C at 5 °C.min^{-1}, and, finally held at 220 °C for 10 min. A solvent delay of 10 min was applied. Detection and quantitation were carried out in electron ionization (70 eV) in SIM mode. The quantifier (underlined) and qualifier ions were 107 and 122 for EP, 113 and 131 for EP-d_{10}, 120, 91, and 119 for VP, 137 and 152 for EG, 150, 107, and 135 for VG. The MS source was set at 230 °C, the quadrupole at 150 °C, and the transfer line between GC and MS at 280 °C.

2.5. Data interpretation

All results were expressed as means of triple replicates. As the experimental design was unbalanced, the discussion of the data is based on descriptive analyses of the concentration levels and the diversity of the samples. Pearson correlation tests and Hierarchical Cluster Analysis (HCA) were performed using XLSTAT software version 2022.3.1 (Addinsoft, Paris, France). HCA was performed on mousy *N*-heterocycles to see a potential existence of wine clusters corresponding to each wine mousy taste composition. Euclidean distance and Ward's method were used on centered reduced data and a dendrogram based on dissimilarity was generated.

3. Results and discussion

3.1. Development of the quantification method

3.1.1. Identification of target compounds

Previous studies suggested the presence of two ATHP isomers (Fig. 1); 2-acetyl-1,4,5,6-tetrahydropyridine (A-1,4,5,6-THP) which is the enamine form and 2-acetyl-3,4,5,6-tetrahydropyridine (A-3,4,5,6-THP) which is the imine form (Strauss et al., 1984, Hayasaka, 2019). Firstly, we identified 2 major peaks for ATHP with the described GC conditions (section 2.3.3) with the MS in scan mode (*m/z* 50 to 300) when injecting after 1 h of SBSE a milli-Q water sample adjusted at pH 10 (1 M NaOH aqueous solution) of ATHP at 1 mg.L^{-1} and by extracting

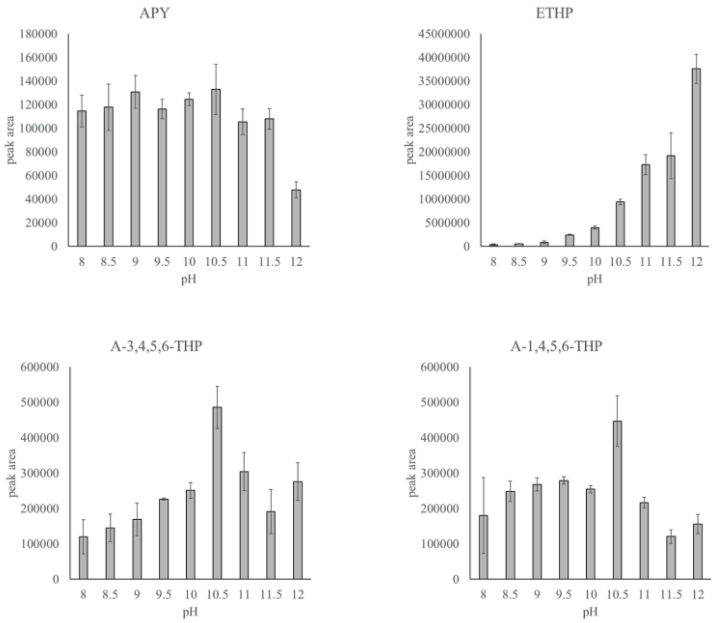

Fig. 2. Comparison of the peak area of *N*-heterocyclic compounds spiked in white wine between pH 8 and 12. Error bars represent ± standard deviation (n = 3).

selected specific ions (extracted ion chromatogram and mass spectra provided as Fig.S1 & Fig. S2 in supplementary material). The first peak at a retention time of 13.7 min was identified as 2-acetyl-3,4,5,6-tetrahydropyridine (A-3,4,5,6-THP) and the second peak at a retention time of 17.3 min was identified as 2-acetyl-1,4,5,6-tetrahydropyridine (A-1,4,5,6-THP) using the NIST database, which is also supported by the fact that the enamine form is less volatile than the imine form. The presence of two isomers was also taken into consideration for APY and ETHP. However, only a single peak for each of these compounds was observed with the described GC and MS conditions (section 2.3.3). The octanol/water partitioning coefficient (log P) of the enamine form of APY, 2-acetyl-2-pyrroline, is given as 0.05 (Table 1). This may explain that 2-acetyl-2-pyrroline is not extracted by SBSE because its polarity is too high for sorption on the apolar PDMS-coated twister. Moreover, the tautomeric equilibrium may be strongly shifted towards the imine form as stated by Adams & De Kimpe (2006). For ETHP, there have been no reports of chemical properties in 2-ethyl-1,4,5,6-tetrahydropyridine, but it can be hypothesized that the log P value of its enamine form is also lower than that in its imine form, as is the case for APY and ATHP, if ever both tautomers coexist.

3.1.2. SBSE optimization for wine sample

3.1.2.1. Effect of pH. SBSE is suitable for the extraction of apolar analytes. Good extraction yields, close to 100 %, are generally obtained for compounds with a log P higher than 2.5 (Baltussen et al., 1999) whereas for compounds with a lower log P the extraction yield is lower. As a result, strongly polar analytes such as ionic species are not extracted from the aqueous matrix. By properly adjusting the pH, weak acids and bases can be converted from their ionic form to their neutral (less polar) form, in which they can be extracted by the PDMS coating (Lancas, Queiroz, Grossi, & Olivares, 2009). It is well known that to completely shift the equilibrium in favor of one or the other species, 2 pH units above or below pKa are needed. As mentioned in the introduction, at pH below pKa, imines and enamines can be protonated due to the presence of a free doublet on the nitrogen atom. It is therefore important to shift the equilibrium towards the deprotonated forms by increasing the pH 2 units above pKa to favor the SBSE. Regarding the three *N*-heterocycles studied, no conforming data was found for pKa. Therefore, the influence of pH was examined between 8 and 12 by steps of 0.5 unit with spiked wines. Spiked concentrations of APY, ATHP, ETHP standards were respectively 71.3 μg.L^{-1}, 105.0 μg.L^{-1} and 303.0 μg.L^{-1}, and as described in the Material and Methods section, 2 g of NaCl were added in samples. Fig. 2 illustrates a comparison of peak areas of each compound for the different pH conditions. The peak area of ETHP increases substantially when the pH is higher than 10 and reaches its maximum at pH 12, indicating that the pKa of ETHP may be around 10. The fluctuation of the peak areas of APY and ATHP was smaller than that observed for ETHP. For ATHP, the largest peak area was obtained at pH 10.5, whereas a slight decrease was observed for APY at pH 11. On the other hand, the response for ETHP is much higher, as illustrated in the y-axis scale of Fig. 2. Moreover, APY and ATHP have a lower olfactory threshold than ETHP and thus have to be detected at lower levels. Therefore, to obtain the best sensitivity for APY and ATHP, pH 10.5 was selected.

3.1.2.2. Effect of salt addition. Previous studies have shown that NaCl addition increases the recovery of polar solutes with low log P values (e.g., log P < 2.5) (Prieto et al., 2010; Kawaguchi, Ito, Nakazawa, & Takatsu, 2013; Ochiai, Sasamoto, & Kishimoto, 2015). To optimize the SBSE conditions of *N*-heterocyclic compounds in wine matrices, the influence of the amount of NaCl was examined using spiked wines. The pH of the wine was adjusted to 10.5 with a 1 M aqueous solution of NaOH and spiked APY, ATHP and ETHP concentration standards were respectively 255.5 μg.L^{-1}, 630.0 μg.L^{-1} and 141.4 μg.L^{-1}. SBSE was performed for 1 h in triplicate on 0, 0.5, 1 and 2 g of NaCl. The addition of 2

Table 2
Performance parameters of the analytical method for white, rosé and red wine.

	White wine							Rosé wine							Red wine						
Compound	Linear range (μg.L^{-1})	r^2	slope	Fcalc[b]	LOD[a] (μg.L^{-1})	LOQ[a] (μg.L^{-1})	Recovery (n = 3) (%)	RSD[a] (n = 3) (%)	r^2	slope	Fcalc[b]	LOD[a] (μg.L^{-1})	LOQ[a] (μg.L^{-1})	Recovery (n = 3) (%)	RSD[a] (n = 3) (%)	r^2	slope	Fcalc[b]	LOD[a] (μg.L^{-1})	LOQ[a] (μg.L^{-1})	Recovery (n = 3) (%)	RSD[a] (n = 3) (%)
APY	3.6 – 71.3	0.9947	0.0008	0.0016	0.8	2.7	103	10.6	0.9924	0.0004	0.0007	0.9	3.1	88	11.0	0.9962	0.0007	0.0043	0.5	1.8	101	4.8
ATHP	5.3 – 105.0	0.997	0.0028	0.0009	–	–	104	6.8	0.998	0.0027	0.0006	–	–	100	2.5	0.9974	0.0031	0.001	–	–	93	3.4
A-3,4,5,6-THP		0.997	0.0028	0.0052	0.8	2.6	102	2.9	0.9689	0.0029	0.0332	0.9	3.2	110	3.7	0.9918	0.0033	0.0346	1.5	4.9	94	14.8
A-1,4,5,6-THP			0.9868	0.0029	0.0008	5.2	17.5	104	12.9	0.9868	0.0024	0.0067	5.0	16.8	89	5.4	0.9949	0.0029	0.001	10.7	36.0	112	5.1
ETHP	14.1 – 282.7		0.9979	0.0079	0.0016	0.6	2.0	96	5.1	0.9986	0.0049	0.0005	1.2	4.2	110	5.1	0.9996	0.0052	0.0004	2.7	8.9	103	5.3

[a] LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation.
[b] Ftab was 5.41 for all compounds (Lack-of-fit F-test).

g of NaCl was the maximum for rapid and complete dissolution in the wine matrix. Figure S3 (supplementary material) illustrates a comparison of peak areas of each compound as a function of the amount of NaCl. As expected, the responses for all *N*-heterocyclic compounds increased with the addition of NaCl, with the largest responses for 2 g of NaCl. Therefore, to obtain optimal sensitivity in the studied compounds, the addition of 2 g of NaCl per 10 mL sample was implemented.

3.1.2.3. Optimized SBSE conditions. The Twisters® with the maximum amount of PDMS coating (126 µL, 20 mm × 1.0 mm; length × film thickness) were chosen among the four sizes of the commercially available range, as it was previously shown that increasing the amount of coating increases extraction efficiency and thus the peak area of analytes with low log P (Franc et al., 2009). The procedure uses wine sample volumes of 10 mL as per the theory of SBSE, whereby increasing sample volume increases the phase ratio, β, resulting in a decrease in extraction efficiency (Baltussen et al., 1999). For analytes with low log P, this effect counteracts the fact that the absolute amount of compound in the vial is increased (Franc et al., 2009). Indeed, for an analyte with a log P of 1.5, the theoretical extraction efficiency, using a 126 µL PDMS Twister® and 10 mL of sample, is 28.5 % whereas for 20 mL of sample it is just 16.6 %. Therefore, for a sample containing 100 µg.L^{-1} of this analyte, the maximum extractable amount possible would be 0.28 µg from a 10 mL sample and 0.33 µg from a 20 mL, which is a negligible increase. Moreover, with larger sample volumes, diffusion and extraction kinetics are modified and the time required to reach equilibrium, i.e. the maximum extraction efficiency, is lengthened (David & Sandra, 2007). A volume of 10 mL of wine was thus extracted for 1 h.

3.2. Performance of the analytical method

Calibration samples of the three *N*-heterocyclic compounds were prepared using young commercial sauvignon blanc white wine, syrah rosé wine and cabernet sauvignon red wine. The cabernet sauvignon red wine contained ETHP at extremely low levels close to the limit of detection. The wines were spiked with the three *N*-heterocyclic compounds and IBMP-d_3 as the internal standard. The linearities of the calibration curves (five levels in duplicate) were confirmed between 3.6 and 71.3 µg.L^{-1} for APY, between 5.3 and 105 µg.L^{-1} for ATHP, and between 14.1 and 282.7 µg.L^{-1} for ETHP through a "lack of fit" test (Van Loco, Elskens, Croux, & Beernaert, 2002). Calculated F values turned out to be lower than F critical values for all the compounds (Table 2). Peak areas of A-1,4,5,6-THP and A-3,4,5,6-THP were added for calculation. Correlation coefficients (r^2) were above 0.994 for all three analytes in white, rosé and red wine (Table 2). The limits of detection (LODs) and the limits of quantification (LOQs) were defined as the concentration of each *N*-heterocyclic compound that gave signal-to-noise ratios (S/N) of 3:1 and 10:1, respectively, and were calculated for white, rosé and red wines spiked with 5.0 µg.L^{-1} of APY and ATHP, and 15.0 µg.L^{-1} of ETHP. LODs and LOQs for the three heterocyclic compounds are shown in Table 2. The recoveries (n = 3) of APY at a level of 15.0 µg.L^{-1}, ETHP at a level of 30.0 µg.L^{-1} and ATHP at a level of 15.0 µg.L^{-1} for white and rosé wines, and 30.0 µg.L^{-1} for red wine were between 96 and 104 % in white wine, 88–110 % in rosé and 93–112 % in red wine. Relative Standard Deviations (RSDs, n = 3) for intra-day precision were below 15 % which was considered as fully acceptable. Each parameter of A-1,4,5,6-THP and A-3,4,5,6-THP is given in Table 2. These analytical data were obtained by calculation under the assumption that the presence ratio of A-1,4,5,6-THP and A-3,4,5,6-THP at pH 10.5 was approximately 1:1 (Fig. S1 in supplementary material). For red wine, recoveries and intra-day precision were further assessed by means of the extraction of 10 replicates at two levels of spiking (spiking 2 being five times spiking 1). Data are available in table S1 in supplementary material. Recoveries ranged from 83 to 119 % and RSD of intra-day precision ranged from 18 to 26 %.

Table 3
Ranges and frequencies of presence (in brackets) of mousy analytes in the studied wines categorized according to SO_2 use and wine color.

Wine color	SO_2 category	APY (µg.L^{-1})	ETHP (µg.L^{-1})	ATHP (µg.L^{-1})
WHITE	CUSTOMARY*	nd** (0/2)	nd (0/2)	nd (0/2)
	LIMITED*	nd to 5.1 (2/13)	16.4 to 55.9 (13/13)	nd to 35.2 (7/13)
RED	CUSTOMARY	nd (0/4)	nd to < LOQ (3/4)	nd (0/4)
	LIMITED	nd to 7.7 (8/31)	14.0 to 120.5 (31/31)	nd to 54.5 (20/31)
ROSE	LIMITED	nd to 1.8 (1/24)	nd to 32.2 (23/24)	nd to 29.1 (12/24)

* CUSTOMARY: usual concentrations, usual use of sulfur dioxide during winemaking; LIMITED: limited or no use of sulfur dioxide during winemaking, final free SO_2 < 10 mg.L^{-1}.
**nd: not detected.

3.3. Analysis of wine samples

The developed method was applied to measure the concentration of the three *N*-heterocyclic compounds in 74 commercial wines. At the same time, 4-ethylphenol (EP), 4-ethylguaiacol (EG), 4-vinylphenol (VP) and 4-vinylguaiacol (VG) were quantified. The panel of 74 wines was composed of 68 items produced without added sulfur dioxide or with limited addition of SO_2, of which 48 were suspected of mousiness. The production of the other six wines involved the customary addition of SO_2 and are referred to as "control wines". The quantitative data are available in supplementary material, table S2. The classification into 'limited' and 'customary' addition of SO_2 is based on indications about SO_2 use on the bottle label or from the producer.

Initial investigation of the results shows that the main compounds present in wines were ETHP and ATHP. Table 3 shows the ranges and frequencies of presence of mousy odorous compounds. In "control wines", the concentrations of APY and ATHP were lower than LOD in all wine samples, while ETHP was detected below LOQ in some cases. Nevertheless, the concentration of ETHP in wine was below the previously reported olfactory detection threshold for all samples. ATHP was detected in 39 wines ranging from below LOQ to 54.5 µg.L^{-1}. ETHP was quantified in all wines except one among the 68 wines produced with the limited use of sulfur dioxide and ranged from 14.0 µg.L^{-1} to 120.5 µg.L^{-1}. By contrast, APY was only detected in 11 wines ranging from below LOQ to 7.7 µg.L^{-1} (Table S2, supplemental data).

The levels of mousy heterocycles were compared to those of volatile phenols. According to Romano et al. (2008), the production of ATHP could be linked to the presence of *B. bruxellensis*, which is also capable of producing volatile phenols in wines. The investigation was performed using correlation x/y projection on a 2D space using wines with the same color and compounds one by one. There was no correlation (Spearman's test was used after analysis of the data distribution by Shapiro-Wilks test) between the concentration of the mousy compounds and the concentration of the volatile phenols, so these investigations were taken no further.

A set of other tests (Spearman's test after analysis of the data distribution by Shapiro-Wilks test) were performed without significant results. For these samples, there is no link between the ETHP and ATHP concentrations. Similarly, there is no link between the accomplishment of malolactic fermentation (MLF) and the heterocycle levels even if it was already demonstrated that lactic acid bacteria were able to produce *N*-heterocyclic compounds in wines (Costello et al., 2002), nor between wine ages and heterocycle levels. Furthermore, there is no link between the color of the wines or the grape varieties and the presence of *N*-heterocyclic compounds.

Finally, the analysis of the results, with ascending hierarchical classification (AHC) of dissimilarities using Euclidean distances and

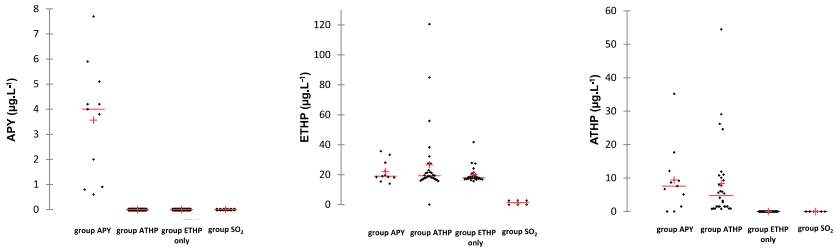

Fig. 3. Distribution of *N*-heterocyclic compound contents in the four clusters (Hierarchical Cluster Analysis). Each dot corresponds to a wine sample. The cross indicates the mean concentration of the compound in the corresponding group. The line indicates the median concentration of the compound in the corresponding group.

Ward's aggregation method, enables the proposal of 4 groups of wines (Fig. 3). The first group contains 6 wines without detectable quantities of APY and ATHP; 3 out of these 6 wines contain very weak quantities of ETHP (below LOQ). After analysis of the results, the groups were compared to the intrinsic data of the wines. All of the wines from the first group (group SO_2), were vinified with traditional levels of sulfur dioxide. These are the "control wines". All the wines produced involving the customary use of SO_2 were classified in this group by the AHC approach. The second group (group APY) contains 11 wines of different colors and origins but all of them contain detectable APY levels. There is no correlation between APY levels and other heterocycle levels. Nine wines in this group of 11 contain both ETHP and ATHP. The third group (group ETHP only) includes 27 wines containing only detectable ETHP and undetectable ATHP. The last group (group ATHP) contains 30 wines with detectable levels of ATHP.

To sum up, our initial results highlight several points. The first is that the customary use of SO_2 may limit the occurrence of mousy heterocycles. The second is that the presence of APY is associated with the presence of the two other heterocycles, ATHP and ETHP. The third is that suspect wines may contain APY or ATHP or even both but sometimes none of them (i.e. concentrations below LOD) while non-suspect wines generally contain neither APY nor ATHP but this needs further investigation. ETHP was present in suspect and non-suspect wines produced with a limited use of SO_2.

The main conclusion is the absence of correlation between the levels of heterocycles. The possibility of different origins in mousy deviations must be explored. As already mentioned, various compounds may be involved in this spoilage and several parameters such as their concentrations, the matrix effect, individual detection abilities, the composition of a subject saliva, and the pH of the tongue surface may impact the perception of this defect in wine leading to a variation in descriptors and a lack of consensus for detection, identification, and characterization of mousiness in wine.

4. Conclusion

A simple method was developed to determine concentrations of *N*-heterocyclic compounds of mousy off-flavor in wines using SBSE-GC–MS. This method allows the simultaneous quantification of three mousy off-flavors: 2-acetyl-1-pyrroline (APY), 2-acetyltetrahydropyridine (ATHP) and 2-ethyltetrahydropyridine (ETHP). As regards ATHP, two isomers were detected and measured. The method exhibits good performances as low LODs were obtained, recoveries ranged between 88 and 112 % and, RSDs were below 15 %. The analysis of wine samples, suspected of mousiness, generally revealed the presence of ATHP and / or APY, but this needs to be investigated further. Further studies to understand the character and mechanisms of mousiness are underway. This easy-to-use tool can now be deployed to quantify mousy compounds in a large set of wines. The influences of certain oenological parameters such as microbial spoilage, wine ages, wine composition, oxidation and antioxidants will be explored. This tool will become a key feature of "mousy odor spoilage" studies and could represent an important milestone in efforts to prevent aroma deviation.

CRediT authorship contribution statement

Daiki Kiyomichi: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **Céline Franc:** Conceptualization, Methodology, Writing – review & editing. **Pierre Moulis:** Investigation, Writing – review & editing. **Laurent Riquier:** Validation, Investigation. **Patricia Ballestra:** Conceptualization, Writing – review & editing. **Stéphanie Marchand:** Conceptualization, Formal analysis, Writing – review & editing. **Sophie Tempère:** Conceptualization, Writing – review & editing. **Gilles de Revel:** Conceptualization, Supervision, Project administration, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.135454.

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The method has also been developed for the quantification of AP proposed by Hayasaka as a compound responsible for mousiness. AP was extracted with the coated magnetic stir bars (Twister®) with a good result of performance. The linearity of the calibration curves was confirmed between 6.97 µg/L and 139.4 µg/L in three different wines ($r^2 = 0.999$). The limit of detection and of quantification (LOD, LOQ) were 1.72 µg/L and 5.74 µg/L respectively. However, it has not been found in any of the 74 wines studied. It was therefore decided to remove it from our assay method. Indeed, the presence of ions in the method can induce interferences and thus decrease the quality of separation of other compounds.

II.2. Quantification of the *N*-heterocycles in model culture media

An analytical method to quantify APY, ATHP, and ETHP in the *N*-heterocycle assay medium (*N*HAM) was developed in parallel with those previously described (*Cf*: II.1 Quantification of the *N*-heterocycles in wines by SBSE-GC-MS; p. 41).

II.2.1. Materials and Methods

II.2.1.1. Chemicals

The chemical compounds used were the same as for the quantification method developed in wine (*Cf*: II.1 Quantification of the *N*-heterocycles in wines by SBSE-GC-MS; p. 41).

Sodium hydroxide pellets (99 % purity) were purchased from VWR chemicals (Fontenay-sous-Bois, France), sodium carbonate (>99.5 % purity) was supplied by Fluka.

Milli-Q quality water was obtained from a Milli-Q 132 Reference system (Merck Millipore, Fontenay-sous-Bois, France).

II.2.1.2. Extraction of mousy compounds

The SBSE process was carried out using Twister® (polydimethylsiloxane (PDMS) coated stir bars (20 mm length x 1.0 mm film thickness) supplied by Gerstel (Mülheim an der Ruhr, Germany). A volume of 20 µL of the internal standard solution (IBMP-d_3) at 91 µg/L in ethanol/ water (1/1) was added to 10 mL of each *N*HAM samples. The pH was adjusted to 10.5 using sodium carbonate or NaOH (5 M). Ten millilitres of the basified sample were then transferred to a brown bottle (30 mL), a Twister® was dropped into the sample and the bottle was capped with a PTFE-faced rubber stopper. The closed bottles were stirred for 60 min at 900 rpm. The stir bar was taken out, rinsed, dried, and then transferred into a glass tube for thermal desorption.

II.2.1.3. SBSE-GC-MS instrumentation and analytical conditions

The SBSE-GC-MS instrumentation and analytical conditions were the same as for the quantification method developed in wine (*Cf*: II.1 Quantification of the *N*-heterocycles in wines by SBSE-GC-MS; p. 41).

II.2.2. Results and discussions

II.2.2.1. SBSE optimization for NHAM sample

To obtain the best sensitivity for APY, ATHP and ETHP, the influence of pH was examined between 8 and 12 by steps of 1 unit with spiked *N*HAM. The pH was adjusted with a NaOH solution (5 M). Figure 21 illustrates a comparison of peak areas of each compound for the different pH conditions. As in wine, for APY the largest peak area was obtained at pH 10, whereas a decrease was observed at pH 11. Samples were analysed only one time.

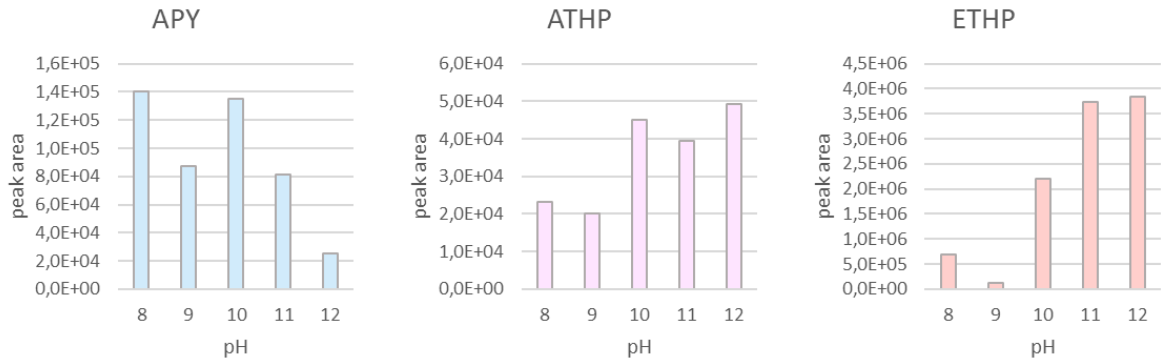

Figure 21 : Comparison of peak areas of N-*heterocycles compounds spiked in a* N*HAM between pH 8 and 12 basified with NaOH solution (5 M).*

II.2.2.2. Effect of sodium hydroxide vs sodium carbonate

To obtain the best repeatability, we compared two chemicals for alkalinization of the medium at 2 different pH. Samples were adjusted to pH 8 and pH 10.7 by adding NaOH or sodium carbonate. Each sample was analysed 2 times, we noted that the extraction of the 3 compounds was greater at pH 10.7 than at pH 8 regardless of the base used. The analysis showed less variability when sodium carbonate was used (Figure 22).

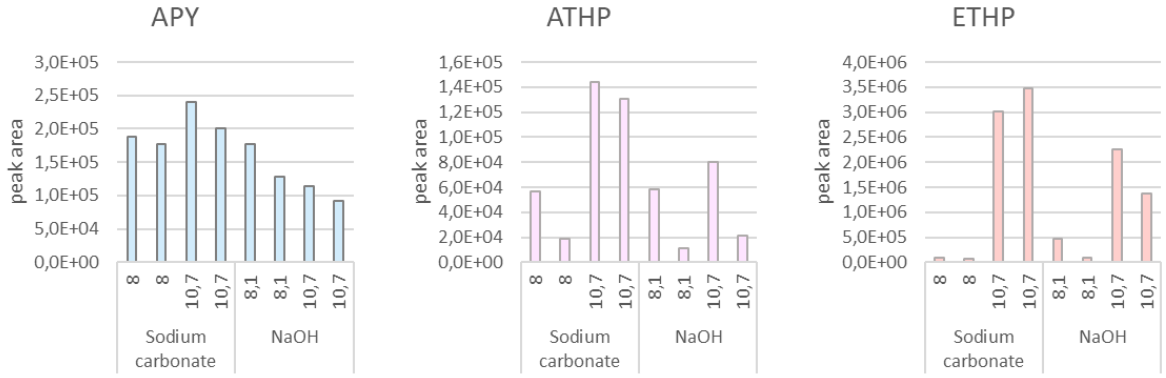

Figure 22 : Comparison of peak areas of N-*heterocycles compounds spiked in a* N*HAM alkalinized at pH 8 and 10.7 using NaOH or sodium carbonate.*

II.2.2.3. Performance parameters of the analytical method in the NHAM.

The performance parameters of the analytical method in the *N*HAM are presented in Table 4. Limits of detection and quantification are sufficiently low to detect and quantify all three compounds in our experimental samples. For intra-day precision, *N*-heterocycles spiking was chosen close to the LOQ for APY, but RSD is still fully acceptable, as it does not exceed 20 %. Recoveries range from 83 to 125 % are also acceptable for an experimental medium.

Table 4 : Performance parameters of the analytical method in NHAM.

			intra-day precision		
Compound	**LOD [a]**	**LOQ [a]**	**Level in µg/L**	**RSD[a] % n = 10**	**Recovery % n = 10**
APY	0.6	1.9	2.5	16.8	112
ATHP	4.4	14.6	26.4	12.6	125
ETHP	0.4	1.2	5.7	11.3	83

[a] LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation.

II.3. Study of tautomeric forms of ATHP potentially related to pH

In this part, we wanted to focus on the proportion of the two tautomeric forms and to compare our results with the hypothesis that the presence of the two tautomeric forms would be pH dependent.

As mentioned before, mousy compounds can show two tautomeric forms. In the assay proposed in this chapter, it was possible to analyse both isomers of ATHP. In this part, both forms have been integrated separately. The imine form, 2-acteyl-3,4,5,6-tetrahydropyridine, came out first with a retention time of 13,667 min, the enamine form, 2-acetyl-1,4,5,6-tetrahydropyridine, came out later at 17,323 min. Regarding APY and ETHP, only imine forms were found in wines, as Costello & Henschke (2002) had shown. Snowdon *et al.*, (2006) theorized that the presence of two tautomeric forms of ATHP could be pH-dependent, explaining why mousy taint is not detected when wine is smelled. The enamine form could be favoured in acidic pH (condition of wine) and the imine form would be present at oral pH. Due to their structure, the enamine form might be more polar than the imine form, which would explain the greater volatility and the perception of the defect only retro nasally (Snowdon *et al.*, 2006). LogP is a measure of differential solubility of chemical compounds in two solvents and is calculated by the logarithm of the partition coefficient between octanol and water. The lower the logP, the more the compound will end up in the aqueous phase and will be hydrophilic, polar. On the other hand, the higher the logP, the more the compound will be in the organic phase and will be non-polar. Since it is not possible to measure logP for all substances, various models have been developed. According to Chemicalize (http://chemicalize.com/) the imine form would have a higher logP (1.30) than the enamine form (0.50), so the imine form would be less polar and therefore more volatile as proposed by Snowdon

et al. (2006). When developing dosing methods (*Cf*: II.1 Quantification of the *N*-heterocycles in wines by SBSE-GC-MS; p. 41) the 2 tautomeric forms were integrated at pH ranging from 8 to 12. On the other hand, the pH balance of which Snowdon spoke would be at more acidic pH, from 3 pH of the wine to 8 pH of the maximum of oral pH (according to Larsen *et al.*, 1999) .

II.3.1. Materials and Methods

200 mL of crystalline® water were supplemented with 100 µL of a solution of ATHP (940 mg/L, in ethanol 100 %) and 400 µL of a solution of IBMP-d_3 (91 mg/L, in ethanol/water 50/50). The mixture was gradually acidified by the addition of tartaric acid. 20 mL were taken for analysis at different pH (7.72; 6.50; 5.11; 4.22; 3.17; 2.18; 1.53).

The parameters of SBSE extraction and GC-MS analysis developed in part II.1 Quantification of the *N*-heterocycles in wines by SBSE-GC-MS were used to qualitatively compare the response of the imine and enamine forms of ATHP to different pH.

II.3.2. 2-Acetyltetrahydropyridine tautomerism

Studies on the extractability of the two tautomeric forms as a function of pH indicated that at acidic pH (1.57, 2.18 and 3.17) neither of the two forms is detected. The representation of peak areas for enamine and imine forms of ATHP obtained for different pH (1.57, 2.18, 3.17, 4.22, 5.11, 6.50 and 7.72) showed proportionality between the two forms (independent of pH) (Figure 23). A correlation exists between both forms of ATHP, with a Spearman ρ = 0. 96. The presence of this correlation demonstrated that independent of pH, extraction, and response of the two tautomeric forms of ATHP are proportional. The equilibrium of these two forms would therefore not be dependent on pH contrary to the hypothesis of Snowdon *et al.* (2006). According to our results, the imine form of ATHP would have a response 1.3 times higher than the amine form. The proportion between the 2 compounds would therefore not be pH dependent. On the other hand, we cannot rule on the concentration of each tautomers.

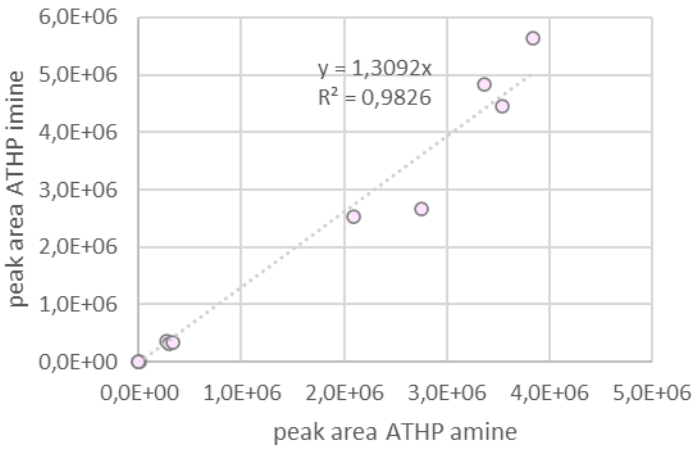

Figure 23 : Peak area of the imine form in function of enamine form of the ATHP in acidic condition (pH 1.57, 2.18, 3.17, 4.22, 5.11, 6.50, and 7.72), with a Spearman ρ = 0.96.

The presence of an *N*-heterocycle in the compound makes the compound acidifiable under certain conditions. Nitrogen is a proton-acceptor. The pK_a of these compounds have been not experimented and their theoretical pK_a differ according to the sources and calculation methods. According to Rayne & Forest (2016) pK_a associated with imine and enamine forms would be 7.75 and 5.31 respectively. Both values were estimated using the SPARC software programme (http://archemcalc.com/sparc/) with the full speciation option for ionizable functional groups, including nitrogen moieties acting as both acids and bases. Surprisingly, Chemicalize website (http://chemicalize.com/) indicates lower pK_a values, i.e., 1.69 and 3.60 (in July 2022) for imine and enamine forms respectively. However, a year earlier (in July 2021), the website gave values of 3.89 and 4.10. The model for calculating the pK_a of these compounds is therefore not completely reliable. Anyway, if the pH drops below their pK_a then the protonated form of the molecule will predominate, which implies a positive charge on the compound therefore an increase of polarity and a loss of volatility. In addition, the compound will no longer be extracted by the coated stir bar (Twister®) because the extraction efficiency of this latter is depending on logP of the compounds. This phenomenon may explain the absence of peak found at pH 1.57, 2.18, 3.17. For an efficient extraction, logP should be above 2. The increase in pH (beyond both pK_a) leads to a loss of that proton and a gain in the extractability of the *N*-heterocycles. At higher pH (4.22, 5.11, 6.50 and 7.72) both forms of ATHP were extracted and analysed by our method. This would mean that we find some deprotonated form above pH 4.22. In view of these results, the most likely pK_a would be close to those given by the Chemicalize website in July 2021.

The sample was allowed to stand for 15 min before the analysis of the compound. The balance between the different forms could be slower. A kinetic study to see the behaviour of the two tautomers over a longer period would be very interesting.

It would be interesting to do the same study using proton NMR, which would allow us to quantify the two isomers separately. The hydrogen involved in the tautomerism, according to Chemicalize website, would present different chemical shifts depending on its position (Figure 24), which makes it possible to quantify the two compounds separately.

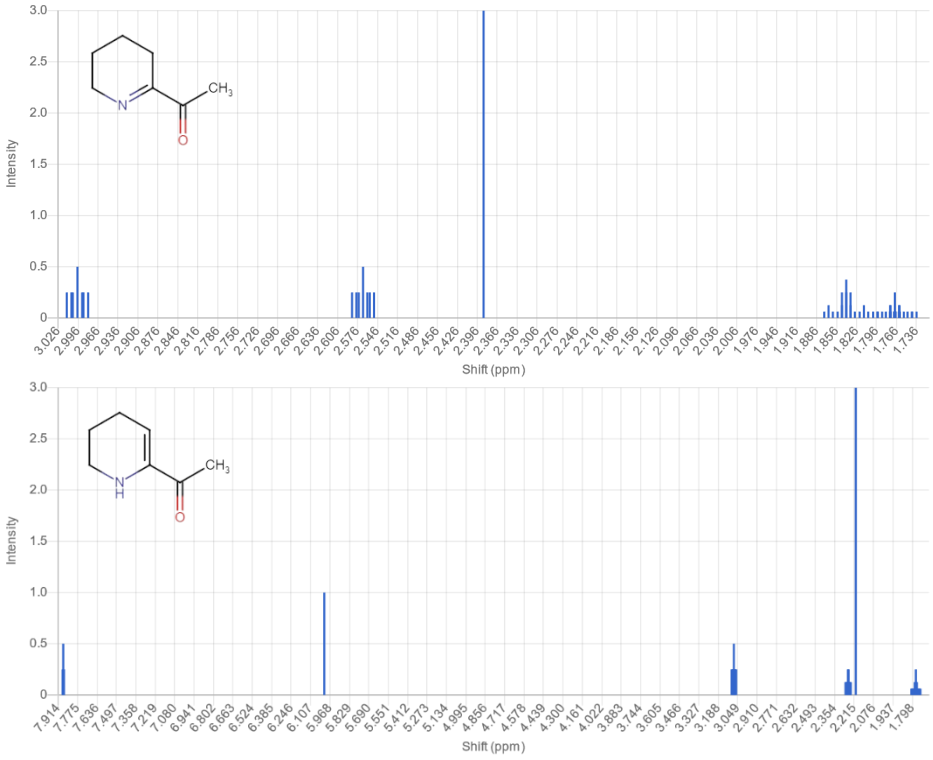

Figure 24 : H1 NMR of both ATHP tautomers plotted as signal intensity (vertical axis) vs. chemical shift (in ppm on the horizontal axis) according to Chemicalize website (http://chemicalize.com/).

II.4. Study of wine under oxidative conditions

A partnership in the UMR Œnologie was made and samples were provided by Dr. Alicia Jouin. As part of her study, Dr. Alicia Jouin is interested in the impact that oxygen additions in different quantities can have on wines without added sulphites. Merlot wines (2021) had experienced 3 oxygenation cycles, at each cycle the wine was supplemented with an oxygen concentration ranging from 0 to 36 mg/L (0, 2, 4, 8, 12, 16, 20, 26 and 36 mg/L), its consumption was then followed, once it was completely consumed, the cycle was then finished. Each sample had 3 oxygenation cycles, the maximum oxygen concentration a sample could have known was 108 mg/L. Samples were collected at the end of the 3 cycles.

The mousy compounds have been analysed 3 times in each sample collected, we did not have the opportunity to have a calibration for this experimentation in the wine at the time of these analyses. The results presented are therefore areas normalized by our internal standard. We then processed the results according to the maximum concentration found. The samples were also analysed by a panel of 4 tasters experienced with mousy wine.

At the tasting, the panel was consensual to say that not all wines had the alteration. Only wines with the addition of oxygen in concentrations 2, 4, 8 and 12 mg/L were associated with the defect. At higher

concentrations, only the event character was recognized in the wines. It was then difficult to know if the mousy off-aroma was no longer there or if it was masked by greater oxidation defects.

On chemical analysis, APY and ETHP were not detected. These compounds were not present in our samples or in concentrations below LOD. On the other hand, ATHP has been found in different concentrations. The relative evolution of the compound is presented in Figure 25. The presence of oxygen seems to greatly impact the production of the compound. The amount of ATHP increases with the concentration of O_2 up to 8 mg/L, then decreases until waiting for a zero at 36 mg/L of oxygen below. This would explain why wines with higher oxygen levels were not felt to be mousy. This decrease may be related to different phenomena, the production of the compound could be inhibited, other oxidation phenomena could take place. The compound itself could be oxidized in the form of AP. Unfortunately, we had not analysed AP, we could then have linked the theory of Kuenzler & Pour Nikfardjam (2013) about the oxidation from ATHP to AP to our samples.

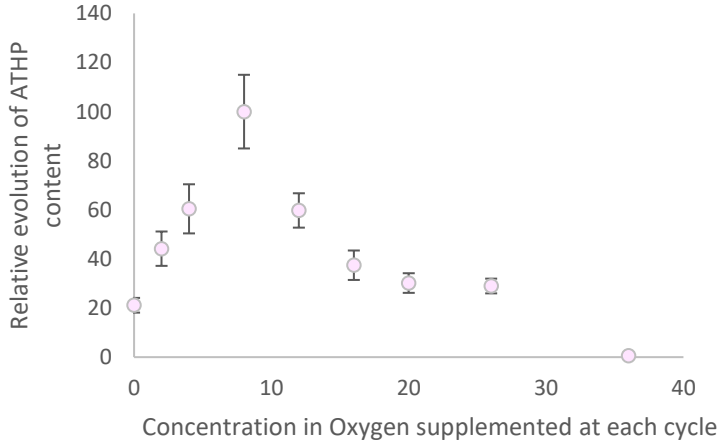

Figure 25 : Relative evolution of ATHP content in a wine in function of the added oxygen; the oxygen was added 3 times.

These samples were also monitored from a microbiological point of view by Microflora®. Although no alterative microorganisms were found, lactic acid bacteria such as *O. oeni* were still present in the environment. We cannot therefore rule on the origin of the production of ATHP. Whether chemical or microbiological, however, we can ensure that oxygen greatly impacts its production.

II.5. Conclusion

Simple methods were developed to determine concentrations of *N*-heterocyclic compounds of mousy off-aromas in wine and in *N*HAM using SBSE-GC-MS. These methods allow the simultaneous quantification of three mousy compounds: 2-acetyl-1-pyrroline (APY), 2-acetyltetrahydropyridine (ATHP) and 2-ethyltetrahydropyridine (ETHP). As regards ATHP, two tautomers were detected and measured. The proportion between these 2 isomers was looked at pH ranging from 1.57 to 7.72, this one

would not depend on the pH and the difference in volatility of these compounds between the wine and in the mouth would depend on the pH but related to their acid/base balance and not the tautomerism. A collaboration with Dr. Alicia Jouin was also able to shed light on the impact of oxygen and therefore redox potential on the production of mousy compounds. We cannot comment on its direct impact since microorganisms are present in wine.

Chapter III.

Who are the producers?

This chapter corresponds to research studies carried out in the laboratory of the UMR Œnology, University of Bordeaux, Bordeaux, France, and the laboratory of the Department of Microbiology and Biochemistry, Hochschule Geisenheim University, Geisenheim, Germany throughout the 3 years of the project. It is related to the first task of the thesis project and therefore presents the results related to the production of N-heterocycles of different microorganisms in model solution. The interaction between different microorganisms is also discussed.

III.1. Introduction

Today, *B. bruxellensis* yeast is presented as the main species responsible for mousy off-aroma in wines. And yet work has shown that some heterofermentative lactic acid bacteria can produce the alteration (Costello *et al.*, 2001), as well as acetic bacteria. But what are really the microorganisms found in mousy wines today and what are their capacity to produce *N*-heterocycles responsible for this spoilage? To answer this question, we looked at the microbial biodiversity of 25 mousy wines and the ability of their microbial isolates to produce the defect under standard conditions based on the precursors described in the literature. (Costello & Henschke, 2002; A. Romano *et al.*, 2008).

The screening was then extended to strains from different collections representing a large variability considering their genetic diversity (Avramova *et al.*, 2018a; Lorentzen & Lucas, 2019). This screening associated to the results of the preliminary screening enables to better describe the diversity of wine microorganisms producing mousy off-aromas.

III.2. Which microorganisms are bringing the mousy off-aromas in our wines?

The results of this part were written in the form of a scientific article entitled "Which microorganisms contribute to mousy off-aromas in our wines?". This article was submitted to the journal OENO One.

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Which microorganisms contribute to mousy off-flavour in our wines?

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Abstract

Nowadays, the frequency of occurrence of mousy off-flavour, in wines, increases. The causes could be the significant decrease in sulphur dioxide addition during processing, the increase of pH, or even the trend for spontaneous fermentation in wine. This off-flavour was associated with *Brettanomyces bruxellensis* or lactic acid bacteria metabolisms. Three *N*-heterocyclic compounds (APY, ETHP, ATHP) were described as involved in mousiness perception. So far, no study addressed the variability in that *N*-heterocycles production according to microorganism strains from different species. Twenty-five wines presenting mousy off-flavour were analysed. 252 bacteria with 90.5 % of *Oenococcus oeni* and 101 yeasts strains with 53.5 % of *Saccharomyces cerevisiae* were isolated and identified. Their capacity to produce mousy compounds was investigated using Stir Bar Sorptive Extraction-Gas Chromatography-Mass Spectrometry (SBSE-GC-MS) and a standardized *N*-heterocycle assay medium. While four and three species of yeast and bacteria respectively were isolated from mousy wines, only three species of microorganisms were associated to *N*-heterocycles production: *B. bruxellensis* , *Lentilactobacillus hilgardii* and *Oenococcus. oeni*. Screening was then extended to collection strains for these three species to improve their genetic representativity. Our results show that the levels and the ratios of the three *N*-heterocycles present huge variation according to the

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species. In addition, it has been shown that in most mousy wines, *B. bruxellensis* was not found. Finally, an interesting correlation between ATHP and ETHP was identified.

Mousiness, *Brettanomyces bruxellensis*, lactic acid bacteria, *Oenococcus oeni, Lentilactobacillus hilgardii*, Wine off-flavours

Introduction

Taints and off-flavours are one of the major concerns in the wine industry and even if the issues provoked by them are harmless, they can still have a negative impact on the quality or on the visual perception of the consumer (Ridgway *et al.*, 2010). The mousy taint was first reported in cider. It was described as "Peculiarly disagreeable flavour in wine, which is closely resembling to the smell of a residence of mice" (Thudichum, 1894). In the past, it was relatively easy to control it by protecting the wine from microbial spoilage with sulphur dioxide (SO_2) and high acidity (Bartowsky, 2009).

Nowadays, it is more common to detect wines with mousy off-flavour (Tempère *et al.*, 2019). Massini & Vuchot (2015) outlined that the significant decrease in sulphur dioxide, the increase in pH and the trend for spontaneous fermentations in wines could be the reasons behind this. Recently, Pelonnier-Magimel *et al.* (2020), studied the quality of 52 wines vinified without adding SO_2 and 20 wines with classical addition of SO_2: 70.6 % of the wines without SO_2 were described with an off-flavour and 6.2 % of the wines presented a mousy taint.

Mousy off-flavour

Mousiness is a consistent underlying problem for the wine industry (Fugelsang & Edwards, 2007; Grbin *et al.*, 1996). There are three identified *N*-heterocyclic compounds associated with mousiness, 2-ethyltetrahydropyridine (ETHP), 2-acetyltetrahydropyridine (ATHP) and 2-acetylpyrroline (APY) (Costello *et al.*, 2001; Herderich *et al.*, 1995). One particularity that turns this wine taint very unique is that it is difficult to smell it by ortho-nasal perception. The mousy compounds are not sufficiently volatile to be detectable at wine pH (Bartowsky & Henschke, 1995). However, when the wine comes into contact with saliva, the neutral pH of the mouth (Larsen *et al.*, 1999) permits a deprotonation of *N*-heterocycles and increases their volatility. The acid-base equilibrium allows the perception by retro-olfaction. Another specificity is the persistence of mousy compounds in the mouth : more than 10 minutes after swallowing or spitting the wine (Grbin *et al.*, 1996).

Production of mousy *N*-heterocycles in wines is attributed to *Brettanomyces* yeasts and lactic acid bacteria (LAB). However, no extensive investigation has been done to search for other species likely to produce mousy off-flavours in wines and, on another hand, microbial mechanisms leading to the

development of this alteration are still unclear. It has been suggested that the presence of the amino acids such as L-lysine and L-ornithine could be fundamental for the ring formation of ATHP and APY, respectively. Ethanol could also be essential for the formation of mousy compounds considering the acetyl chain of APY and ATHP. Other compounds such as ethanal, metal ions and oxygen may also play an important role in the production of mousy *N*-heterocycles (Costello & Henschke, 2002).

Brettanomyces bruxellensis

B. bruxellensis is the main species from the genus *Brettanomyces* described in wine. This yeast has a good resistance to the low pH and strains show some differences of sensitivity against the SO_2 antimicrobial activity. Nunes de Lima *et al.* (2021) noticed some differences of tolerance regarding the grape variety. It may be explained with the phenolic composition of wines. *B. bruxellensis* is really well known for its negative contribution in wine odor (Chatonnet et al., 1992; Harrouard et al., 2022). *B. bruxellensis* is able to produce volatile phenols, such as 4-ethylphenol and 4-ethylguaicol from cinnamic acid derivatives (Romano *et al.*, 2008). These compounds are undesirable and associated to the "Brett character" in wine, recognised as an off-flavour (Tempère *et al.*, 2014). They have a characteristic odour, variously described as stable, horse sweat, leather, and phenolic, as well as pharmaceutical (Tempère *et al.*, 2019).

B. bruxellensis have also been associated with other spoilage. They can produce a high amount of acetic acid (Peynaud & Domercq, 1956; Schanderl, 1951). They can also induce a turbidity in wine (Van Der Walt & Van Kerken, 1958, 1959; Van Zyl, 1962) and are sometimes associated with spoilage of other beverages such as soft drinks (Kolfschoten & Yarrow, 1970), beer (Rainbow, 1981; Smith *et al.*, 1981) and cider (Beech, 1958; Cabranes *et al.*, 1990; Tucknott, 1977).

Several strains of *B. anomalus and B. bruxellensis*, known to be associated with the spoilage of wine or other fermented beverages, have been shown to produce a mousy taint when fermenting grape juice or by contamination on finished wine (Grbin & Henschke, 2000; Heresztyn, 1986; Romano et al., 2008). Their ability to produce ATHP and ETHP has been confirmed using different chemically defined media containing among others ethanol and lysine (Grbin, 1998).

Lactic acid bacteria

The LAB are crucial in the vinification process of wines: some strains of *Oenococcus oeni* promote most of the time the progress of the malolactic fermentation (MLF) in red wines and part of white wines. They belong to the must and wine indigenous microbiota and develop spontaneously during or after the alcoholic fermentation (Lonvaud-Funel, 1999; Lonvaud-Funel *et al.*, 1991). Most of the

time, the MLF proceeds satisfactorily but harsh conditions (low temperatures, low pH, etc.), or a low population of native bacteria can cause late onsets of MLF or languid fermentations.

In order to facilitate the triggering of MLF it is possible to use malolactic starters (Kunkee *et al.*, 1964). These are selected for their technological properties and their adaptation to the environment, therefore tolerating difficult physicochemical conditions, as acidic pH, relatively low temperatures and the presence of ethanol (Torriani *et al.*, 2011). In that time, the ability to produce the mousy off-flavour is not verified before commercialisation.

The LAB are also known as mousy off-flavour producers. *L. hilgardii and Levilactobacillus brevis* known to be responsible for the "amertume" in wine (Ribéreau-Gayon *et al.*, 2006), were the first LAB species linked to mousy off-flavour (Heresztyn, 1986; Tucknott, 1977). These bacteria have been shown to produce large amounts of ATHP and smaller quantities of APY and ETHP when incubated in a synthetic medium (Costello & Henschke, 2002). *O. oeni*, the preferred bacterium for MLF, has been highlighted to produce APY, ATHP and ETHP (Costello, 1998; Costello *et al.*, 2001). Several *O. oeni* strains have also been found to be capable of producing strong mousy off-flavour during growth in an ethanolic grape juice medium (Romano *et al.*, 2008). Indeed, preliminary unpublished results showed that 13 strains of *O. oeni* out of 14 tested produced APY in a model medium under standardized conditions at concentrations above the threshold of detection.

The objective of the present work was to describe the variability of the production of mousy *N*-heterocycles by wine microorganisms considering their genetic diversity. Bacteria and yeasts isolated from a large collection of mousy wines were identified and screened for their capacity to produce mousy compounds under standard conditions. Screening was extended to collection strains belonging to species known as producers of mousy *N*-heterocycles.

Material and methods

1. Microbiology

1.1 Mousy wine sampling

Twenty-five wines were collected in the Rhône Valley and Beaujolais areas (France). All of them were produced without sulphite addition. The wines were tasted by a panel trained for the detection of defect in wines. All of them was qualified as "mousy wines".

1.2 Microbial strains

Microbial strains used for the study were isolated from mousy wines or were chosen among collection strains to represent the phylogenetic diversity of *B. bruxellensis* (Avramova, 2018), *O. oeni*

(Lorentzen & Lucas, 2019) or *L. hilgardii* species. They have been delivered by the Centre de Ressources Biologiques Oenologiques (CRBO, Bordeaux, France), the YJS collection (Laboratory for Molecular Genetics, Genomics and Microbiology, Strasbourg University, France), the AWRI collection (Australian Wine Research Institute, Adelaide, Australia) and the DSM collection (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). The fifty-two collection strains are listed in supplementary data (Table S2).

Initially stored at -80° C in glycerol 30 % (v/v), microbial strains were routinely maintained on grape juice agarose medium (5 g/L yeast extract, 250 mL/L of red grape juice, 1 mL/L of Tween80, 25 g/L agar, pH 4.8).

1.3 Isolation of microorganisms from mousy wines

Microorganisms were isolated from 25 mousy wines using 3 different media on Petri dishes. A specific YPG-based medium (10 g/L yeast extract, 10 g/L peptone, 20 g/L glucose and 25 g/L agar, pH adjusted to 4.8) named LT (supplemented with 0.15 g/L of biphenyl (Sigma Aldrich, Saint-Quentin Fallavier, France) and 0.1 g/L of chloramphenicol (Sigma Aldrich, Saint-Quentin Fallavier, France)) was used for the yeasts. Two specific red grape juice based media (5 g/L yeast extract, 250 mL/L of red grape juice, 1 mL/L of Tween80, 25 g/L agar, pH 4.8) named LAB (when supplemented with 0.1 g/L of pimaricine and put in anaerobic conditions using GasPak™ EZ sachets (Thermo Fisher 120 Scientific, Bordeaux, France)) and named AAB (when supplemented with 0.1 g/L of pimaricine and 0.0125 g/L of penicillin) were used to isolate lactic acid bacteria (LAB) and acetic acid bacteria (AAB) respectively. From each wine, 10 LAB, 5 AAB and 5 yeast clones have been collected when it was possible.

1.4 Identification of microbial isolates

Identification of isolates was done using a MALDI-TOF MS Biotyper (Bruker, Karlsruhe, Germany). A fresh colony was spotted onto an MSP 96 target polished steel BC and allowed to dry at room temperature. The spot was overlaid with 1 µL of a 70% formic acid aqueous solution and dried at room temperature. Each sample was then overlaid with a-cyano-4-hydroxycinnamic acid (HCCA) (1 µL) matrix (Bruker, Germany). MALDI-TOF MS analysis was performed on a Microflex™ LT/SH MALDI-MS System using Flex Control, MTB Compass (Version 3.1), and a MALDI-Biotyper™ application (Bruker Daltonics, Bremen, Germany), which allows the similarity of the mass profile of

an unknown microorganism to be calculated with the mass profiles in a database (Vallet-Courbin *et al.*, 2022).

1.5 PCR typing

PCR were performed to differentiate clones of *Saccharomyces cerevisiae* and of *O. oeni*.

The Whatman® FTA Clone Saver card technology (Cat. No. WB120028; Whatman, Sigma-Aldrich, France) has been used for the bacterial genomic DNA extraction from cultures. Regarding the strains of *Saccharomyces cerevisiae*, a colony was transplanted in 20 µL of ultra-pure filtered water in a microtube, heated for 15 min at 95° C and then frozen to extract DNA from the cells.

O. oeni clones were genotyped using the multi-locus variable number analysis of tandem repeat on 5 loci (MLVA) described previously (Claisse & Lonvaud-Funel, 2012, 2014). PCR products were compared by using a MultiNA system (Microchip Electrophoresis System for DNA/RNA Analysis, Shimadzu).

Saccharomyces cerevisiae strains were screened by inter-delta sequence analysis (Legras & Karst, 2003) combined with the MultiNA system to evaluate genetic diversity and to determine their clonal relationships.

When the strains came from the same sample and had similar PCR profiles then the isolates were assimilated to clones.

1.6 Cell preculture

The microorganisms were removed from the Petri dish and inoculated in 10 mL of grape juice medium (250 mL/L commercial grape juice, 5 g/L yeast extract, pH 4.8) inside of a 15 mL tube during 3 days for *B. bruxellensis* and 5 days for the LAB at 25°C. After this period, they were again inoculated (2% v/v) in 50 mL tubes containing 45 mL for the LAB and 40 mL for the yeast of grape juice medium.

1.7 Screening of microbial strains for the ability to produce mousy *N*-heterocycles in synthetic media

The microbial preculture was centrifuged (11600 g for 9 min at 4 °C), the cell pellet was washed with sterile phosphate-KCl buffer (KH_2PO_4 5.5 g/L, KCl 4.25 g/L, pH 4.5). For *B. bruxellensis*, the population was measured by flow cytometry (Cytoflex, Beckman Coulter Inc., Brea, California USA). The equivalent volume to 1.10^9cells was then centrifuged and washed a second time. The cell pellet was resuspended in 22 mL of an *N*-Heterocycle Assay Medium (*N*HAM) (Table 1) (prepared

182 in 3 solutions: an amino acid, an ethanal and a *N*HAM base; these solutions were sterilized by
183 filtration through 0.22 µm membranes).

184 *Table 1 : Composition of 1L for* N-*Heterocycles Assay Medium(*N*HAM) (Romano, 2008).*

Component	For 1 L
Ammonium citrate tribasic	2 g
Calcium chloride	1 g
Citric acid	2 g
D-Fructose	50 g
Ethanal	100 mg
Ethanol (96%)	52 mL
Iron sulphate	43 mg
L-Lysine	5 g
L-Ornithine	5 g
Magnesium sulphate	12.5 mg
Malic acid	5 g
Manganese sulphate	25 mg
Potassium chloride	4.25 g
Potassium phosphate monobasic	5.5 g
Tween 80	1 mL
pH	4.5

185

186 The LAB population was standardized using optical density (λ=650 nm) indice (OD=1) in the *N*HAM
187 after the two rinses with sterile phosphate-KCl buffer. Cell cultures were incubated at 25 °C for 24 h
188 and reactions were performed on a 22 mL volume in 50 mL screw capped tubes.

189 **2. Chemical analysis**

190 The analysis of the *N*-heterocycles (APY, ATHP, ETHP) was adapted and optimized in *N*HAM in
191 parallel with the method proposed for wine by Kiyomichi *et al.* (2023). The performance parameters
192 of the analytical method in the *N*HAM are presented in Table 2.

193 *Table 2 : Performance parameters of the analytical method in the* N*HAM.*

Compound	LOD [a]	LOQ [a]	intra-day precision		Recovery % n = 10
			level in µg/L	RSD[a] % n = 10	
APY	0.6	1.9	2.5	16.8	112
ATHP	4.4	14.6	26.4	12.6	125
ETHP	0.4	1.2	5.7	11.3	83

[a] LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation.

2.1 Sample preparation

Stir bar sorptive extraction (SBSE) was used in the following way. To increase the pH to 10.5 and thus the extractability of the compounds, sodium carbonate (0.7 g) was poured into a 30 mL brown glass vial and 10 mL of the supernatant of the cell culture in the synthetic media was added. 20 µL of the deuterated internal standard were spiked (2-isobutyl-3-methoxypyrazine-d_3; IBMP-d_3 at 91 µg/L in ethanol/water 1/1, CAS registry No. 588732-63-2, 99.9 % purity, supplied by cluzeau info labo (Sainte-Foy-La-Grande, France)). A stir bar coated with polydimethylsiloxane (PDMS; Twister™, dimensions: length: 20 mm, film thickness: 1.0 mm, Gerstel, Mülheim an der Ruhr, Germany) was dropped into the sample. The vial was capped with a PTFE-faced rubber stopper and the closed vials were stirred for 60 min at 900 rpm. After extraction the Twister was taken out and then rinsed with demineralised water, wiped with a lint-free tissue and put into a desorption tube.

2.2 Gas chromatography-mass spectrometry with selected ion monitoring (GC-MS-SIM)

The loaded Twister in the desorption tube was thermodesorbed into the thermodesorption unit (TDU, Gerstel, Germany) operating in splitless mode (initial temperature 40 °C, rate 60 °C/min to 280 °C, held for 10 min) with simultaneous cryofocusing with a Cooled Injection System (CIS 4, Gerstel, Germany) in an empty and straight glass liner at -100 °C using liquid nitrogen. The desorbed analytes were then transferred (initial temperature -100 °C, rate 12 °C/s to 280 °C, held for 5 min) to a HP-5MS fused silica capillary column (30 m × 0.25 mm, 0.25 µm film thickness, Agilent Technologies, Les Ulis, France) in the gas chromatograph (Agilent 6890 Agilent Technologies). Helium was used as carrier gas at constant flow rate (1.1 mL/min). The GC temperature was programmed from 40 ºC to 80 ºC at a rate of 3 ºC/min, then up to 150 ºC at a rate of 6 ºC/min and finally up to 240 ºC (held for 5 min) at a rate of 10 ºC/min.

An Agilent 5975 mass selective detector (Agilent Technologies, Les Ulis, France) operating in electron ionization (70 eV) was used for detection (source temperature: 23 °C, quadrupole temperature: 150 °C and transfer line between GC and MS at 280 °C) in selected ion monitoring mode (SIM) using the following m/z ions (quantifier in bold): APY: 68/83/**111**; ATHP: 82/83/97/**125**; ETHP: 96/**110**/111; IBMP-d_3: 95/**127**/154.

3. Statistical analysis

Kruskal-Wallis statistical test (agricolae package, R, p value < 0.05), Anova 1-way (p-value <0.05), Student *t*-test (p-value <0.05), Spearman test (p-value <0.05) were performed using R and R-packages agricolae (Mendiburu, 2021), ade4 (Dray & Dufour, 2023), ggplot2 (Wickham *et al.*, 2023).

Results and Discussion

Identification and screening of indigenous mousy wine strains. Considering the 25 mousy wines, a large collection of 353 isolates have been collected (results of enumeration are presented in the supplementary data, Table S1). Concerning yeasts, 4 different species, i.e. *B. bruxellensis*, *Pichia manshurica*, *Priceomyces carsonii* and *Saccharomyces cerevisiae* were identified among the 101 isolates. Concerning LAB, 3 different species, i.e. *O. oeni*, *L. hilgardii* and *Pediococcus parvulus* were identified among the 252 isolates. The proportions of each species are presented in Figure 1 A (for yeasts), and Figure 1 B (for LAB). Moreover, 38 AAB have been isolated but these microorganisms could not be identified using the MALDI-TOF MS. Finding various microorganisms in such spoiled wine samples was expected. SO_2 is known to be a potent antimicrobial, so in absence or at low levels, microorganisms can grow more freely in wines.

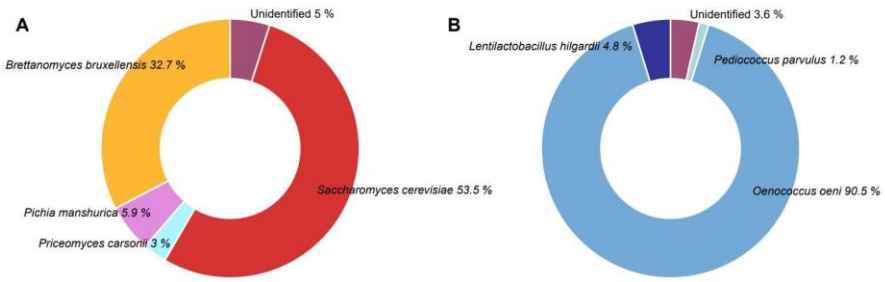


*Figure 1 : Frequency of species of yeast (**A**) and of lactic acid bacteria (LAB) (**B**) in all the isolates from twenty-five mousy wines.*

The most prevalent species (Table 3) were *Oenococcus oeni* (90.5% of LAB) and *Saccharomyces cerevisiae* (53.6% of yeasts). This is not surprising in a finished wine; these microorganisms have developed during fermentations. No information was available concerning the filtration of wines before bootling. One of our samples did not present any microorganisms, we can assume that it was filtered for sterilization before bottling. The second most isolated yeast in these samples was *Brettanomyces bruxellensis* (32.7% of yeasts). The low level of SO_2 may explain the presence of this spoilage microorganism. On the other hand, it is difficult identify the origin of the contamination, indeed it is possible to find *B. bruxellensis* in the cellars in bioadhered forms, but also in winemaking environment (Connell *et al*., 2002; Fugelsang & Edwards, 2007; Le Montagner *et al.*, 2023; Lebleux *et al.*, 2020). Finally, it should be noted that some species are uncommon and were found only in few samples. *Lentilactobacillus hilgardii*, *Pediococcus parvulus*, *Priceomyces carsonii* and *Pichia manshurica* were only found in about 10% of the wines studied.

Out of the 228 *O. oeni isolates*, 25 different genetic profiles were discriminated against by VNTR PCR and 16 different profiles by Delta PCR on the 54 isolates of *S. cerevisiae* identified.

Table 3 : Prevalence of isolated species in 25 mousy wines.

		Wine samples	Prevalence
LAB		23	92 %
	Oenococcus oeni	21	84 %
	Lentilactobacillus hilgardii	3	12 %
	Pediococcus parvulus	2	8 %
AAB		8	32 %
Yeast		16	64 %
	Saccharomyces cerevisiae	12	48 %
	Brettanomyces bruxellensis	5	20 %
	Priceomyces carsonii	3	12 %
	Pichia manshurica	2	8 %
Without any microorganisms		1	4 %
Total		25	

When more than two clones had similar PCR products and came from the same sample then only two clones of this sample were tested for their ability to produce mousiness in the *N*HAM. The other ones were assimilated as the same strain.

Concerning AAB, that were present in 32 % of the spoiled wines, their capacity to produce mousy off-flavour in a model medium was also tested. None of them was able to produce the mousy compounds. However, the screening method may not be suitable for such bacteria. AAB were forming clusters of cells it was difficult to know exactly how many microorganisms were inoculated into the environment. That is why these microorganisms are not studied.

Figure 2 presents the number of yeast and LAB clones tested per species. Strains identified as able to produce at least one of the 3 compounds responsible for the mousiness in *N*HAM are represented in solid color, while clones not presenting any of the three mousy compounds in the medium after 24 hours are hatched. All the isolates of *O. oeni*, *L. hilgardii* and *B. bruxellensis* screened in this media produced mousy compounds, confirming previous studies (Snowdon *et al.*, 2006). Isolates tested in the same media, from other species, *P. parvulus*, *P. manshurica*, *P. carsonii* and *Saccharomyces cerevisiae*, were not able to produce the mousy off-flavour. Concerning these species they are most of the time known as undesirable yeast or bacteria and they are often identified in spoiled wines, in the presence of insufficient SO_2, and at pH values above 3.5 (Cordero-Bueso *et al.*, 2013; Jackson, 2008; Saez *et al.*, 2011; Wade *et al.*, 2019). Our results did not allow to identify new species producing the alteration, but they confirm the ability of *O. oeni*, *L. hilgardii* and *B. bruxellensis* to

produce the mousiness in various proportions depending of the strain. Although *Saccharomyces cerevisiae* was present in more than half of the wine samples, no clones were producers.

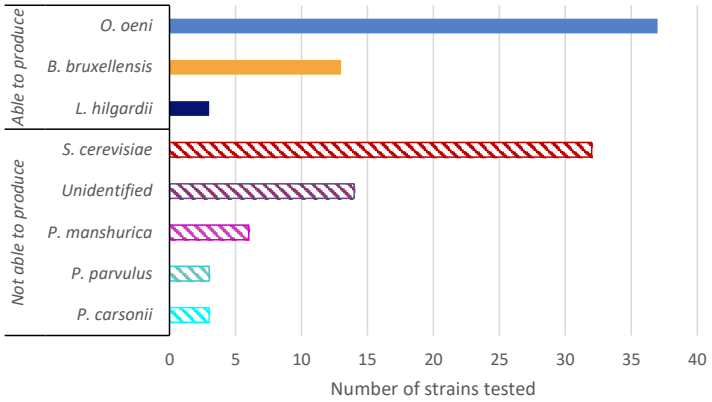


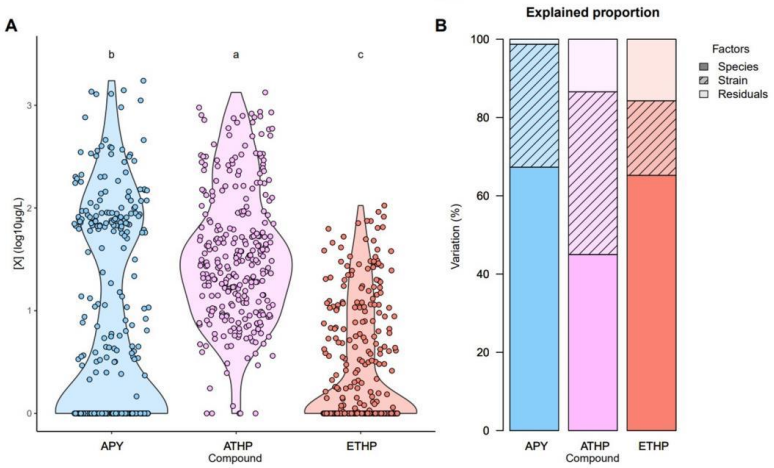
Figure 2 : Ability of production of at least one mousy compound in a NHAM for all the screened strains depending on their species.

The fact to study only strains isolated from mousy wine may influence the representativeness of the diversity of *Brettanomyces bruxellensis*, *Lentilactobacillus hilgardii* and *Oenococcus oeni* strains. To overcome this and investigate more intensely the different species the screening has therefore been extended to different collection strains on 22 *Brettanomyces bruxellensis*, 20 *Oenococcus oeni* and 10 *Lentilactobacillus hilgardii*.

Screening of the collection strains. Production of mousy *N*-heterocycles was characterized in microbiological triplicates with duplicates of analysis. Six quantifications were therefore carried out for each strain. Figure 3 A shows the concentrations obtained. First, a great variability can be noticed in the production of the different compounds. For the sake of scale, the results are presented on a logarithmic scale. ATHP and APY are produced at significantly different concentrations (from 1µg/L to 1mg/L), but they are in the same order of magnitude and can rise to more than 1 mg/L while ETHP is produced at significantly lower concentrations. Whatever the strain studied, this latter was never produced at more than 100 µg/L. In addition, all concentrations obtained below the limits of quantification are represented by a zero value. It can be noticed that for APY and ETHP a high proportion of individuals did not produce these compounds, while all microorganisms could produce ATHP. The variability is mainly due to the species (Figure 3 B, Table 4) followed by the strain factors. The concentrations of APY produced are explained for more than 67% by the species studied and almost for 31% by the strains. Therefore, almost 100% of its variation is explained while the explained

296 variances of ATHP and ETHP are below 90% leaving a share of residual variation unexplained.
297 Pushing the analysis further, no variability can be explained in *B. bruxellensis* by genetic groups nor
298 in *O. oeni* by phylogenetic groups. Therefore, these problematics are not dealt with here.

299



300

301 *Figure 3 : (**A**) Concentration of log10 µg/L produced by 52 different microorganisms in an NHAM medium (all concentrations below*
302 *the limit of quantification replaced by 0); and (B) variability explained (with a Pvalue < 0.05) according to species and strain.*

303

304 *Table 4 : Summary table of the analysis of variance (ANOVA) of APY, ATHP and ETHP.*

		APY		ATHP		ETHP	
	Df[a]	Sum Sq[a]	P-value	Sum Sq[a]	P-value	Sum Sq[a]	P-value
Species	2	94.972	$<2.2 \times 10^{-16}$	47.109	$<2.2 \times 10^{-16}$	24.7334	$<2.2 \times 10^{-16}$
Strain	49	44.411	$<2.2 \times 10^{-16}$	13.790	1.762×10^{-4}	22.8072	5.73×10^{-14}
Residuals	95	1.773		11.337		7.3955	

[a] : Df, Degrees of freedom; Sum Sq, sum of squares

305

306 Figure 4 shows the concentrations measured according to the three species tested in the form of Violin
307 plots. The impact of the species on the production of the three compounds is easily noticeable here.
308 As Grbin (1998) highlighted, the *Brettanomcyces bruxellensis* tested were not able to produce APY
309 in this media but they were able to produce ATHP and ETHP with significantly higher levels than
310 the LAB. The variability of intraspecies production remains particularly important and regardless of
311 the compound studied for *B. bruxellensis* and *O. oeni*. On the other hand, *L. hilgardii* seem to produce
312 amounts quite similar between strains.

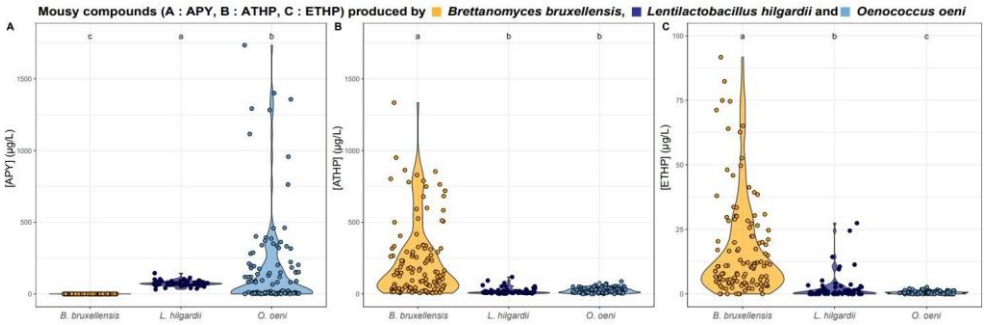


*Figure 4 : Mousy compounds (**A**: APY, **B**: ATHP, **C**: ETHP) in µg/L produced by* Brettanomyces bruxellensis, Lentilactobacillus hilgardii *and* Oenococcus oeni *in a NHAM.*

By representing the production of each microorganism (Figure 5) we noticed that the different strains have a lack of repeatability for producing the mousy compounds. Although they are observed under the same conditions and at theoretical identical population levels for *B. bruxellensis* (mesured using flow cytometry) and similar population levels for LAB (OD_{650}=1), there appears to be considerable experimental variability. The production of these microorganisms may depend on their physiological state at the time of inoculation into *N*HAM. Finally, all the strains studied were likely to produce mousiness under these conditions. Despite this variability, some strains of *B. bruxellensis* and *O. oeni* seem to be able to produce more compounds than others. Psu1 and IOEB-SARCO 1491 were able to produce APY in larger quantities than the other strains. As mentioned above (Figure 3**B**), with associated statistical testing, variations between strains could not be explained by phylogenetic groups. If we look at the strains of *O. oeni*, PSU 1 and CRBO S14, while both are belonging to phylogenetic group A (Lorentzen & Lucas, 2019; Table S2), PSU 1 produces APY at concentrations one hundred times higher than CRBO S14. Similarly, in *B. bruxellensis*, the 2 strains producing the most ATHP, YJS 5319 and YJS 5334, come from two different genetic groups (Avramova, 2018; Table S2). The production of the 3 compounds from the different genetic groups are shown in Figure S1.

On the other hand, the variability of production between tested strains of *L. hilgardii* is low and their production of APY has no significant difference between strains. In addition, their production, and especially DSM 20176, is not comparable to the results presented by Costello & Henschke in 2002. Indeed, his population density was much higher than here. Costello worked with an optical density (650 nm) of 10 whereas we used an optical density of 1.

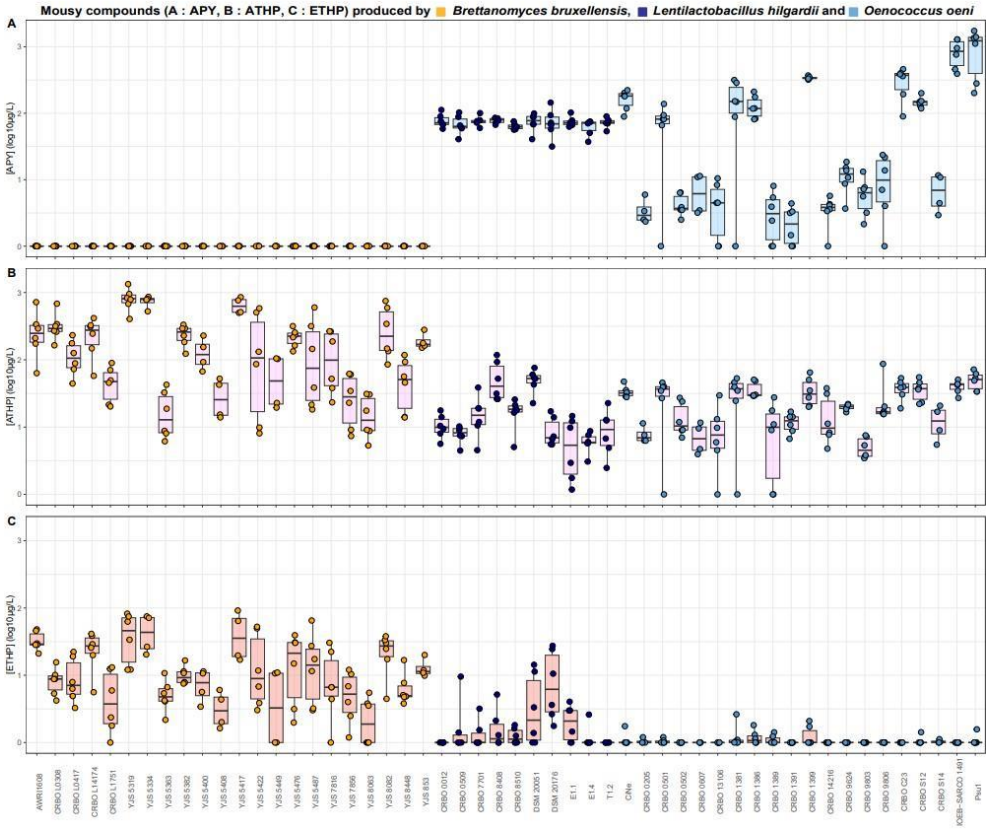


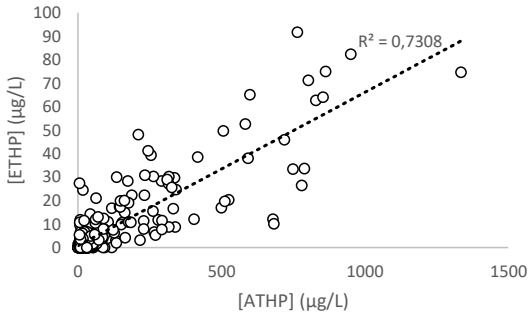
Figure 5 : Mousy compound (A: APY, B: ATHP, C: ETHP) produced by 22 strains of Brettanomyces bruxellensis *(in orange), 10 strains of* Lentilactobacillus hilgardii *(in dark blue) and 20 strains of* Oenococcus oeni *(in sky blue) in 24 h in NHAM expressed in log10 µg/L.*

Recall that the synthetic medium used is a medium extremely far from wine with concentrations of 5 g/L in lysine and ornithine. It is therefore important to note that the concentrations measured for each compound are higher than the concentrations found in wines (Kiyomichi *et al.*, 2023)

It remains interesting to note that here, the proportion of the different compounds produced were in favor of the two acetylated compounds even if the two compounds are produced by *B. bruxellensis* concerning ATHP and by *O. oeni* concerning APY. A correlation even could exist between ETHP and ATHP, with a Spearman $\rho = 0.64$ (Figure 6). This correlation is more obvious true for *B. bruxellensis* strains, where a 1:10 ratio emerges between ETHP and ATHP. The amounts produced for ATHP from *O. oeni* are too low to permit the investigation of this kind of ratio. The concentrations of ETHP that would then be obtained with a 1:10 ratio ([ETHP]/[ATHP]) would be below the limits

of quantification. However, if the ratios were the same between the different species one could propose the idea that the ETHP/ATHP balance is not governed by microorganisms but by chemistry and could depend on the medium and/or the RedOx potential (Pour Nikfardjam & Kunz, 2021).

Figure 6 : Representation of the concentration of ETHP as a function of the concentration of ATHP, with a Spearman ρ = 0.64.



Our results show the microbial diversity in mousy wines, but among all the isolated microorganisms only *B. bruxellensis*, *L. hilgardii* and *O. oeni* were able to produce the mousiness in *N*HAM medium. This medium is containing all the precursors known to be implicated in the development of mousy off-flavour. *B. bruxellensis* has always been blamed by winemakers as being responsible for the appearance of the alteration. The fact that this yeast species was found in only 20 % of the wines studied and was not isolated from 80 % of the mousy wines is a novelty and shows that the alteration is mostly produced by other microorganisms or produced chemically.

Extending the research to collection strains, all strains of *B. bruxellensis*, *O. oeni* and *L. hilgardii* can produce at least 2 of the mousy compounds. On the other hand, no link could be shown either between the genetic material of these strains or the phylogenetic groups already pre-existing (Avramova, 2018; Lorentzen & Lucas, 2019). Large variations persist in strain production, although the conditions and the number of cells inoculated into *N*HAM media are controlled. It could be that other microbial factors impact production, such as the physiological state of microorganisms at the time of the screening.

Expanding the number of strains tested could provide new elements of response and could confirm or refute hypothesis regarding the ratio found between ETHP and ATHP. In addition, the strains of collections tested were randomly selected to have different genetic material, an extension would allow to have more data to compare and potentially allow to identify genes that could be associated with production.

Furthermore, this work focused on the ability of species found in finished wines to produce mousiness. Other microorganisms could have an impact on the production of mousiness upstream. Moreover, all strains of *B. bruxellensis*, *L. hilgardii* and *O. oeni* were able to produce in our conditions of culture. They were screened only in a synthetic medium, the *N*HAM, which is far from the wine composition. It would be interesting to compare these strains and their production in a wine matrix and/or during the whole wine making process. In addition,the capacity of each strain was tested in monoculture, we can very well imagine potential interactions between different strains whether of the same species or different species.

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553 *Table S1 : Enumeration of the 25 studied wines.*

				Enumeration (Cells/mL)		
	Color	**Grape variety**	**Millesime**	**Yeasts**	**AAB**	**LAB**
O wine 1	Orange	ND	ND	<10	130	2.56E+05
R wine 1	Red	ND	ND	<10	<10	1.76E+05
R wine 2	Red	ND	2013	30	2.20E+04	6.00E+05
R wine 3	Red	Gamay	2014	<10	<10	2.00E+03
R wine 4	Red	ND	2012	1.70E+05	<10	ND
R wine 5	Red	Grenache + Syrah	2015	<10	<10	<10
R wine 6	Red	Pinot noir	2015	5.00E+03	<10	10
R wine 7	Red	Cabernet	2015	1.70E+02	4.30E+04	3.88E+05
R wine 8	Red	Gamay	ND	50	<10	6.10E+04
R wine 9	Red	Gamay	ND	10	<10	1.45E+05
R wine 10	Red	Gamay	2015	8.00E+03	ND	3.60E+06
R wine 11	Red	Gamay	2015	30	ND	2.12E+05
R wine 12	Red	Gamay	2016	220	<10	3.16E+07
R wine 13	Red	Gamay	2015	9.50E+04	30	180
R wine 14	Red	Gamay	2016	10	<10	3.96E+07
R wine 15	Red	Gamay	2016	6.70E+04	<10	4.36E+07
R wine 16	Red	Gamay	2015	<10	<10	6.00E+05
R wine 17	Red	ND	ND	6.00E+04	<10	6.30E+06
R wine 18	Red	ND	ND	6.70E+02	2.00E+04	3.00E+04
R wine 19	Red	ND	ND	5.50E+02	1.44E+03	2.00E+05
W wine 1	White	ND	ND	<10	1.40E+05	6.20E+04
W wine 2	White	ND	ND	40	2.00E+05	>10e6
W wine 3	White	ND	ND	<10	<10	2.04E+05
W wine 4	White	Chardonnay	ND	<10	<10	1.10E+06

554

555

Table S2 : List of the 52 strains from the collection and their associated group (according to Avramova, 2018; Lorentzen & Lucas, 2019).

B. bruxellensis		***O. oeni***		***L. hilgardii***	
Name	**Group**	**Name**	**Group**	**Name**	Group
AWRI 1608	Beer[a]	CiNe	A[b]	CRBO 0012	NG[c]
CRBO L0308	Wine 2[a]	IOEB-SARCO 1491	A[b]	CRBO 0509	NG[c]
CRBO L0417	Wine 1[a]	Psu1	A[b]	CRBO 7701	NG[c]
CRBO L14174	Wine 1[a]	CRBO S14	A[b]	CRBO 8408	NG[c]
CRBO L1751	Wine 3[a]	CRBO 0205	A[b]	CRBO 8510	NG[c]
YJS 5319	Wine 3[a]	CRBO 0607	A[b]	DSM 20051	NG[c]
YJS 5334	Kombucha[a]	CRBO 9624	A[b]	DSM 20176	NG[c]
YJS 5363	Kombucha[a]	CRBO 9806	A[b]	E1.1	NG[c]
YJS 5382	Wine 2[a]	CRBO C23	B[b]	E1.4	NG[c]
YJS 5400	Beer[a]	CRBO S12	B[b]	T1.2	NG[c]
YJS 5408	Wine 1[a]	CRBO 0501	B[b]		
YJS 5417	Kombucha[a]	CRBO 0502	B[b]		
YJS 5422	Wine 3[a]	CRBO 9803	B[b]		
YJS 5449	Wine 3[a]	CRBO 14216	B[b]		
YJS 5476	Wine 1[a]	CRBO 1399	C[b]		
YJS 5487	Wine 1[a]	CRBO 1381	C[b]		
YJS 7816	Beer[a]	CRBO 1386	C[b]		
YJS 7853	Wine 2[a]	CRBO 1389	C[b]		
YJS 7856	Wine 3[a]	CRBO 1391	C[b]		
YJS 8063	Wine 2[a]	CRBO 13106	C[b]		
YJS 8082	Wine 1[a]				
YJS 8448	Beer[a]				

[a] Genetic group defined by Avramova (2018)

[b] Phylogenetic group defined by Lorentzen & Lucas (2019)

[c] NG : No existing group for *Lentilactobacillus hilgardii*

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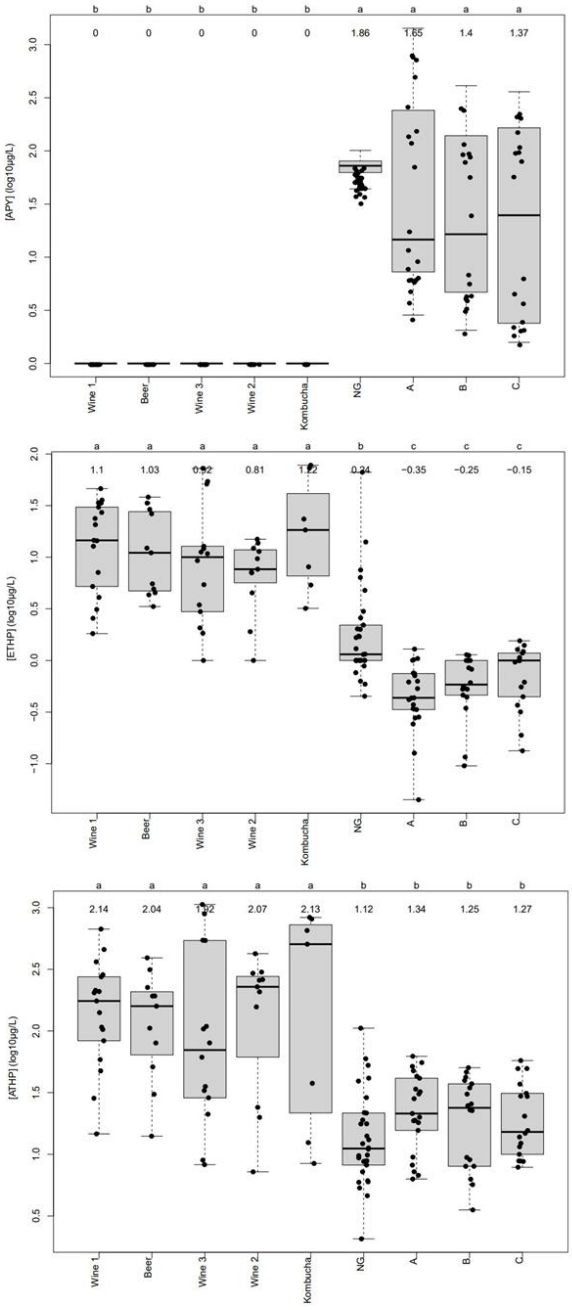

Figure S1 : Production of APY, ETHP and ATHP by the different groups of B. bruxellensis *(Wine 1, Beer, Wine 2, Wine 3, Kombucha),* L. hilgardii *(NG) and* O. oeni *(A, B, C) in a NHAM.*

III.3. Co-inoculation

Pediococcus is a genus of homofermentative lactic acid bacteria belonging to the family *Lactobacillaceae*. It contributes to the process of malolactic fermentation in sauerkraut but is considered as a contaminant in beer and wine. It is not used in the field of œnology. Currently there are 11 recognized species of the genus *Pediococcus* including *Pediococcus parvulus.* This species was isolated in wine in 1962 by Gunther *et al.* and owes its name to its very small size. *Pediococcus spp*. have been isolated from wines around the world and these lactic acid bacteria are generally associated with wine spoilage because they lead to the production of bad aromas whose descriptors are too buttery, or even "dirty socks". More specifically, an alteration related to the excessive synthesis of diacetyl (Davis *et al.*, 1988) and the synthesis of biogenic amines (Landete *et al.*, 2005). *Pediococcus* are also involved in the production of extreme bitterness due to acrolein, a compound that then reacts with phenolic compounds. This species is also responsible for the production of exopolysaccharides giving ropy wines with a very viscous appearance (Dimopoulou & Dols-Lafargue, 2021). The production of all these molecules has a detrimental effect on the quality of the wine. In 1998, Costello showed in his study that at least one strain of *P. parvulus* (P6b) can weakly produce the 3 mousy compounds given the general lack of information regarding the impact of *Pediococcus* on wine quality.

Interestingly, Strickland conducted experiments to investigate the interaction between *Pediococcus* and *B. bruxellensis* and the potential effects on the growth and production of volatile phenols in Pinot Noir. Indeed, he noted that the co-inoculation of *P. parvulus* and *B. bruxellensis* made it possible to obtain wines with lower concentrations of 4-ethylphenol than during separate inoculations. (Strickland *et al.*, 2016).

The objective of this part was first to investigate the ability of 3 strains of the species *P. parvulus* to produce compounds responsible for mousiness alteration and then to study a possible interaction between *P. parvulus* and *Brettanomyces* about mousy compounds production.

Possible interactions between *S. cerevisiae* and *B. bruxellensis* or *O. oeni* have also been considered. The first results of this study are presented in the section.

III.3.1. Materials and methods

III.3.1.1. Microbial strains

3 *B. bruxellensis* strains (CRBO L0417, CRBO L1751 and YJS 7816), *3 O. oeni* strains (CRBO 0502, CRBO 1399 and Psu1), 3 *P. parvulus* strains (CRBO 0202, CRBO 0601 and DSM 20332) and 3 *S. cerevisiae* strains (FX10, GN, SB) were used. They have been delivered by the Centre de Ressources Biologiques Œnologique (CRBO, Bordeaux, France), the YJS strain was from YJS collection (Laboratory for Molecular Genetics, Genomics and Microbiology, Strasbourg University, France).

III.3.1.2. Isolation from stock glycerol

For the different phenotyping experiments, individuals were isolated from biomass stored at -80 ° C, on YPD agar medium (for yeast) or RGJ agar medium (for bacteria) and incubated for 7 to 10 days at 24 ° C.

III.3.1.3. Thoma's chamber counting yeasts

Thoma's chamber is a specific hemacytometer that allow yeast counting under a microscope. It is a gridded glass slide with numbering chambers. Thoma's chamber presented 16 squares of 25 small squares. 10 µL of culture are placed between slide and slide and the cells present are counted under a microscope (x40). Depending on the population density, the culture was diluted to 1/2 – 1/10 with physiological water before microscopic observation to perform a more accurate count.

The average of yeasts contained in several groups of squares was calculated. Then the number of cells in the culture was calculated according to the formula:

$$N = \left(\frac{\bar{n}}{V_i}\right).f$$

N : number of cells/mL

$\bar{n}$: average number of cells in the several groups of 25 little squares

V_i : volume in the several groups of little squares

$V_{Thoma} = 6.25 \times 10^{-6}$ mL

f : dilution factor

III.3.1.4. Cell preculture and culture

The microorganisms studied were stored on a solid YPD medium (10 g/L yeast extract, 10 g/L peptone, 20 g/L glucose, pH adjusted to 4.8 ; supplemented with 25 g/L agar) (for yeasts) and solid RGJ medium (5 g/L yeast extract, 250 mL/L of red grape juice, 1 mL/L of Tween80, pH 4.8 ; supplemented with 25 g/L agar) (for bacteria) at 4°C. Each strain undergoes a pre-culture step in 3 mL of RGJ medium. Precultures were incubated at 25°C for 5 days for *B. bruxellensis* and *O. oeni*, and 3 days for *P. parvulus* and *S. cerevisiae*. The culture step was performed in 90 mL of RGJ medium inoculated at a rate of 2 % from the preculture, the incubation time was like the previous step.

III.3.1.5. Screening of microbial strains co-inoculated for the ability to produce mousy N-*heterocycles in* N*HAM*

The cultures were centrifuged (11600 g for 9 minutes at 4°C), then the pellets were washed twice with a buffer solution (20 mL then 10 mL). To standardize the amount of population inoculated in the *N*HAM cells were added at different concentration (10^9 cells/mL for yeasts (using Thoma's chamber measurement) and with an optical density (λ=650 nm) of 1 for bacteria). This microbial load was inoculated into 22 mL of medium *N*HAM then incubated for 24 h at 25 ° C. Each microorganism has

been screened in these conditions alone. And the co-inoculation of each couple of *Brettanomyces/Pediococcus* have been studied. This screening has been done on two microbiological duplicates.

The co-inoculation of each couple of *Brettanomyces/Saccharomyces* and *Oenococcus/Saccharomyces* has been studied. Unfortunately, due to technical issues, only preliminary results are discussed here.

III.3.1.6. Analysis of acetaldehyde by enzyme kit

The acetaldehyde has been quantified by enzymatic assay (K-ACHYD, Megazyme, Bray, Ireland).

III.3.2. Brettanomyces bruxellensis and *Pediococcus parvulus*

The objective of this part was to study if there were synergic or antagonistic effects between strains of *B. bruxellensis and P. parvulus* in the production of mousy compounds in *N*HAM.

The concentrations produced by each individual microorganism are presented in Table 5. It is observed that the 3 studied strains of *P. parvulus* can produce APY in large quantities and ETHP and ATHP in smaller extent. These results highlight the ability of *P. parvulus* to produce mousy compounds. This was already observed for one strain by Costello in 1998 but could not been confirmed by the screening carried out on isolates from mousy wines (III.2 Which microorganisms are bringing the mousy off-aromas in our wines?; p. 61). None of the three isolates of *P. parvulus* screened had produced mousy compounds. Such results could be explained by the fact that the three isolates could come from a single non-producing strain. Similarly, as seen in the previous part, the 3 strains of *B. bruxellensis* can produce ATHP and ETHP in different amounts depending on the strain studied.

Table 5 : Concentration of the 3 N-*heterocycles (APY, ETHP and ATHP) produced by 3* Brettanomyces bruxellensis *and 3* Pediococcus parvulus *independently in 24 h at 25° C in* N*HAM.*

		[APY] in µg/L		[ETHP] in µg/L		[ATHP] in µg/L	
Species	Strain	av[a]	sd[a]	av[a]	sd[a]	av[a]	sd[a]
Brettanomyces bruxellensis	CRBO L0417	<LOD[b]	<LOD[b]	61	55	123	70
	CRBO L1751	<LOD[b]	<LOD[b]	6	4	49	27
	YJS 7816	<LOD[b]	<LOD[b]	25	23	138	114
Pediococcus parvulus	CRBO 0202	185	64	10	5	<LOQ[b]	-
	CRBO 0601	363	99	12	6	24	4
	DSM 20332	574	271	10	7	23	3

[a:] av, average of the microbial duplicates; std, standard deviation of the microbial duplicates;
[b:] LOD, limit of detection; LOQ, limit of quantification, according to II.2.2.3

Figure 26 represents the production of ATHP and ETHP when strains were separately inoculated (cumulated amount) or co-inoculated. The cumulated production is the sum of the production of the 2 microorganisms taken separately. ETHP (B, D, F) was produced in a similar way (between separately

inoculated or co-inoculated) for 8/9 couples screened, except, when the strain CRBO L1751 was inoculated with CRBO 0601 (D)ETHP was produced in larger quantities.

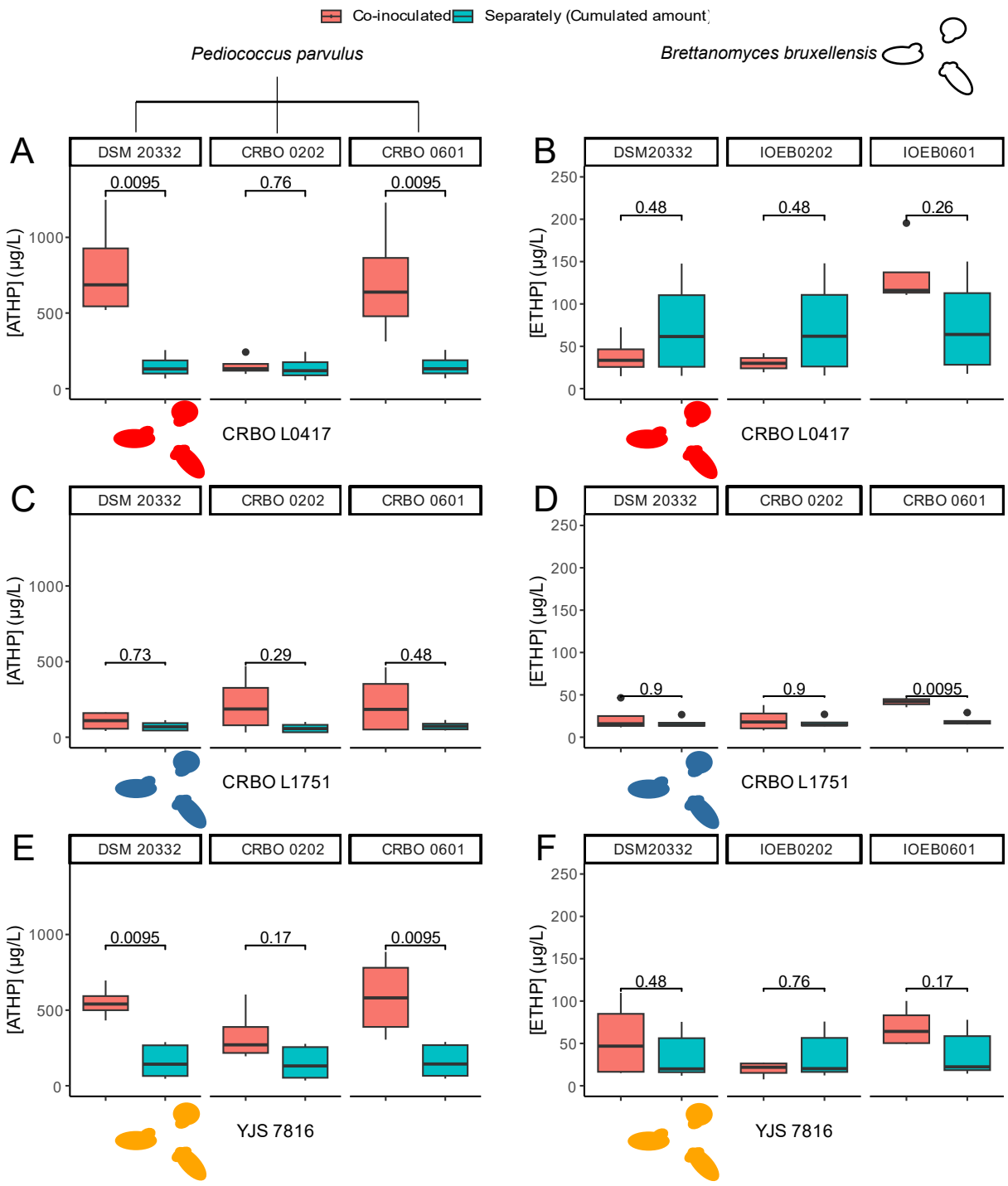

Figure 26 : Comparison of the production of ATHP (A, C, E) and ETHP (B, D, F) by the 3 strains of Brettanomyces bruxellensis *and 3* Pediococcus parvulus *co-inoculated or separately; comparisons were made by a Wilcoxon test, the associated p-values are indicated.*

Production of ATHP occurred in a different manner when *B. bruxellensis* was inoculated alone or co-inoculated with *P. parvulus*. Synergic effect seems to emerge on the production of ATHP for 4 *Brettanomyces/Pediococcus* pairs. Indeed, significant differences emerge for couples CRBO

L0417/DSM 20332, CRBO L0417/CRBO 0601, YJS 7816/DSM 20332 and YJS 7816/CRBO 0601. The ATHP level produced were significantly higher when the strains were inoculated together rather than separately inoculated. The production behaviour during this co-inoculation could be impacted by both strain and the couple.

We can note that simultaneous inoculation of strain CRBO 0202 with any strain of *B. bruxellensis* did not appear to influence the production of both ATHP and ETHP. CRBO 0202 is the only strain of *P. parvulus* studied that was referenced as a ropy strain (Walling *et al.*, 2005). We can assume that the exopolysaccharide network that could physically separate the yeasts from the bacteria and catch an interaction between the 2 different microorganisms. Similarly, the production of *B. bruxellensis* CRBO L1751 strain does not appear to be influenced by the co-inoculation of *P. parvulus* strains.

The difference in behaviour between strain CRBO L1751 and the other 2 strains of *B. bruxellensis* in the face of co-inoculation is very little explained. The 3 strains are of different genetic groups (Avramova *et al.*, 2018a) and the production in the species *B. bruxellensis* depends mainly on the strain and not on the genetic group. Indeed, of the 3 strains tested, CRBO L1751 is the only diploid *Brettanomyces* strain, the other two are triploid. The character of ploidy could have an impact on the production of *Brettanomyces* when co-inoculated.

Similarly, the amounts of APY produced separately and when the microorganisms were together are shown on Figure 27. First, *B. bruxellensis* does not produce APY, the cumulative production of APY is ultimately represented only by the production of the different strains of *P. parvulus*. On the other hand, APY was not produced when these lactic acid bacteria were put in the presence of *B. bruxellensis* revealing an inhibitory effect of yeast on the production of APY by *Pediococcus* and this even for strain CRBO 0202 having a ropy character.

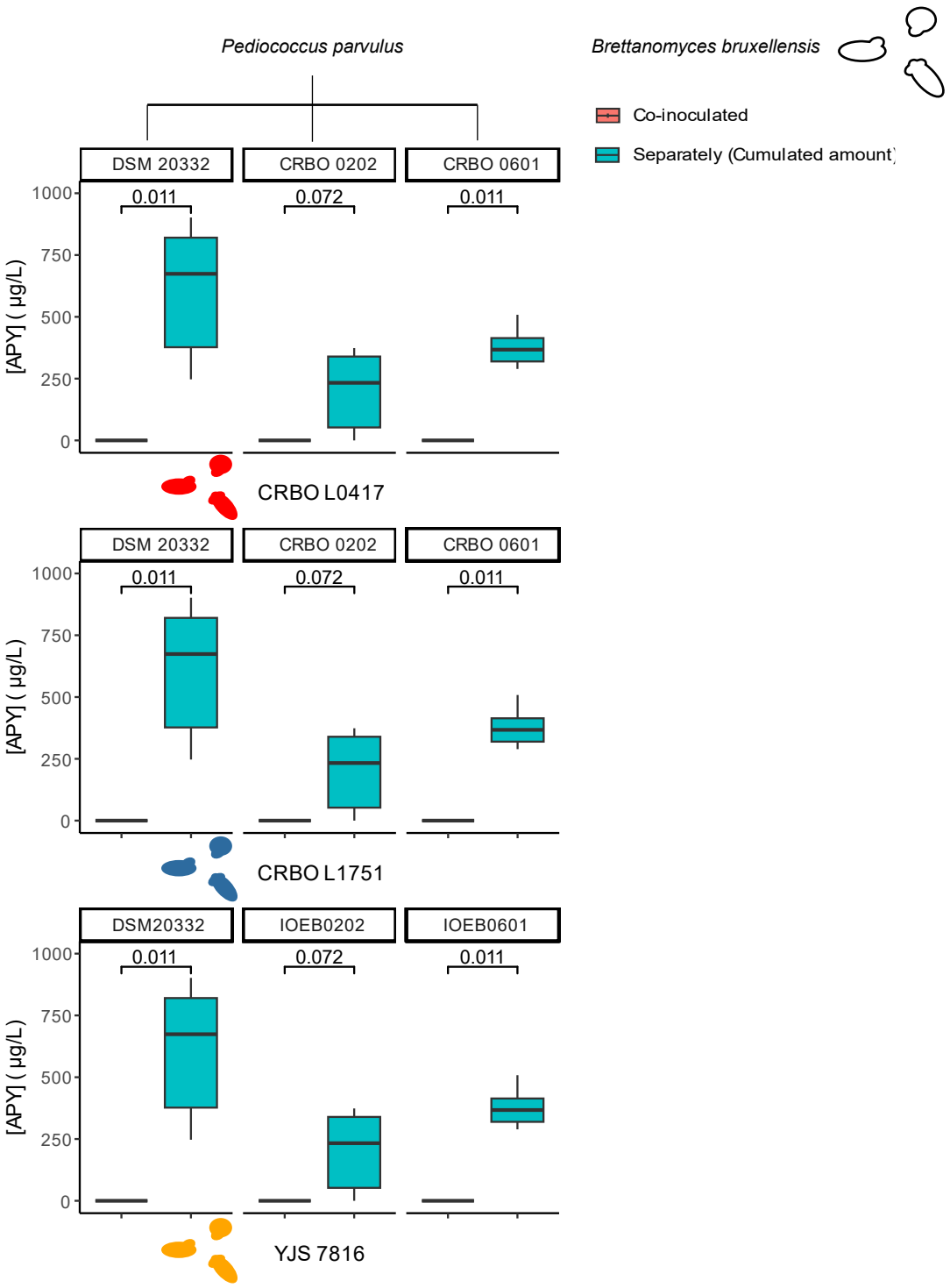

Figure 27 : Comparison of the production of APY by the3 strains of Brettanomyces bruxellensis *and 3* Pediococcus parvulus *co-inoculated or separately; comparisons were made by a Wilcoxon test, the associated p-values are indicated.*

III.3.3. Exploration of the interaction including other yeast/bacteria pairs

Other co-inoculation tests have been done. For example, we studied the possible impact of the presence of *S. cerevisiae* on production of the mousy compounds by *O. oeni* and *B. bruxellensis*.

Unfortunately, due to technical problems and a loss of sensitivity of the GC-MS used, many analyses could not be considered and the results we have are only preliminary. Despite this, some results can be presented as preliminary results.

Due to the technical issue, we do not have a calibration line associated with these manipulations and the results of the relative areas will be presented. The strains of *S. cerevisiae* used in this experience were also screened, none of the three N-heterocycles could be produced in the *N*HAM by this species when inoculated alone in the environment. Therefore, only the results of other microorganisms, with the presence or not of *Saccharomyces*, are shown here.

Figure 28 shows the relative areas of ATHP and ETHP of samples of 3 strains of *B. bruxellensis* (YJS 7816, CRBO L0417, CRBO L1751) with or without *S. cerevisiae* strain (FX10, GN and SB). Since only one microbiological replicate could be traced, the standard deviations are those of the chemical analysis and no statistical tests have been performed on these results. However, we can notice that the 3 strains of *Brettanomyces* were producing more ATHP and ETHP when they were in presence of the strain SB.

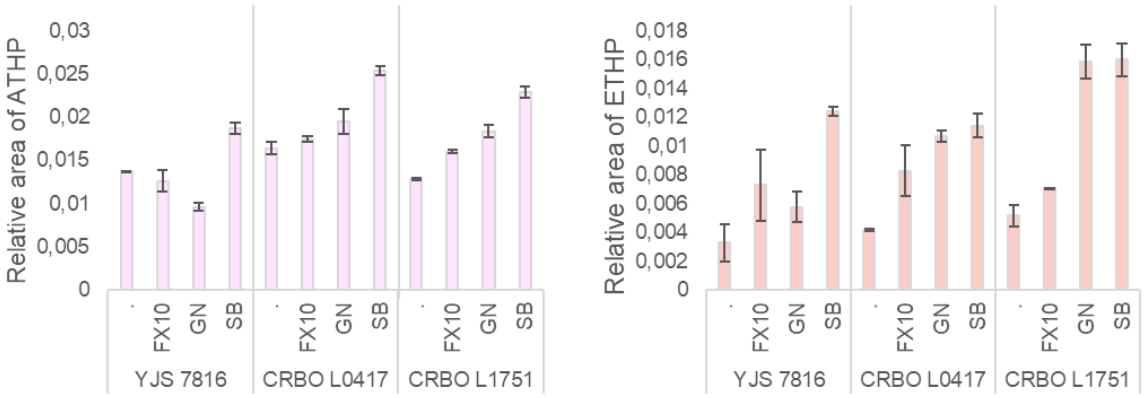

Figure 28 : Relative areas of ATHP and ETHP produced by 3 strains of Brettanomyces bruxellensis *co-inoculated (or not) with 3 strains of* Saccharomyces cerevisiae; *the standard deviation represents the deviation between the 2 analyses.*

Similarly, co-inoculation tests of 3 strains of *O. oeni* (CRBO 0502, CRBO 1399 and Psu1) in the presence of the same 3 strains of *S. cerevisiae* were performed. Unfortunately, no chemical results could be recovered. All the samples co-inoculated with SB appeared to have a higher intensity of the mousiness than with the other 2 strains of *S. cerevisiae*. Without chemical analysis and sensory analysis, it is difficult to frame this observation, but it would be along the same lines as for the strains of *B. bruxellensis*.

Ethanal being produced in different quantities depending on the strains of *S. cerevisiae* (Ochando *et al.*, 2020) and having been recognized as one of the precursors of mousy compounds, we wanted to be interested in the amount of ethanal in *N*HAM after incubation of *S. cerevisiae* strains. This has been quantified by enzyme kit.

Initially, the acetaldehyde is added in the *N*HAM at a level of 100 mg/L. The amounts of the ethanal after the incubation of *S. cerevisiae* are shown in Table 6. We did not take a control sample and no microbiological duplicate was treated. On the other hand, we can see that the FX10 and GN strains would tend to consume acetaldehyde present in the medium while the SB strain would produce it. The fact SB could produce some acetaldehyde may explain the synergic effect observed before, even though the behaviour of the strains in the presence of another microorganism could be totally different.

Table 6 : Acetaldehyde levels after the incubation of 3 strains of Saccharomyces cerevisiae for 24h *in* N*HAM.*

	Acetaldehyde (mg/L)	
	av[a]	sd[a]
FX10	50	6
GN	77	8
SB	114	8

[a]: av, average; sd, standard deviation of the technical duplicate

Another possibility, which may explain the interaction between *S. cerevisiae* and other microorganisms is the presence of lees at the bottom of the tubes, thus uncontrollably bringing more nutrients, amino acids into the environment that can be used by the producers of mousy compounds. Finally, each microorganism could independently participate in the metabolism of production of these compounds. One of the production routes proposed by Grbin *et al.* (2007) involves cadaverine as a biosynthetic intermediate. Lactic acid bacteria such as *O. oeni or P. parvulus* are known to produce large quantities of biogenic amines (Granchi *et al.*, 2005; Wade *et al.*, 2019), they could biosynthesize cadaverine that will be metabolized by *B. bruxellensis*.

Therefore, there seems to be a real effect between different microorganisms when they are tested together in the same sample. It must also be remembered that in real conditions these microorganisms are never found alone in the environment, other species may be present in a minority way that can thus be impacted by synergistic or antagonistic effects the production of mousy compounds. This interaction seems to be mainly synergistically impacted by the production of mousiness Although APY production by strains of *P. parvulus* appear to be inhibited by the presence of *B. bruxellensis*. The presence of certain strains of *S. cerevisiae* in the environment could also impact the formation of mousy off-aroma by other microorganisms.

III.4. Conclusion

In this part, we were mainly interested in microbial production of mousy compounds under standard conditions. To do this, we first studied the microorganisms present in mousy wines. We noted that the presence of *B. bruxellensis* in these wines was of the order of one wine in five. May leave the idea that the alteration was produced in most cases by other microorganisms. It was then shown that 22 strains of *B. bruxellensis* of different origin and genetic group can produce ATHP in very large quantities under standardized conditions. Under the same conditions, APY is produced preferably by all 20 strains of *O. oeni* of the 3 different genetic groups.

Microbial interactions were shown. Synergistic effects on the production of ETHP and ATHP could be observed between different microorganisms. Inhibitory effects of *B. bruxellensis* on the production of APY by *P. parvulus* has been demonstrated.

Furthermore, this work focused on the ability of species found in finished wines to produce mousiness. Other microorganisms could have an impact on the production of mousiness upstream. Moreover, all strains of *B. bruxellensis*, *L. hilgardii* and *O. oeni* were able to produce *N*-heterocycles during a high cell density incubation in a *N*HAM. They were screened only in a synthetic medium, the *N*HAM, which is far from the wine composition. It would be interesting to compare these strains and their formation of mousiness in a wine matrix and/or during the whole wine making process.

Chapter IV.
Mousy taint production: Kinetic studies and oenological conditions

This last chapter deals with the second and the third task of the project. The first kinetic studies let to unexpected results and kinetics and therefore three independent experiments were carried out during the doctoral period at the two research institutions. The results associated with each kinetic are discussed here. Finally, the production of 5 microorganisms was tested on a white Ugni blanc must vinified or not by two strains of *S. cerevisiae*.

IV.1. Introduction

The production mechanisms of mousy compounds are not yet elucidated, and other yeast species may be involved in the development of spoilage. Similarly, no research has been carried out on the link between the physiological state of strains and their production. During his thesis, Grbin (1998) followed the taint compound production by a strain of *B. anomalus* during 16 days. In the same way, Costello (1998) studied the production of mousy taint (determined by the alkaline strip test) in a GJ medium of different genus and species of LAB at different time after inoculation (after 2, 5, and 13 days).

Strains of *S. cerevisiae* do not have the ability to produce mousy compounds under tested conditions. However, preliminary results from co-inoculation showed that one strain of *S. cerevisiae* studied tended to increase concentrations of ATHP produced by *B. bruxellensis.* The production of 5 microorganisms (2 *B. bruxellensis,* 1 *L. hilgardii*, 2 *O. oeni strains*) was studied in matrices of Ugni blanc vinified by 2 strains of *S. cerevisiae.*

IV.2. Materials and methods

IV.2.1. Microbial strains

3 *B. bruxellensis* (CRBO L0417, CRBO L1751 and YJS 7816), 1 *L. hilgardii* (DSM 20176), 2 *O. oeni* (CRBO 0502 and Psu1), and 2 *S. cerevisiae* strains (GN, SB) were used. They have been delivered by the Centre de Ressources Biologiques Œnologique (CRBO, Bordeaux, France), the YJS were from YJS collection (Laboratory for Molecular Genetics, Genomics and Microbiology, Strasbourg University, France) and the DSM 20176 were supplied by the DSM collection (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany).

IV.2.2. Counting

IV.2.2.1. Malassez chamber counting yeasts

Malassez as Thoma's chamber is a specific hemacytometer that allow cell counting under a microscope. It is a gridded glass slide with numbering chambers. Malassez chamber has 25 rectangles containing 20 small squares. 10 µL of culture are placed between slide and slide and the cells present are counted under a microscope (x40). Depending on the population density, the culture was diluted to 1/2 – 1/10 with physiological water before microscopic observation to perform a more accurate count.

The average of yeast contained in several groups of squares was calculated. Then the number of cells in the culture was calculated according to the formulas:

$$N = \left(\frac{\bar{n}}{V_i}\right).f$$

N : number of cells/mL

$\bar{n}$: average number of cells in the several groups of 20 little squares

$V_{Malassez} = 1 \times 10^{-5}$ mL

f : dilution factor

IV.2.2.2. Cytometry counting

The population of *B. bruxellensis* was also measured by flow cytometry (Cytoflex, Beckman Coulter Inc., Brea, California USA). To carry out the count, 50 µL of sample containing the yeasts are taken and added to 3 µL of propidium iodide (PI) and 947 µL of MCIlvaine. PI is a nucleic and fluorescent intercalating agent. It binds to the basics of DNA and helps mark dead cells. MCIlvaine is a pH 4 marking buffer.

IV.2.2.3. Petri dishes counting

The viable population was determined on selective agar medium or not. The counts were either carried out by spreading a volume of 100 µL of sample, diluted or not, with previously sterilized beads or by drops, the sample is several times diluted and drops are deposited on the agar medium. For each sample, two to three counts were recorded. The boxes were incubated for 7-10 days at 24°C. In the absence of colonies after 3 weeks of incubation, the sample was considered negative for the presence of culturable population on the selective medium.

IV.2.3. Experiments in wine

Figure 29 is a schema of the workflow of the inoculation and aliquotation of the experimentation in wine.

IV.2.3.1. Must

The Ugni Blanc must was from the 2022 vintage and the Charente area (16 France).

IV.2.3.2. Preparation of yeast starter

Yeast starters were prepared by transplanting a dozen colonies of *S. cerevisiae* (GN or SB) in an Erlenmeyer flask containing 100 mL of Ugni Blanc wort (the assimilable nitrogen adjusted to 160 mg / L), 100 mL of sterile water, a magnetic bar and closed with sterile carded cotton. The suspension was then stirred for 24 hours. The microbial population was then estimated using a flow cytometer (Cytoflex, Beckman Coulter Inc., Brea, California USA). The volume needed to inoculate 2.10^6 cells/L was then taken.

IV.2.3.3. Vinification in the laboratory

Nano-vilifications were carried out in 1 L fermenters. The freshly grapes collected by hand was destemmed, crushed, and delicately pressed using a pneumatic press, thanks to the equipment of the nano-vinification platform of the UMR Œnologie, in order to separate under standardized conditions, the liquid phase from the solid phase (skins and seeds).

All fermenters were evenly filled with 1 L of must. The assimilable nitrogen adjustment was made in two stages, at the beginning of alcoholic fermentation and at mid-AF up to 160 mg/L with THIAZOTE® (Laffort, Bordeaux, France). The yeast starter (GN or SB) was added to the fermenters.

The course of the alcoholic fermentation was controlled by measuring the density every 24 hours at 20° C using an electronic densimeter DMA 35 (Anton Paar, Courtaboeuf, France) until two consecutive samples were taken without the density changing. Once a day, the fermenters were stirred by rotation to resuspend the yeasts. At the end of alcoholic fermentation, the amount of residual sugar (glucose and fructose) was measured using the automatic analyser with enzyme kits Y400 (Biosystems, Barcelona, Spain). When the number of residual sugars was less than 2.5 g/L, then the wines were racked of and frozen in the form of 22 mL aliquot in 50 mL tubes. At the same time, tubes of must without AF were aliquoted.

For each starter, the experimentation was repeated on 3 biological duplicates.

IV.2.3.4. Acclimation of strains for the inoculation in wine

For each strain, from a fresh culture on agar medium, a colony was suspended in a 13 mL tube containing 9 mL of grape juice medium and 1 mL of 100 % ethanol (purity > 99.9 %). The precultures were incubated for 6 days at 25° C, without agitation. At 6 days, the culture was transplanted into a 50 mL tube containing 35 mL of grape juice medium and 5 mL of 100 % ethanol (purity > 99.9 %) inoculated at 2 %.

IV.2.3.5. Inoculation of wine

Prior to inoculation, the aliquoted tubes of 22 mL of post-FA wine (*Cf*: IV.2.3.3 Vinification in the laboratory; p. 101) were placed overnight at 4° C in order to thaw. Two strains of *B. bruxellensis* (CRBO L0417 and YJS 7816), one strain of *L. hilgardii* (DSM 20176) and two strains of *O. oeni* (CRBO 0502 and Psu1) were inoculated at 5.10^5 cell/mL each in 30 separately tubes per alcoholic fermentation modality. Half of them were stirred (180 rpm) and the other half kept static. Each 22 mL tube was then incubated for up to 24 days at 24°C. At each sampling a tube was sacrificed to carry out the measurements of culturable population and concentration of mousy compounds. Similarly, control tubes without added microorganisms were studied as controls.

IV.2.3.6. Monitoring the production of mousy taint compounds

The production of mousy compounds in wine was monitored over 24 days with samples taken at 0-3-9-18-24 days. For monitoring, in order not to agitate the cultures, not to resuspend at the time of collection, not to modify the oxygenation of the samples, a tube was removed from the device at each sampling.

For each tube sampled, we measured the population by spreading on specific medium of *non-Saccharomyces* yeast when *B. bruxellensis* was inoculated and the population of lactic acid bacteria when *L. hilgardii* or *O. oeni* was inoculated, and the concentrations of APY, ATHP and ETHP.

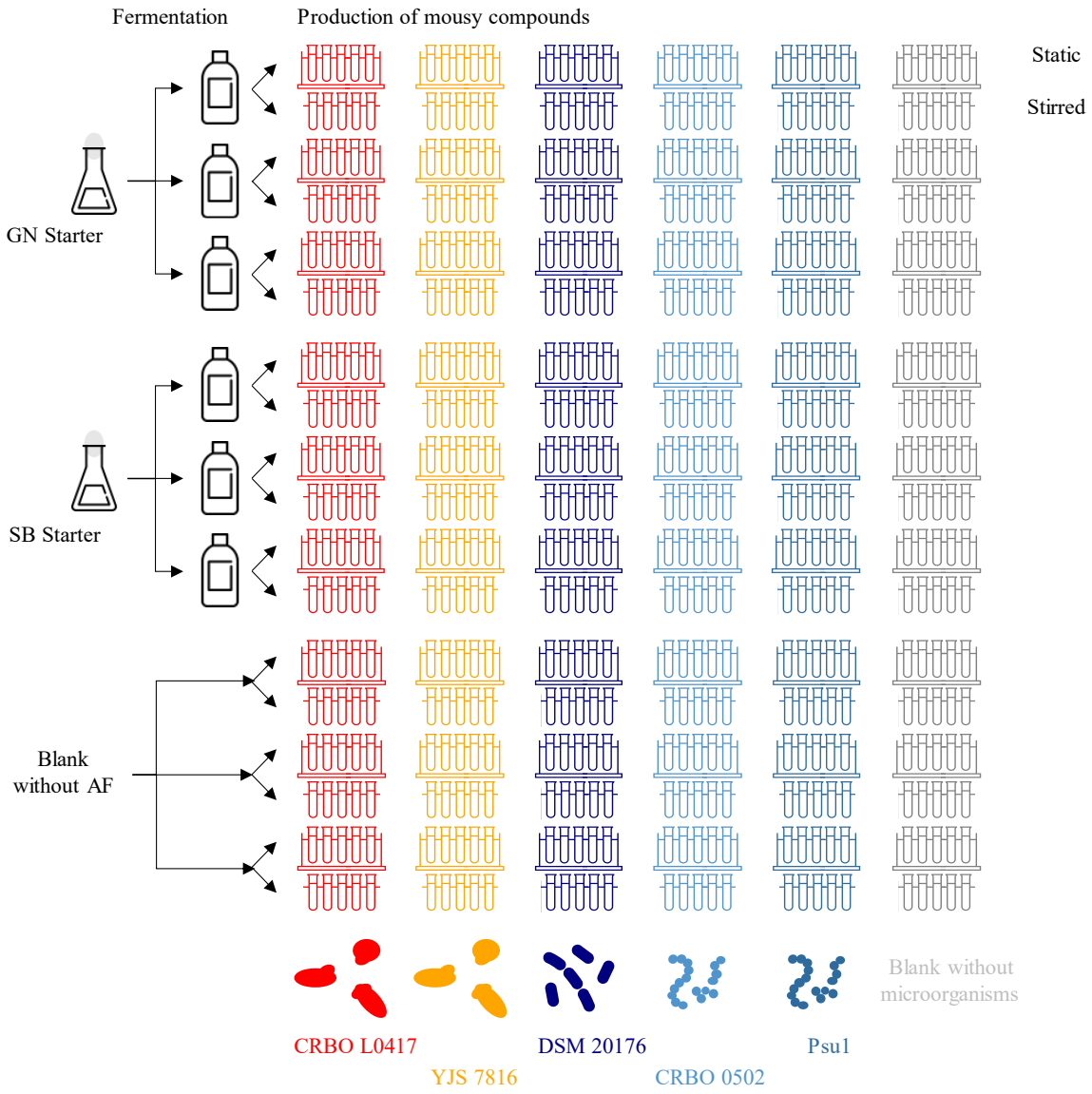

Figure 29 : Workflow of the inoculation of 2 strains of Brettanomyces bruxellensis *(CRBO L0417, YJS 7816), 1 strain of* Lentilactobacillus hilgardii *(DSM 20176), and 2 strains of* Oenococcus oeni *(CRBO 0502 and Psu1), and of the aliquotation of cultures for Ugni blanc trials.*

IV.2.4. Kinetics in *N*HAM – growth and mousy compounds production

IV.2.4.1. Sample preparation for kinetics

For three strains of *B. bruxellensis* (CRBO L0417, CRBO L1751 and YJS 7816), the growth and production capacities of *N*-heterocyclic compounds in *N*HAM medium were studied. Each strain was tested in biological duplicates (different precultures for each replica). After isolation on agar YPG medium from stock glycerol (5-7 days at 24 ° C), a colony was used to inoculate 5 mL of preculture in *N*HAM (3 days). Population density and number of cells are quantified using a flow cytometer (until 10^7 cells/tube). The tubes are then centrifuged for 9 minutes at 11600 g and at 4 ° C. The yeast pellet is taken up in 22 mL of *N*HAM to obtain 5.10^5 cells/mL. 10 tubes per stump are made. As there are 3 strains and a replica for each of them, there are a total of 60 tubes for the entire study. Indeed, at each analysis, 2 tubes per stump are sacrificed (Figure 30).

To better control the population at the beginning of the kinetic, a count on Malassez chamber is carried out by staining the dead cells with Methylene Blue.

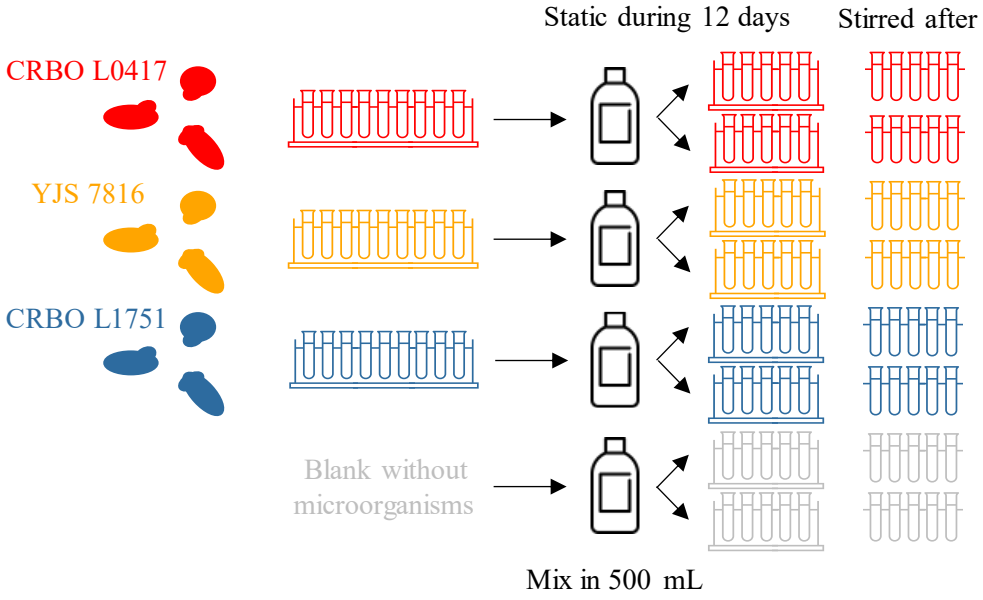

Figure 30 : Inoculation and aliquotation of cultures of 3 strains of Brettanomyces bruxellensis *in* N*HAM for the first kinetic.*

For the second and the third kinetic studies, similar approach has been used. The difference is that the precultures of the 3 strains of *B. bruxellensis* were carried out in RGJ medium. Each strain was tested in organic triplicates during the third kinetic study.

IV.2.4.2. Measured parameters during kinetics investigations

The production of mousy compounds was monitored over few weeks (23 to 50 days) with samples taken regularly (Figure 31).

For each tube sampled, we measured the population by flow cytometry, the *N*-heterocycles were measured according to II.2 Quantification of the *N*-heterocycles in model culture media (p. 50). At each sampling a tube was sacrificed to carry out the measurements of population by flow cytometry and concentration of mousy compounds by SBSE-GC-MS.

During the second kinetic, some samples were kept for quantifying malic acid, acetic acid, fructose, and glycerol by semi-automated enzymatic assay (K-ACTGK, K-GCROLGK, K-LMAL-116A, Megazyme, Bray, Ireland). A sample volume of 800 µL was manually transferred in Micronics tubes (Novazine, Lyon, France, ref: MP32033L) and stored at -20° C. The measurement has been done following 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). Fructose was determined assayed by using the enzymatic method described by Stitt *et al.* (1989).

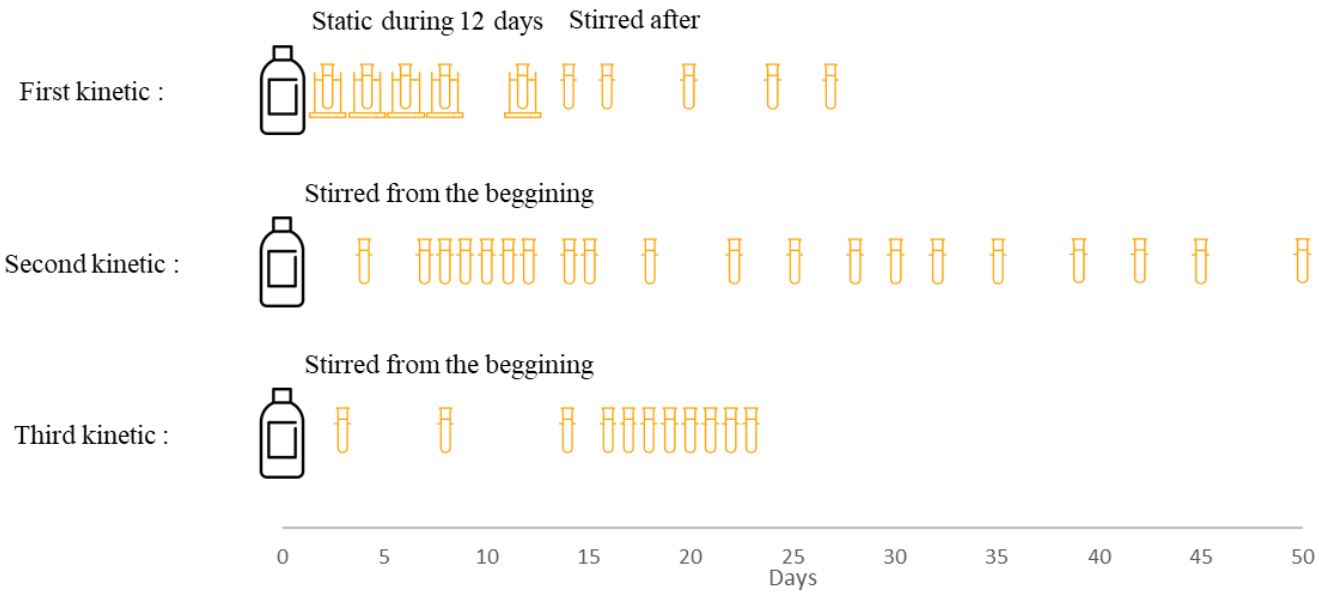

Figure 31 : Example of the kinetic monitoring of strain YJS 7816, strains CRBO L0417 and CRBO L1751 have been sampled in the same way. The first kinetics were performed in duplicates, the second and third in triplicates. After the second kinetic, one more sample were also measured on the 161st days.

IV.2.4.3. Determination of kinetic parameters

The different kinetic parameters were determined by local polynomial regression using the method of locally estimated scatterplot smoothing (LOESS) on the viable cells. From this model, we were able to calculate the growth rate, the maximum population, and the duration of the lag phase.

The growth rate:

$$r_{(t)} = \frac{N_{(t)} - N_{(t-1)}}{d_{(t)} - d_{(t-1)}}$$

$N_{(t)}$: number of cell/mL at t.

r is expressed in cell/mL/d.

r is the day associated with r_{max}.

The maximum population has been noted K_{max} and is expressed in cells/mL.

The duration of the lag phase is expressed in day and has been associated with:

$$lag(t) \leq \frac{max_N - min_N}{20}$$

$\max/\min_N$: number maximum/minimum of cells/mL.

IV.3. *Brettanomyces bruxellensis* Kinetics

IV.3.1. First kinetic

Once inoculated into the tubes, the population was checked by drops and by Malassez Chamber. The results are presented below (Table 7). CRBO L0417 appears to have had lower growth than the other 2 strains of *B. bruxellensis*. The results of the drops do not confirm those obtained through the Malassez cell count. This difference can be explained by an error when counting on Malassez's cell. Indeed, some cells may not have been counted. If these were slightly bluish, they could be considered dead and thus not considered during the count. The population density is comparable for YJS 7816 and CRBO L1751.

Table 7 : Counting by Malassez chamber and by drops of the first day of the kinetic.

B. bruxellensis strain	Malassez chamber counting (cells/mL)	CFU/mL
CRBO L0417	$3.2\ 10^5$	$5.0\ 10^5$
CRBO L1751	$5.6\ 10^5$	$2.3\ 10^5$
YJS 7816	$5.0\ 10^5$	$2.26\ 10^5$

Regarding the growth kinetics of the three strains (Figure 32), we can observe that they are all in the latency phase between day 0 and day 8. Indeed, there is no increase in population. Subsequently, between the 10^{th} and 16^{th} day there is an acceleration phase, the population has almost tripled from $2.8.10^6$ to $8.8.10^6$ cells/mL. Between the 16^{th} and 20^{th} day, CRBO L1751 shows an exponential growth characteristic of the logarithmic phase. Indeed, there is a factor of 9 between the amount of population

on days 16th and 20th. After the 20th day, we noticed that the growth slowed down, the strain is in the phase of slowing down. It can therefore be assumed that it has entered the stationary phase thereafter. Unfortunately, the study was not long enough to observe it.

If we look at the other two strains (YJS 7816 and CRBO L0417), agitation from the 12th day seems to have had a real impact. Indeed, the population shows only a very small increase until the 16th day. Regarding strain CRBO L0417 (wine 1), the acceleration phase is observable between the 14th and 20th day. We can assume that the exponential phase takes place between the 20th and 23rd day. Indeed, the population only tripled during this phase. Subsequently, there was a slowdown in growth, reflecting the transition to a deceleration phase. Other studies have shown that red group strains require the greatest period of adaptation.

YJS 7816 (beer) seems to have grown less than the others. Indeed, the acceleration and exponential phases seem to be confused between days 16th and 20th. There is then a stationary phase between days 20th and 23rd and then a phase of decline. Finally, throughout its growth the population increased by a factor of 7. The advantage is that we were able to observe all the stages of growth of this strain during this first kinetic.

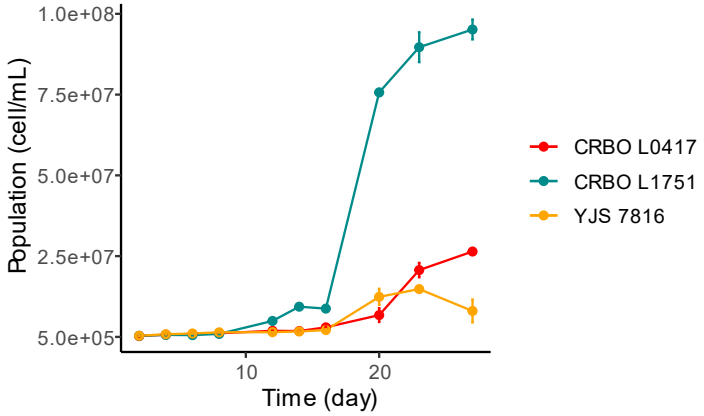

Figure 32 : Growth kinetics of 3 strains of Brettanomyces bruxellensis in NHAM.

For the N-heterocyclic compounds ETHP and ATHP (Figure 33), a link is sought between yeast growth kinetics and the production of these compounds. We can observe that all strains have the same tendency. Indeed, there is a very slight production of N-heterocyclic compounds at the time of the latency phase and the acceleration phase. On day 16th, strain CRBO L1752 has produced ATHP at concentrations close to 2000 µg/L. After the exponential phase, there is a sharp increase in production for ATHP by almost a factor of 10. Once in the stationary phase, ATHP amount drops very quickly. Regarding ETHP, only strains CRBO L0417 and CRBO L1751 produce it in observable quantities and this only from the exponential phase but also during the stationary phase.

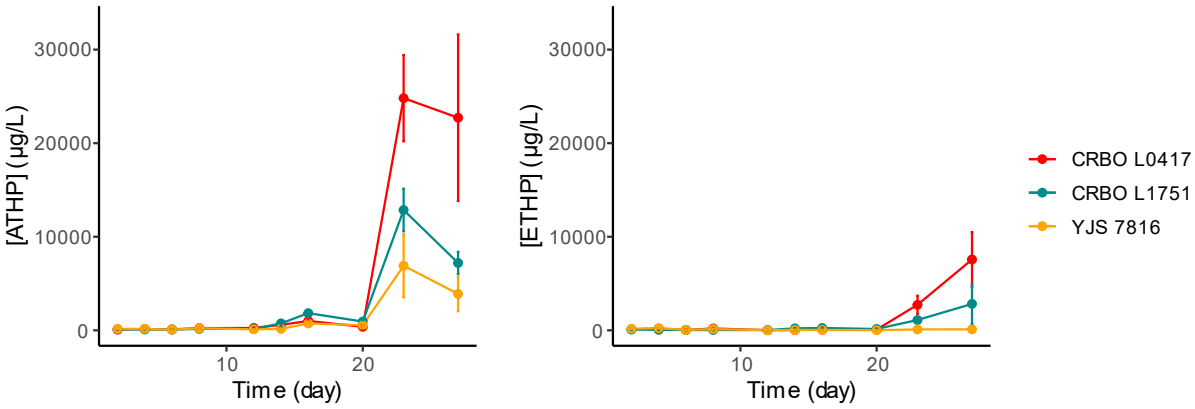

Figure 33 : Production of N-*heterocycles ATHP (A) and ETHP (B) by 3 strains of* Brettanomyces bruxellensis *in a* N*HAM during the first kinetic.*

We can therefore conclude that strains capable of the formation of *N*-heterocyclic compounds produce ATHP, from the latency phase (in small quantities) and mainly in the stationary phase. Between these two phases, the sampling method did not allow the presence of these compounds to be seen. In contrast, ETHP is formed at the beginning of the exponential phase and even during the stationary phase. Differences in the production capacity of the two compounds can therefore be observed.

Another important point is that CRBO L1751 is a strain that grows most easily in *N*HAM but does not produce the highest ATHP and ETHP levels.

However, it should be noted that the stirring from the 12th day represent a bias. Indeed, we do not have the ability to determine what the impact of agitation was on yeast growth. Similarly, we do not know the effect of this oxygenation on the mechanism of production of the mousy taint. The previous conclusions should then be verified over a longer period, without agitation or with continuous stirring from the beginning.

Grbin (1998) explored the production of ATHP and ETHP by a strain of *Brettanomyces anomalus*, for 16 days (in the presence or absence of 100 mg/L of Lysine exogenous to the cell). ATHP was produced during the exponential growth phase of the yeast and stabilized once the stationary phase was reached. The ETHP was produced only after the stationary phase had already been established.

Our first kinetics studies showed some production that could be similar to those shown by Grbin (1998) (Figure 34) with ATHP maximum concentrations once the stationary phase is reached. On the other hand, the concentrations obtained are not comparable, our medium is much richer in L-lysine (5 g/L) while in his study, its medium was supplemented with 100 mg/L. Production is done almost in parallel with the different growth phases of the 3 strains studied. However, additional studies have been conducted to remove the biases we faced during this first kinetics. This study was only a preliminary study, and it has already made it possible to highlight some interesting points. Indeed, it can be assumed

that the decrease in ATHP content on the last day is related to the reduction of the latter in ETHP. To find out, another kinetic study was done over a longer period.

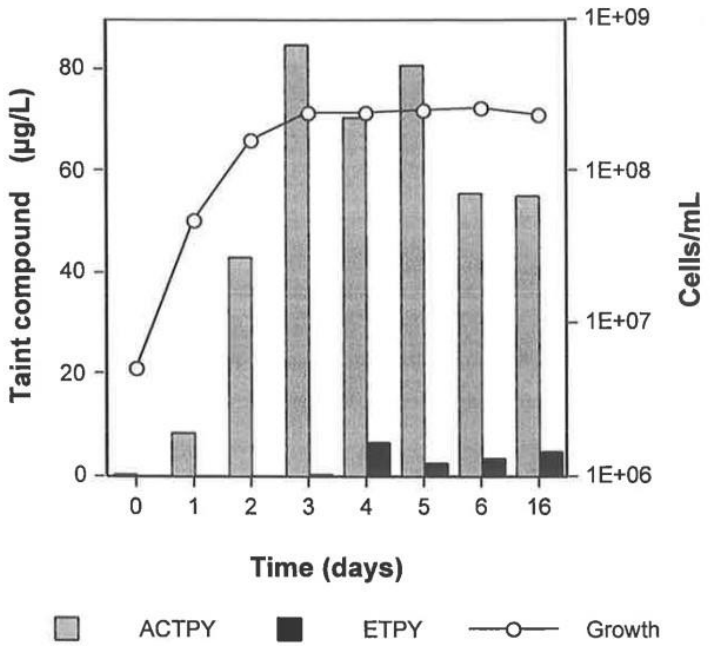

Figure 34 : Production of ATHP (ACTPY) and ETHP (ETPY) by Brettanomyces anomalus *(AWRI 1101) during growth cycle with 100 mg/L of L-lysine added to his medium (from Grbin, 1998).*

IV.3.2. Second kinetic study

The second kinetic investigation therefore focused on a longer time than the first to determine what happened after the 27th day. Would ATHP and ETHP stabilize or would there be a redox exchange between the two compounds. During this second kinetic study, we were also able to analyse by enzymatic kit several compounds present in the environment. Figure 35 shows the results of the cells (alive, dead, and total) quantification by the flow cytometry.

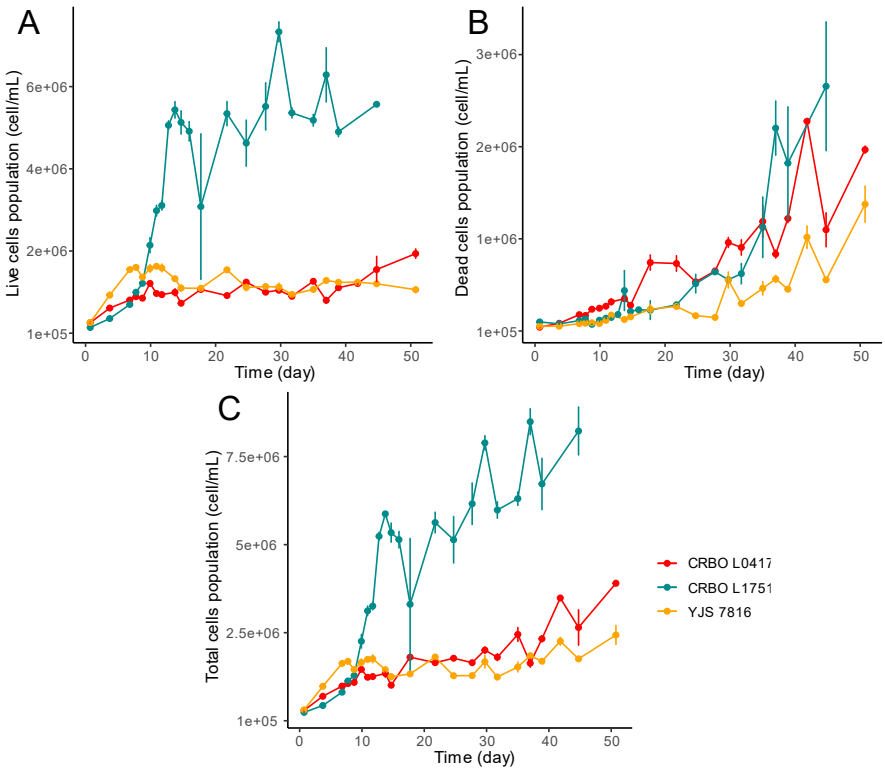

Figure 35 : Second growth kinetics of 3 strains of Brettanomyces bruxellensis *in* N*HAM;*
A*: Live cells population,* B*: Dead cells population,* C*: Total cells population*

During this kinetic study, all the tubes were stirred from the beginning. If we compare the maximum population (Table 8), it can be noticed that it is much lower, although the 3 strains of *B. bruxellensis* have taken less time to reach 5% of their total growth.

During the first kinetics the precultures were done directly in a *N*HAM while for it, the yeasts were previously cultured in an RGJ medium. Which could show a better fit in the first experiment. As the first kinetics, CRBO L1751 had the highest growth rate and population maximum level.

About the production of the mousiness, we can see that there is ultimately more than one peak in ATHP production and that it seems to disappear very quickly (Figure 36 A). The concentrations of ATHP and ETHP (Figure 36 B) found in the medium are much lower between the first kinetics and this one. This could be explained by the much lower population density than during the first survey. On the other hand, the hypothesis that it is degraded to ETHP following its production does not seem convincing here. Indeed, the samples that had been agitated since the beginning of the experiment, it might be able to induce an oxygen supply and a medium with a higher redox potential.

Table 8 : Kinetic studies parameters of B. bruxellensis *strains.*

	Strain	K_{max}[a] (cell/mL)	Lag_{φ}[a] (day)	r_{max} [a] (day)
First kinetic	CRBO L0417	2.75e07	12	23
	CRBO L1751	9.47e07	12	20
	YJS 7816	1.37e07	4	20
Second kinetic	CRBO L0417	1.95e06	0	4
	CRBO L1751	5.87e06	0	12
	YJS 7816	1.50e06	0	4
Third kinetic	CRBO L0417	7.92e06	3	17
	CRBO L1751	7.14e06	3	20
	YJS 7816	5.29e06	3	21

[a]: K_{max}, population maximum; Lag_{φ}, needed time to reach 5 % of the total growth; r_{max}, day with the maximum growth rate

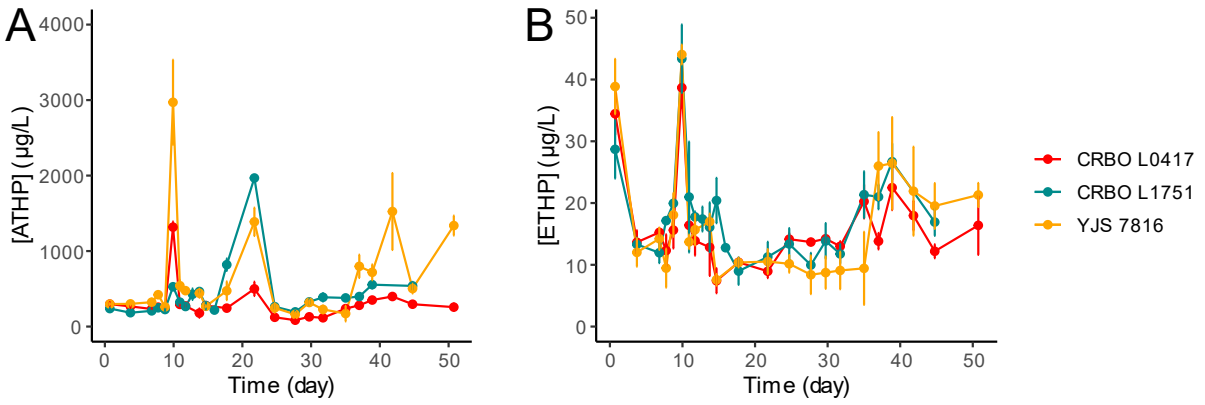

Figure 36 : Production of N-*heterocycles ATHP (A) and ETHP (B) by 3 strains of* Brettanomyces bruxellensis *in a* NHAM *during the second kinetic.*

The ATHP presents 2 different local maximums for each strain. A first maximum is observed on the 10th day. It is not the strain that grew the best that would have presented the most amount of ATHP. We may have missed them during the first kinetic investigation since we sampled the 8th day and then the 12th day. Moreover, the samples were not agitated during the first 12 days Indeed, the appearance and

disappearance of the compound is very fast, its presence in high concentration therefore seems very punctual. A second maximum was observed on the 22nd day. This time, it was CRBO L1752 (Wine 3) which presented the highest level of ATHP.

Figure 37 represents the growth rate (r) in function of the time (A) and the amount of ATHP presents in the samples (B). As described before, the CRBO L1752 had the best growth in the *N*HAM. It is interesting to compare it with the amount of ATHP in the sample. First of all, the two strains allotriploid (CRBO L0417 and YJS 7816) had a higher level of ATHP for the first pic than the second one. Conversely, the diploid strain (CRBO L1752) has its maximum production on the 22nd day. The production of ATHP seems to be correlated with specific moment of the growing culture. The first pic of ATHP may correlate with the decreasing of the growth rate (µ). It is noteworthy, by comparison of the two graphs that the pace of the curves is similar although shifted by a few days. For each maximum of growth, a maximum level of ATHP in the medium is found shortly after.

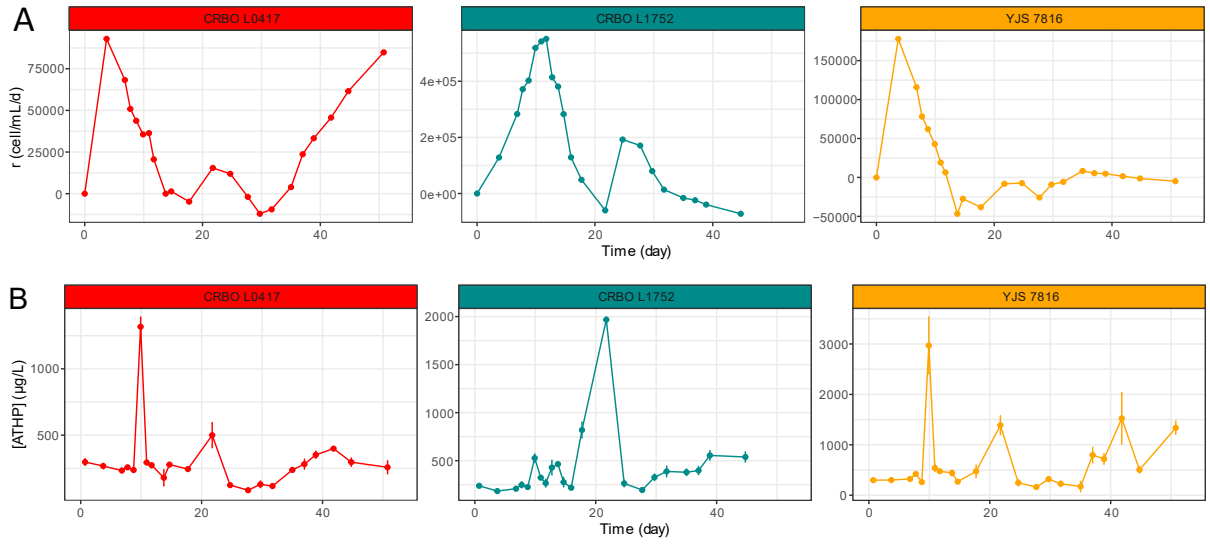

Figure 37 : Growth rate (r) and ATHP levels in function of time of 3 strains of Brettanomyces bruxellensis *during the second kinetic in* N*HAM.*

The presence of these peaks was entirely unexpected. During the first kinetics, the compound decreased on the last day, but did not disappear entirely. Similarly, Grbin (1998) showed a maximum of the compound related to an accumulation of production but the compound did not seem to disappear. We expected increasing concentrations with time and accumulation of the compound. *B. bruxellensis* is considered as a spoilage microorganism because it converts hydroxycinnamic acids from the grape berry into volatile phenols (I.1.4.3 Volatile phenols; p. 14). Kinetic work has shown that the production of compounds responsible for this alteration is cumulative (Cibrario, Perello, *et al.*, 2020).

During this second kinetic we also measured the malic acid, acetic acid, fructose, and glycerol. Results are presented in Figure 38. Malic acid (D) is almost constant and does not seem to be consumed

or produced during this experiment. Acetic acid and glycerol are mainly produced by the CRBO L0417 strain, this can be explained by its consumption of fructose. One of the characteristics of *B. bruxellensis* is its ability to produce acetic acid when it adopts fermentation metabolism in the presence of oxygen. It can also produce very small amounts of glycerol which allows it to regenerate part of the NADH pool (Aguilar Uscanga *et al.*, 2003). The more a strain ferments, the more it will tend to produce these two compounds Regarding the concentration of fructose added at an initial concentration of 50 g / L, we found lower amounts. This could be explained to a lower concentration by poor dilution at the time of analysis with the enzyme kit. It must have been saturated resulting in a poor estimate of its concentration at the time of dosage. On the other hand, the fructose consumption of strain CRBO L0417, does not seem to have any correlation with the production of the 2 compounds. Therefore, the analysis was not repeated.

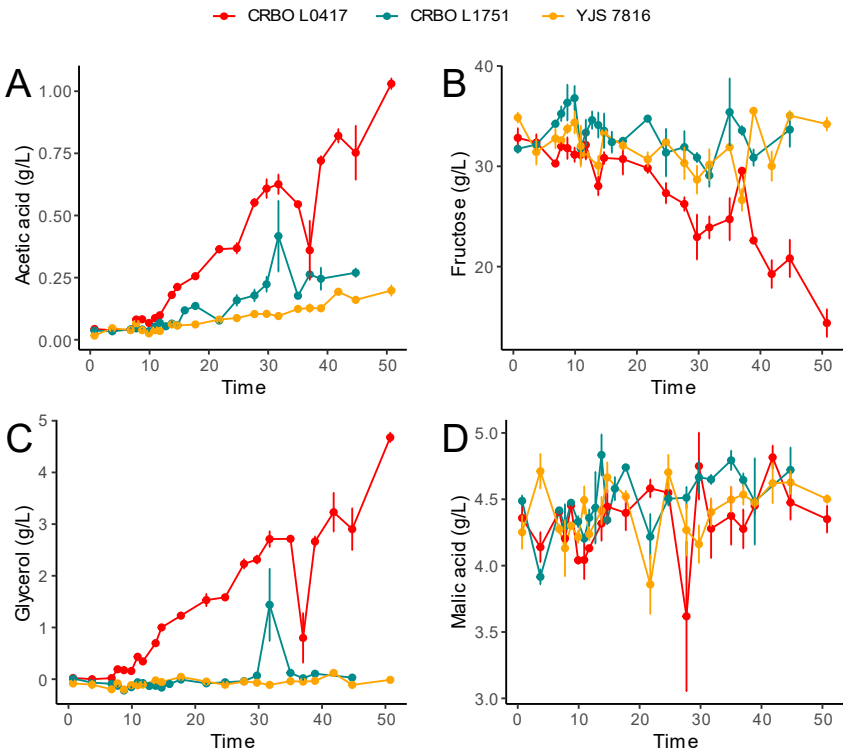

Figure 38 : Production and consumption of acetic acid (A), fructose (B), glycerol (C) and malic acid (D) by 3 strains of Brettanomyces bruxellensins *in a NHAM during the second kinetic.*

No link could be found between these substances and mousy compounds. This work was done in sacrificed tubes and not in the same sample taken regularly generate a bias. We cannot be sure that each sample has experienced almost the same evolution in microbial growth. On the other hand, it makes it possible to have no intrusive sampling. And the samples have the same ratio of air/liquid in the tubes throughout the experiment, without having oxygen inputs through the sampling procedure. Moreover, the fact of having followed these compounds throughout this second kinetics, could have shown us aberrant values on the days concerned by the ATHP peaks. No abnormal developments seem to have

occurred in the samples of days 10 and 21 days showing that the experiment was reproducible between the different tubes.

IV.3.3. Third kinetic study

During this third kinetics we increased the sampling frequency around the day of the second peak seen during the first two kinetics. It would have been interesting to check the pic at day 10th, but we did not have enough space on our shaking plate to increase the number of samples, so we preferred to check this second peak.

The kinetic parameters are presented in Table 8, value of r_{max} is not to be considered, having taken only a few points in the first days we were able to miss the exponential phase and therefore the maximum growth rate associated with these samples. On the other hand, we can note that just as in the second kinetics we do not reach the same population densities as in the first experiment. The population remains below 10^7 cells/mL.

Once again, we observe the production and disappearance of ATHP in the environments. And for the second time despite a delay of a few days, similar graphs are observed between growth rate and ATHP concentrations (Figure 39).

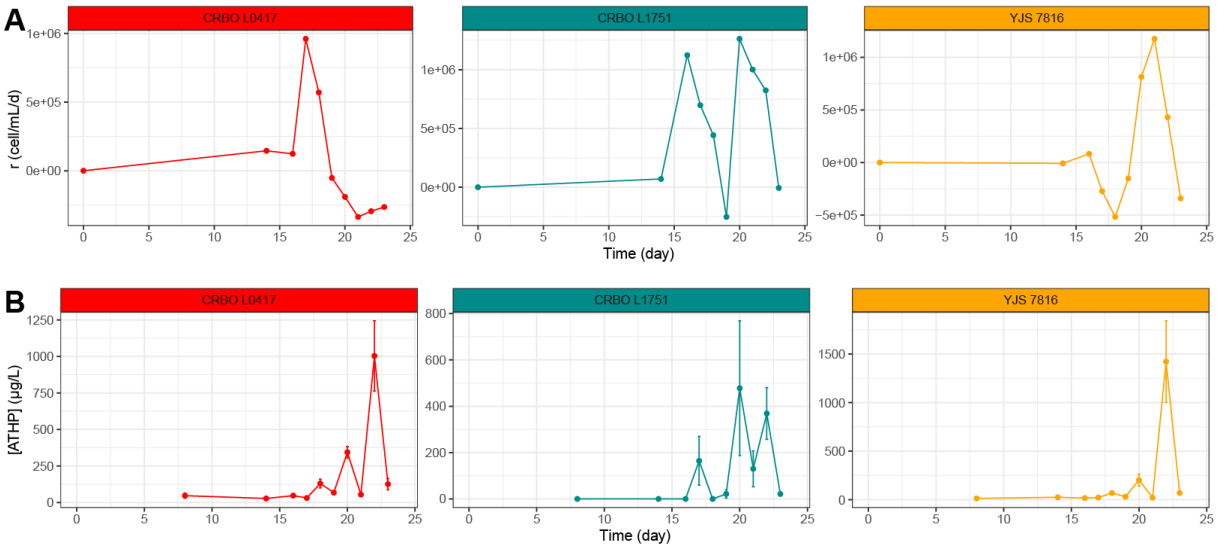

Figure 39 : Growth rate (µ) and ATHP levels in function of time of 3 strains of Brettanomyces bruxellensis *during the third kinetic in a* NHAM.

To our knowledge, no chemical compound has this kind of production/consumption curves in wine. We can only speculate. The fact that the compounds are found in maximum concentrations at the time of the drop-in growth rate is astonishing. This could correspond to a signal or a response to a signal between the different cells in order to alert the slowing of microbial growth, the appearance of stress. Quorum sensing (QS) is mainly studied in bacteria and is now more than recognized as a chemical

communication ability between different microorganisms impacting their genetic regulation (Miller & Bassler, 2001). QS signals differ depending on its nature, its mechanisms, and the target genes. In every case it allows a communication between different bacteria and can result in a modification of the behaviour, of the entire community. It is mainly studied in bacteria for the ability to form some biofilm.

This communication between individuals of the same species is much less studied in yeasts although fungal QS systems has been revealed. Farnesol (acyclic alcohol $C_{15}H_{26}O$) has been shown to control the behaviour of some microorganisms. It can control the ability to form mycelia in the species *Candida albicans,* this compound is naturally produced by the yeast strain. In low concentrations, the microbial population will form mycelia. Once the population density is well increased, farnesol will continue to accumulate. Once it is in large quantities, the yeasts grows as budding yeasts (Hornby *et al.*, 2001). The effect of this compound has also been noticed on *S. cerevisiae* yeasts, inhibiting yeast growth without compromising its viability (Machida *et al.*, 1998, 1999). It is not the only compound with a possibility of action on the behaviour of this yeast. The work of Chen & Fink (2006) showed that other aromatic compounds, as phenylethanol, secreted by *S. cerevisiae* could control morphogenetic changes in yeast going from an invasive filamentous form to a budding yeast form. Chen & Fink (2006) also showed that this production is linked to nutrient deficiencies in the environment.

Several studies focusing on *B. bruxellensis* have shown its polymorphism and its ability to bioadhere (Le Montagner *et al.*, 2023; Lebleux *et al.*, 2020). Using flow cytometry, other factors are measured by the device. It also measures certain characteristics of cells, such as size or shape. (Figure 40). Forward scatter (FSC) is based on two properties, the cell size, and its refractive index. Side scatter is based on the cell granularity or internal complexity. We tried to determine whether there was a link between cell morphology and N-heterocyclic compounds. No correlation could be demonstrated between these both size and shape of the cell with ATHP compound.

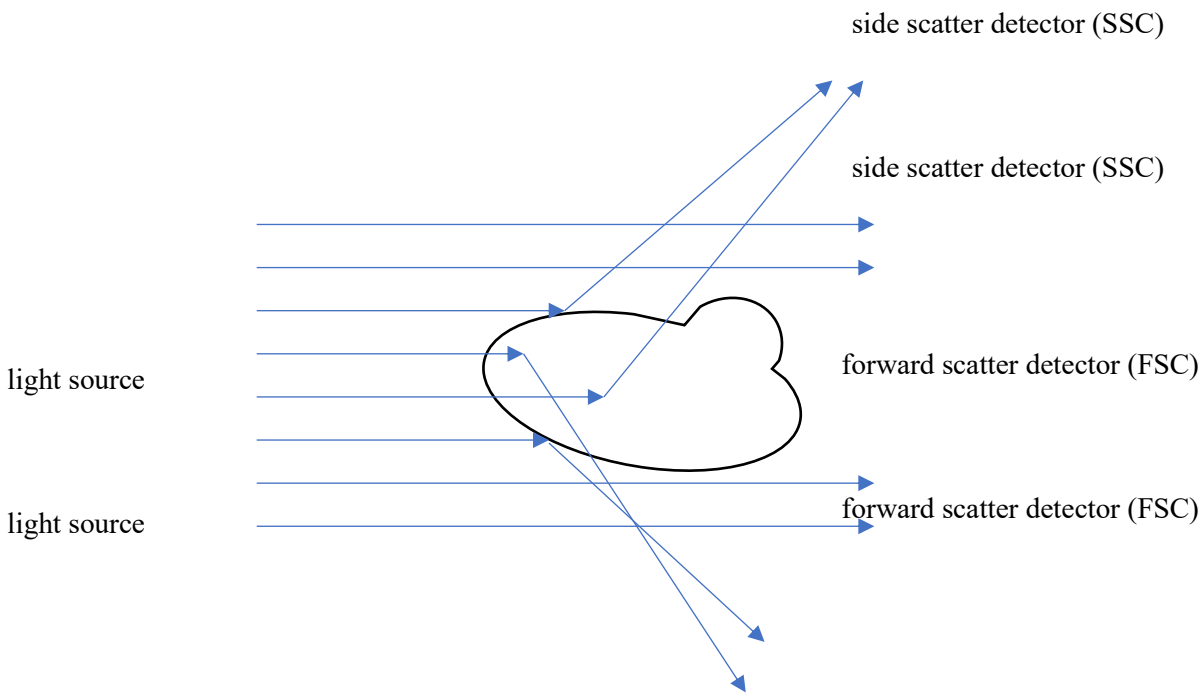

Figure 40 : Principles of flow cytometry; FSC measuring cell size; SSC measuring cell complexity.

IV.4. Ugni blanc

Saccharomyces cerevisiae has never been linked to the production of mousiness. Even if they must be confirmed, the results of co-inoculation trials and the presence of *S. cerevisiae* question on its potential impact. The production of 5 different microorganisms (2 *B. bruxellensis,* 1 *L. hilgardii,* 2 *O. oeni)* was therefore examined in different matrices, a Ugni blanc must, and a Cabernet Sauvignon must, each must be associated with 2 wines, fermented either by the GN strain or by the SB strain.

Work on Cabernet Sauvignon is still ongoing and will not be discussed here, so only the results obtained on Ugni blanc will be presented.

To get as close as possible to œnological practices, the Ugni blanc (collected in Charente area, France) must has deliberately not been pasteurized, filtered, stabilized. The kinetics of alcoholic fermentations are presented in supplementary data. The monitoring of the various producing microorganisms was carried out on the samples, but no implantation control was carried out on them. Additionally, since the wine had not undergone any microbial stabilization, significant indigenous populations of microorganisms were present. These results will not be discussed here. The experiment on Cabernet Sauvignon was treated differently in order to obtain more relevant microbiological results.

Once the alcoholic fermentations finished, the 3 different matrices of Ugni blanc, were supplemented by populations (5.10^5 cells/mL) of *B. bruxellensis*, *L. hilgardii*, or *O. oeni*. The 3 different compounds production was followed at different times (3 days, 9 days, 18 days, 24 days). APY concentrations found

in samples are presented in Figure 41. Similarly, the levels of ETHP and ATHP are shown in the appendix, APY was the compound mainly produced in this experiment. These results are surprising, APY is a compound very rarely found in wines usually (*Cf*: II.1 Quantification of the *N*-heterocycles in wines by SBSE-GC-MS; p. 41). Similarly, under model conditions with the precursors known to date, this compound cannot be produced by *B. bruxellensis*. APY concentrations found are then surprising.

First of all, we can notice that APY is not produced when no alcoholic fermentation takes place. Several authors had previously identified ethanol as a precursor to N-heterocyclic compounds.(Grbin *et al.*, 1996; Heresztyn, 1986; Tucknott, 1977). And a production of APY is observable after fermentation. It seems that this production would be more important after the strain of *S. cerevisiae* SB than GN strain. During the co-inoculation experiments, SB strain seemed to show a synergic effect on ATHP production by *B. bruxellensis* in *N*HAM (*Cf*: III.3.3 Exploration of the interaction including other yeast/bacteria pairs; p. 93). Although preliminary results on the effect of co-inoculation, the three strains of ATHP produced by *B. bruxellensis* tended to be superior in the presence of strain SB.

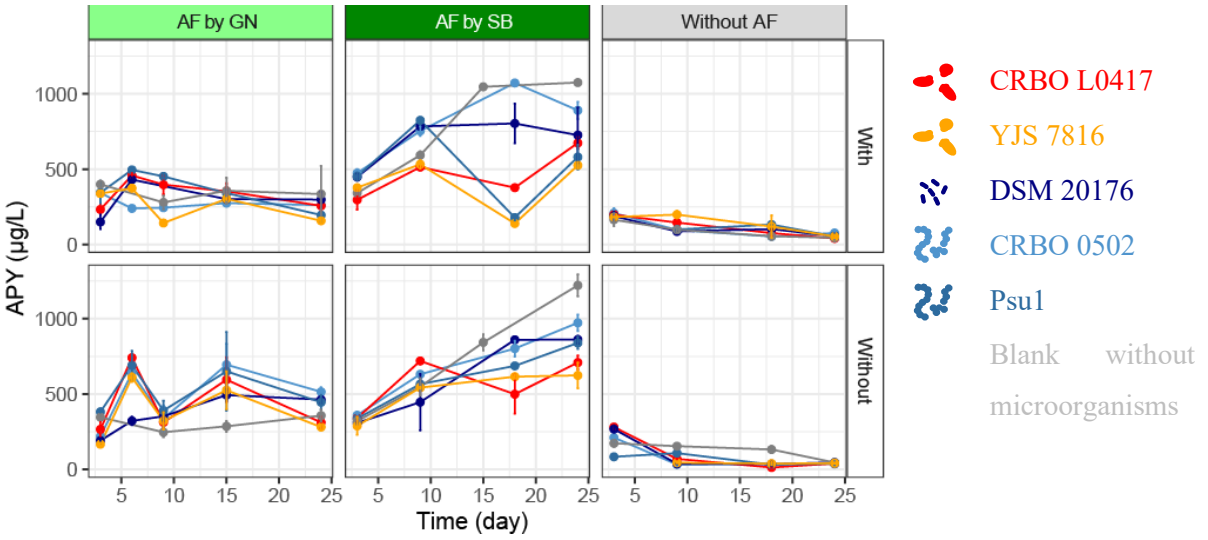

Figure 41 : APY levels in µg/L in function of the Saccharomyces cerevisiae *strains used for the alcoholic fermentation (GN, SB), the stirring action (with or without) and the time by 5 different microorganisms; (2* Brettanomyces bruxellensis*: CRBO L0417 and YJS 7816; 1* Lentilactobacillus hilgardii*: DSM 20176; 2* Oenococcus oeni*: CRBO 0502 and Psu1) and a blank only with the indigenous flora.*

Another element is noteworthy, the indigenous microbial population seems to have produced more APY than the microorganisms we inoculated into the medium (grey line, AF by SB, Figure 41). We can also hypothesize, that the addition of our microorganisms would have inhibited the production of the compound by native microorganisms. We can also hypothesize, that the addition of our microorganisms would have inhibited the production of the compound by native microorganisms.(*Cf*: III.3.2 *Brettanomyces bruxellensis* and *Pediococcus parvulus*; p. 90). Having no information on the indigenous population, we will not be able to go further on these discussions.

If we are interested now, in the explained variance of the different compounds produced. A summary table of the analysis of variance (ANOVA) of APY, ATHP and ETHP for each day is presented in supplementary data. Figure 42, represents a stacked histogram of the variance explained by the different factors on the production of the mousy compounds on day 24th. The first notable element is that the production of different compounds is not explained by the same variables. The variations of APY found in the wine, on this last day, is explained at 60.4 % by the alcoholic fermentation and the *S. cerevisiae* strain used. However, this factor explains only 15 % of the production of ATHP and ETHP. The other factor impacting the production of APY is the strain supplemented in the medium (25.5 %). The variability of APY is therefore mainly explained by simple factors (85.9 %) while ATHP production is related to interaction impacts between different factors (44.4 %) thus showing that the producing microorganisms will not behave in the same way depending on the strains of *S. cerevisiae* (11 %). For ETHP, and for ATHP, a significant part of the variability is not explained by our different factors (respectively 40.8 % and 36.1 %).

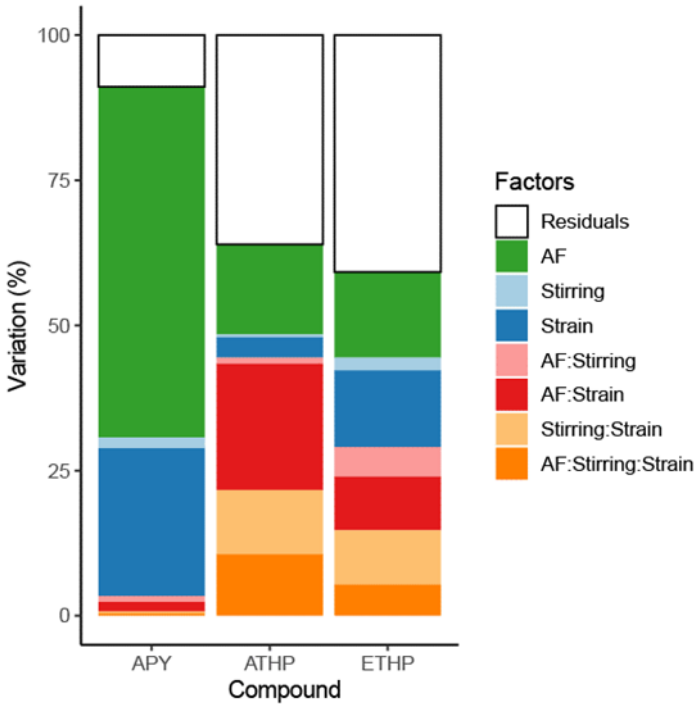

Figure 42 : APY, ATHP, and ETHP variabilities, on the day 24, explained according to the yeast strain used for the alcoholic fermentation (AF), the stirring, the producer microorganisms (strain), and the different interactions.

In order to graphically represent the different samples, the concentrations obtained for each compound were normalized by the maximum production obtained. Then the average concentration of microbiological triplicates was made. Figure 43 represents the relative production of APY, ATHP and ETHP by 5 different microorganisms and the indigenous flora, stirred or not, in function of the *S.*

cerevisiae strains used for the AF. The production of ETHP does not appear to be impacted by the yeast strain used in AF. The proportions of ETHP produced are equivalent for SB and GN strains. The two strains of *S. cerevisiae* differed in the amounts of ATHP and APY found in the samples after 24 days. As a result, both strains of *S. cerevisiae* are well separated on this graph. After fermentation of the SB strain, the amount of relative APY was proportionally higher than in the wines fermented with the GN strain.

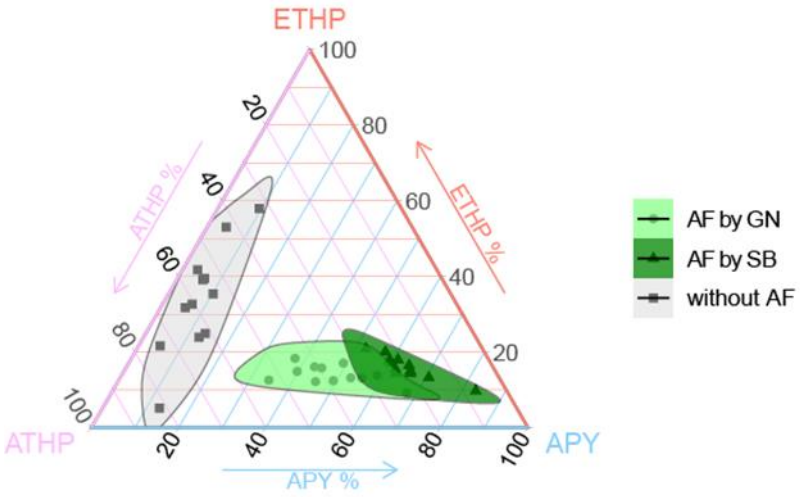

Figure 43 : Ternary plot representing the proportion of the relative amount of APY ATHP and ETHP produced by 5 different microorganisms (2 Brettanomyces bruxellensis*: CRBO L0417 and YJS 7816; 1* Lentilactobacillus hilgardii*: DSM 20176; 2* Oenococcus oeni*: CRBO 0502 and Psu1) and a blank only with the indigenous flora, stirred or not, in function of the* Saccharomyces cerevisiae *strains used for the alcoholic fermentation, after 24 days.*

This representation is only graphic and scientifically provides only few information. A ternary plot (or ternary diagram) is a graph that allows the representation of the relative proportions of three variables. The coordinates of each axis correspond to the proportions of each variable divided by the total value (sum of the values of the three variables):

$$P_x = \frac{V_x}{\sum_{x,y,z} V} \text{ x } 100$$

P_x: proportion of x in %

V_x: value of x

If the obtained concentrations were used directly for the creation of the ternary plot, the graph would not have shown anything. Given that the concentration of APY obtained was significantly higher than those of the other two compounds, the proportions of ATHP and ETHP were negligible.

The results presented represent the proportion of the relative amount of each compound, normalized by the maximum concentration produced. For example, if a strain had been producing all three chemical compounds at maximum capacity, after normalization, the results would show 100% for APY, 100% for

ATHP, and 100% for ETHP results. The creation of the ternary plot will therefore place the previous example at the centre of the triangle with proportions of 33% on each axis ($P_x = \frac{100_x}{100_x + 100_y + 100_z}$ x $100 = 33.3$ %) .

This example is represented by a black dot on Figure 44. This figure illustrates how to read the ternary plot. The coordinates should be compared to a central point which represents the relative production of each equivalent compound. This equivalent production between the three compounds can be maximum, minimum, or average. The previous example was a strain that had produced all three compounds at maximum capacity. Another example could have been a sample that does not present any of the three compounds, which would have the same spatial representation as the previous one.

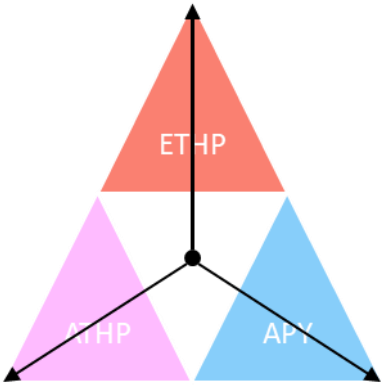

Figure 44 : Ternary plot representing the medium plot (33.3 % APY, 33.3 % ATHP, and 33.3 % ETHP) and 3 zones of surepresentation of one of the compounds.

The results are also represented in a 3D scatter plot with APY, ATHP, and ETHP concentrations on the axes. Figure 45 shows two views of this representation. It is noteworthy that samples that have not undergone alcoholic fermentation are only represented in terms of ATHP/ETHP. Therefore, APY was not produced in these samples. As with the ternary plot representation, the impact of the two strains of *S. cerevisiae* is very noticeable. The post-SB trials contained significantly more than the 3 compounds (especially ETHP and APY). After alcoholic fermentation of Ugni blanc, APY was produced in large quantities in all samples. Once again, correlations emerge from these results. Regarding the obtained results after AF, there is a significant correlation between the amount APY and ETHP (p-value = 0.006793) with a Spearman $\rho = 0.73$.

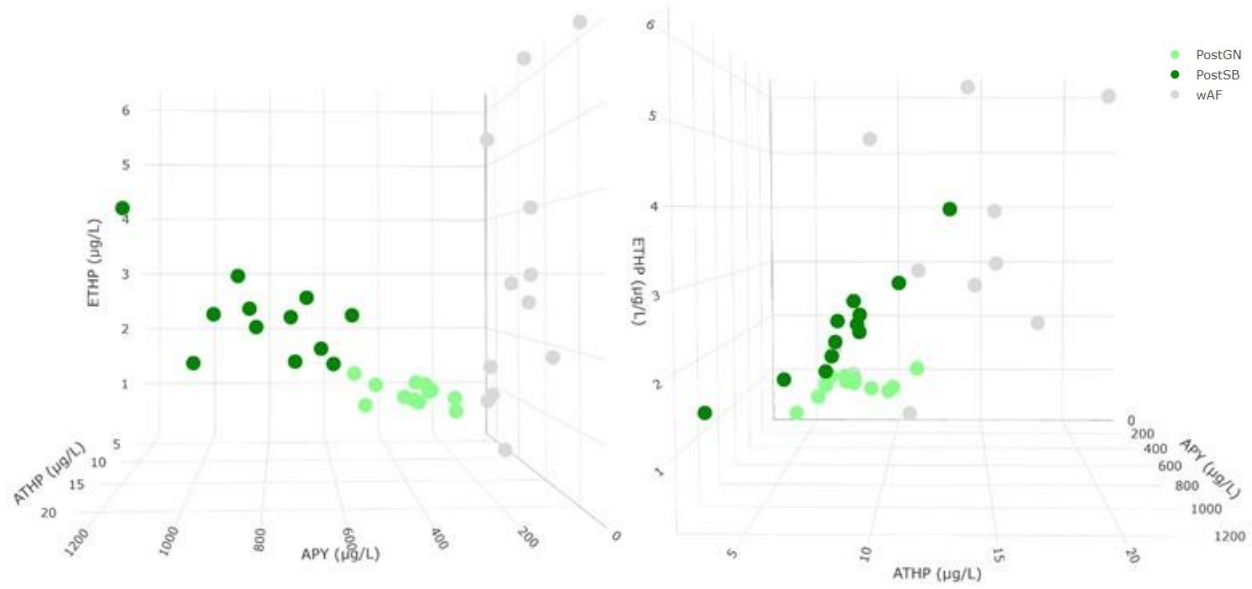

Figure 45 : Scatter 3D plot representing the produced concentration of APY ATHP and ETHP produced by 5 different microorganisms (2 Brettanomyces bruxellensis: *CRBO L0417 and YJS 7816; 1* Lentilactobacillus hilgardii*: DSM 20176; 2* Oenococcus oeni*: CRBO 0502 and Psu1) and a blank only with the indigenous flora, stirred or not, in function of the* Saccharomyces cerevisiae *strains used for the alcoholic fermentation (PostGN; PostSB; wAF: without alcoholic fermentation), after 24 days; each point represents the average of microbiological triplicates.*

As discussed from Figure 41, APY here was mainly produced by the community of indigenous microorganisms. When microorganisms were added to the must, APY production decreased. The concentrations obtained are much higher than those usually found in wines. We have no explanation for that. The strain factor (representing the producing microorganisms inoculated post AF; Figure 42) is significant and explains 6.3 to 25.5% of its variability over the 4 days of sampling (Appendix 10).

Regarding APY, the FA factor (including alcoholic fermentation and the strain of *S. cerevisiae* used) explains most of its variability throughout the experiment (30.1 % on the third day and > 58 % thereafter; Appendix 10).

This is the first time that *S. cerevisiae* has been shown to have an impact on mousy taint production. These observed differences may be chemical, so the SB strain could have produced more favourable conditions for the development of mousy off-aromas. Analyses are underway to chemically compare these two wines (Post SB and Post GN). It would also have been interesting to look at the microorganisms present in the uninoculated samples to identify them and to know which microorganism could have produced N-heterocyclics.

To confirm these results, the experiment is repeated in a must of Cabernet Sauvignon. The experiment was therefore treated differently to have more relevant microbiological results. The stirred tests were replaced by a modality stabilized microbiologically at 80° C for 30 min. In addition, implantation checks are carried out on each sacrificed sample. And microorganisms present in non-inoculated samples are identified using MALDI-TOF MS Biotyper (Bruker, Karlsruhe, Germany).

IV.5. Conclusion

In this chapter we are mainly interested in the production of different microorganisms over a longer period than in the previous part. The kinetics in the model medium were able to highlight peaks of presence of ATHP produced by *B. bruxellensis*, these peaks were present when the growth rates dropped, i.e., at the beginning of the stationary phases. A final kinetics is underway, this time in a Merlot microbiologically stabilised must, in order to compare the results obtained for the 3 strains of *B. bruxellensis* in model and œnological environments.

In a second step, the production of different microorganisms was studied in a Ugni blanc must and two associated wines (fermented by 2 strains of *S. cerevisiae*). The control sample without added microorganisms showed the highest levels of APY production. To better control the microbial population, the same manipulation will be carried out in a matrix of Cabernet Sauvignon, with a batch of pasteurized must. Tests on white Ugni show a real impact of the *S. cerevisiae* strain on the production of mousy *N*-heterocycles after fermentation.

General conclusions

One of the main challenges of œnology is to have a better knowledge of defects in order to avoid all possible organoleptic deviations in wines. The mousiness is one of them. A better understanding of the phenomena related to the production of mousy compounds in wines will allow to assess the risks of wine contamination.

What are the methods today to analyse mousiness?

Until then, assay methods existed to quantify N-heterocyclic compounds separately. SBSE-GC-MS quantification methods have been developed in wine and *N*HAM. These methods make it possible to quantify simultaneously the APY, the ATHP, the ETHP. Although these methods allow less handling time, machine time remains important. We also looked at the presence of the compound AP. It has never been found in our wines and was therefore removed from our method. Recently, a method of 2-acetyl azaheterocycles by UHPLC-MS/MS has been developed and has shown its effectiveness in different matrices, such as coffee, popcorn, roast-beef sauce, cashew (Bösl *et al.*, 2021). It would be interesting to see if this method by derivatization can be applied in wine to save machine time and sensitivity.

These methods were essential for the smooth running of this thesis, but they are also helpful for professionals in the oenological field. Today, only a few laboratories in France and in the world offer the analysis of these compounds in wine. Indeed, to our knowledge, AWRI offers a method for the determination of ATHP developed by Hayasaka (2019).

During this thesis, we could not work on the sensory character of this defect. Although this is an essential focus of oenology research, we wanted to provide more knowledges about the production of the defect and not about its organoleptic impact. It would be necessary to continue the work started by Tempère *et al.* (2019). The aromatic complexity of this defect is now recognized, and its descriptors are multiple. This is explained by the differences in sensitivity due to oral physico-chemical characteristics of each taster. Despite this, after tasting several series of mousy wines during this project, It can be assumed that there is not one mousy off-aroma but several mousy taints with different notes. It would have been interesting to investigate the comparison of the mousiness perception of the 74 different wines analysed in the “II.1 Quantification of the *N*-heterocycles in wines by SBSE-GC-MS”. In this study, we classified the wines into four different clusters according to the compounds found in the wines, would these have been described differently? No work has been done on possible perceptual interactions between the 3 compounds.

What is to blame for the upsurge in mousy off-aroma?

Historically, the origin of this alteration has been discussed at length. In recent decades, and mainly since the work of Tucknott (1977), the defect has been studied primarily from a microbiological point of view. During this project, we were able to study the ability to produce mousy compounds by different microorganisms, and thanks to a collaboration we investigated the impact of oxygen concentrations.

Among all the microorganisms studied, isolated from wine or taken out of collection, all strains of *Brettanomyces bruxellensis, Lentilactobacillus hilgardii* and *Oenococcus oeni* have the ability to produce *N*-heterocycles in a model medium. One of the initial goals of the thesis was to search for producing and non-producing individuals within the same species to use comparative genomic methods. Unfortunately, our results did not allow us to explore proteomic or genetic pathways. Indeed, no link could be shown either between the genetic material of these strains or the phylogenetic groups already pre-existed (Avramova, 2018; Lorentzen & Lucas, 2019) and the tested strains.

The study on mousy wines was able to show that in only 20% of the wines studied, strains of *B. bruxellensis* were isolated, meaning that in 80% of cases, the wines did not contain significant quantities, suggesting that the alteration may have been produced by something else. Large variations persist in strain production, although the conditions and the number of cells inoculated into *N*HAM media were controlled. It could be that other microbial factors impact production, such as the physiological state of microorganisms at the time of the screening. Chemical factors, such as the oxygen rate, or the pH, may also have influenced these variations.

During a collaboration in the laboratory, we were able to show that oxygen had a real impact on the production of ATHP. The presence of microorganisms in the wines prevents us from concluding on a production only by chemical means. This issue of oxidation is omnipresent in wines without the addition of sulphite. Regarding the defect, the winegrowers talk about its appearance and disappearance once the fermentations are finished. These behaviours are not yet explained; however, winegrowers make the parallel between these fluctuations and stages of winemaking where the wines are exposed to oxygen. The study of the impact of oxygen must continue, in order to provide more answers to professionals in the sector.

Are there factors that can increase the mousy taint production risk by microorganisms?

Certain factors could influence the risk of spoilage. The oxygen described above is one of them. The described precursors are present in wines and in sufficient concentrations to form spoilage.

Although results obtained with co-inoculation tests as well as with Ugni blanc trials must be confirmed, they tend to show that the strain of *Saccharomyces cerevisiae* could influence the concentrations of *N*-heterocycles subsequently produced. The same study that was carried out with Ungi blanc is being done for another grape variety, Cabernet Sauvignon. Investigations considering more strains of *Saccharomyces cerevisiae* by including industrial yeasts could be very interesting to determine if the selection factors could have influenced strains less at risk than others.

Studies on the physico-chemical properties of wines fermented by different strains of *Saccharomyces cerevisiae* could lead us to determine new physico-chemical parameters influencing the production of N-heterocyclic compounds.

Other co-inoculation trials were done. In synthetic medium, synergistic effects on ATHP production could be observed while APY production was inhibited when *Brettanomyces bruxellensis* was co-inoculated with strains of *Pediococcus parvulus.* In real conditions, microorganisms are never found alone in wine. Each microorganism tested presented different productions when inoculated alone and accompanied. Further research work on these interactions are needed. In standardized conditions, we have shown that 20 strains of *Oenococcus oeni* from collections, have the ability to produce mousy compounds in different quantities. Can the production of compounds by *Oenococcus oeni* in a wine depend on the strain of *Saccharomyces cerevisiae* previously used? This question remains suspended, moreover, co-inoculation work has been carried out between these two species of microorganisms in a standardized medium. The effect of the two microorganisms in *N*-heterocycles assay medium was explored, for this, the two microorganisms were inoculated together or sequentially. This work was not discussed in this project, unfortunately, we did not obtain any treatable results due to technical issues.

Do we know why and when microorganisms produce these compounds?

During this work, we worked on kinetic studies over a long period of time. Whether in the samples of Ugni blanc, where we were able to show that the strain of *Saccharomyces cerevisiae* used during alcoholic closure could influence the production of mousy compounds afterwards. During this manipulation, the must has not been pasteurized, filtered, or stabilized, leaving all its native microorganisms. Our control was therefore a non-innocent sample, leaving the initially present populations free to develop by the way it was in its samples that the concentrations of mousy compound were the highest.

Production kinetics over longer times were studied in a model medium. These have shown that the kinetics of regulations in the presence of *Brettanomyces bruxellensis* strains of the different compounds are more complex than imagined. Indeed, the production is not linear, it would look more like peaks of ATHP punctual presence. These peaks were present when the growth rates dropped, i.e., at the beginning of the stationary phases. The same kinetics are underway in a Merlot wine to determine if the production profile will be similar.

Do we know how to remove the mousiness from wine?

The main objective was to have a better understanding of wine spoilage in order to apprehend it more effectively. This step is essential before moving forward.

Multiple actors within the wine industry, including technical institutions, have tested over 50 potentially curative treatments by varying their application conditions. Among these tests, chitosan, aeration, plant proteins, PVPP adhesives, etc., were tested to determine their effect on the presence of mousy taint in wines. None of the treatments proved conclusive results in their trials (GroupeICV, 2023).

Once present, there is currently no definitive solution to eliminate the mousy taint from wine.

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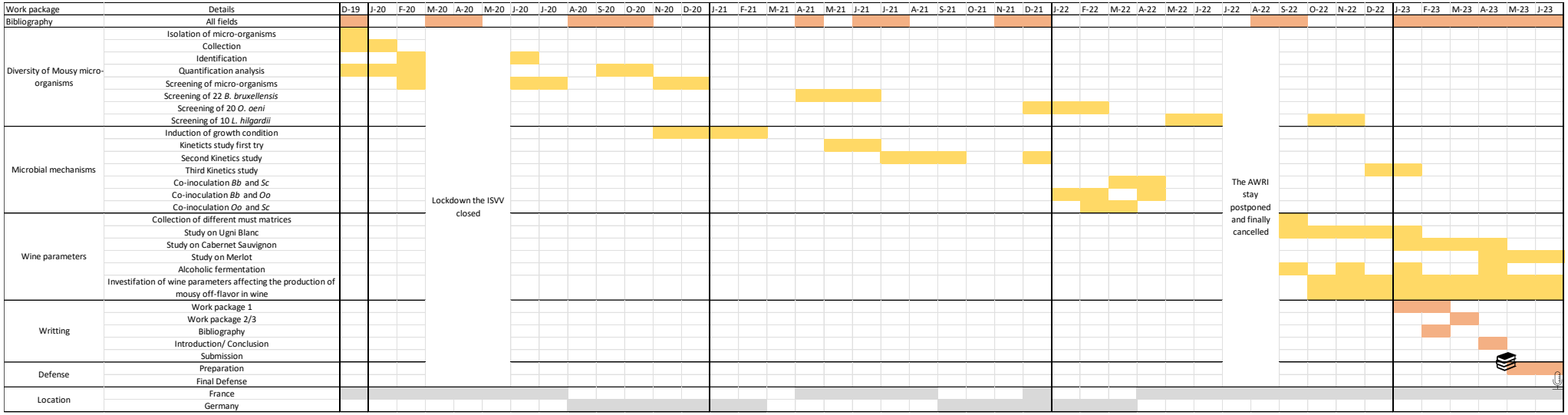
Appendices

Appendix 1 : What was scheduled in the initial workflow.

		Task 1 done by the phD student at Bdx ***Task 1** finalized by a M1 student at Bdx, if necessary					**Task 2-1** done by the phD student				**Task 2-1** done by the phD student **Task 2-2** done by the phD student						**Tasks 2-2** and **2-3** done by the phD student (with the help of a M1 student for **T2-3**) **Task 3** done at Bordeaux (*** T3-1 and T3-2** done by a technician before phD student stay						**Task 3-3** done by the phD student				**Task 2-2** (if necessary) **Tasks 2-4** and **4-1** done by the phD student				**Task 4** done by the phD student					Redaction		
	Planning stays in the different research centers	Bordeaux					Geisenheim				Adelaïde						Bordeaux						Geisenheim				Bordeaux				Geisenheim					Bordeaux		
	beginning of the thesis in December 2019	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov			
		Year 1												Year 2																	Year 3									
Tasks/months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36			
Task 1: Characterization of the diversity of wine microorganisms producing mousy off-flavor		/////	/////	/////	/////	/////	// *//	// *//																															
T1-1	Collect of red and white mousy wines																																						
T 1-2	Isolation of bacteria and yeasts from wines						*																																
T 1-3	Screening of micro-organisms for their ability to produce mousy off-flavor						*	*																															
T 1-4	Identification and collection of wine microorganisms producing mousy compounds						*	*																															
Task 2: Elucidation of the microbial mechanisms leading to the production of mousy compounds							/////	/////	/////	/////	/////	///	/////	/////	/////	/////	/////	/////	/////	/////	/////	/////	/////	/////	/////	/////	/////	/////	/////	/////									
T2-1	Searching for compounds that can contribute to mousy off-flavor in wines (other than the 3 known)																																						
T2-2	Comparative genomic analyses																																						
T2-3	Phenotypic analyses																																						
T2-4	Transcriptomic analyses																																						
Task 3: Determination of key parameters of mousy-off flavor occurrence													//*//	//*//	//*//	//*//	/////	/////	/////	/////	/////	/////	/////	/////	/////	/////													
T3-1	Collection and elaboration of different wine matrices												*	*	*	*																							
T3-2	Selection of ten representative strains of wine microorganisms producing mousy off-flavor												*	*	*	*																							
T3-3	Investigation of wine parameters affecting production of mousy off-flavor																																						
Task 4: Development of diagnostic tools to assess the risk of mousy-off flavor production in wines																											/////	/////	/////	/////	/////	/////	/////	/////	/////				
T4-1	Methods for detection wine microbial strains producing significant mousy off-flavor																																						
T4-2	Methods for quantifying determining precursors of mousy off-flavor in wines																																						

Appendix 2 : What have been done during this thesis.



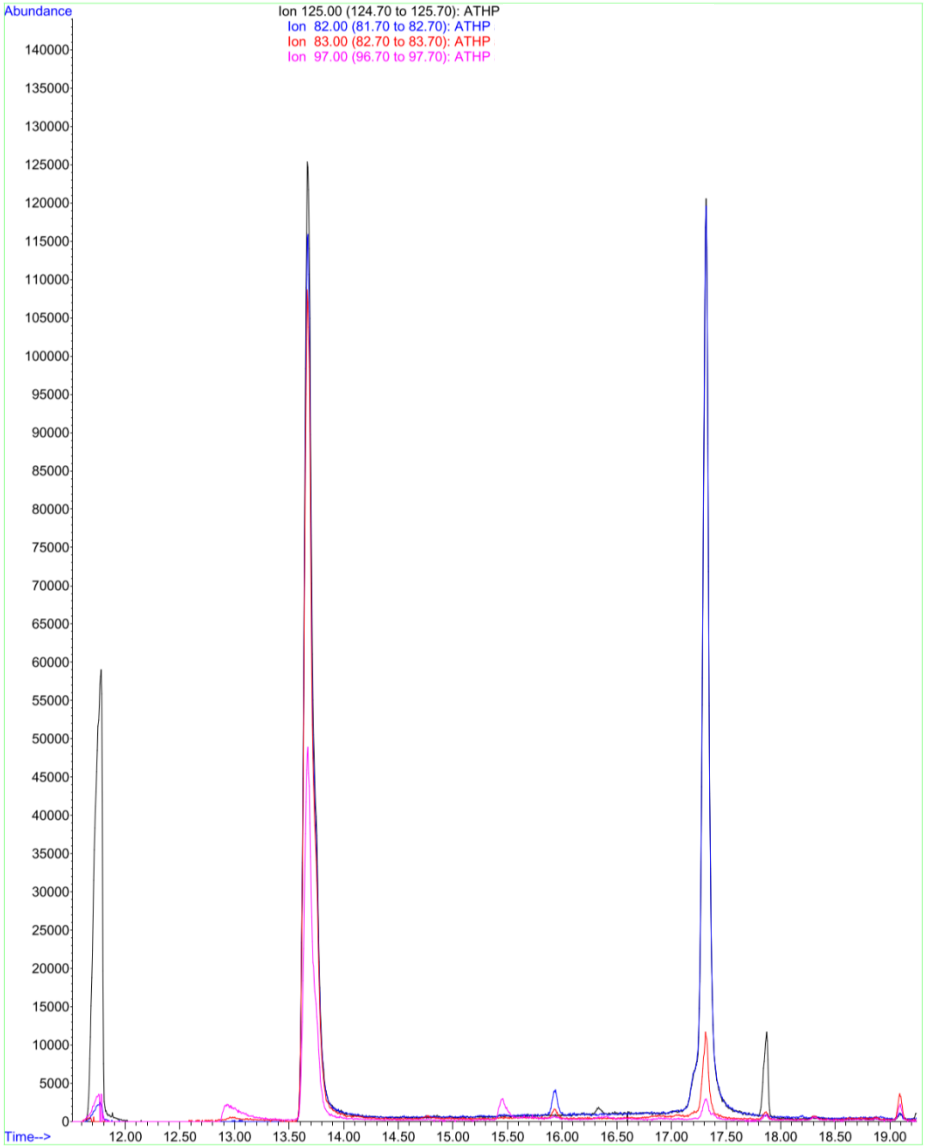
Appendix 3 : Performance parameters of the analytical method for white, rosé and red wine.

		White wine							Rose wine							Red wine						
Compound	Linear range ($\mu g.L^{-1}$)	r^2	slope	Fcalc [b]	LOD [a]	LOQ [a]	Recovery (n = 3) (%)	RSD[a] (n = 3) (%)	r^2	slope	Fcalc [b]	LOD [a]	LOQ [a]	Recovery (n = 3) (%)	RSD[a] (n = 3) (%)	r^2	slope	Fcalc [b]	LOD [a]	LOQ [a]	Recovery (n = 3) (%)	RSD[a] (n = 3) (%)
APY	3.6 – 71.3	0.9947	0.0008	0.0016	0.8	2.7	103	10.6	0.9924	0.0004	0.0007	0.9	3.1	88	11.0	0.9962	0.0007	0.0043	0.5	1.8	101	4.8
ATHP	5.3 – 105.0	0.997	0.0028	0.0009	-	-	104	6.8	0.998	0.0027	0.0006	-	-	100	2.5	0.9974	0.0031	0.001	-	-	93	3.4
A-3,4,5,6-THP		0.997	0.0028	0.0052	0.8	2.6	102	2.9	0.9689	0.0029	0.0332	0.9	3.2	110	3.7	0.9918	0.0033	0.0346	1.5	4.9	94	14.8
A-1,4,5,6-THP		0.9868	0.0029	0.0008	5.2	17.5	104	12.9	0.9868	0.0024	0.0067	5.0	16.8	89	5.4	0.9949	0.0029	0.001	10.7	36.0	112	5.1
ETHP	14.1 – 282.7	0.9979	0.0079	0.0016	0.6	2.0	96	5.1	0.9986	0.0049	0.0005	1.2	4.2	110	5.1	0.9996	0.0052	0.0004	2.7	8.9	103	5.3

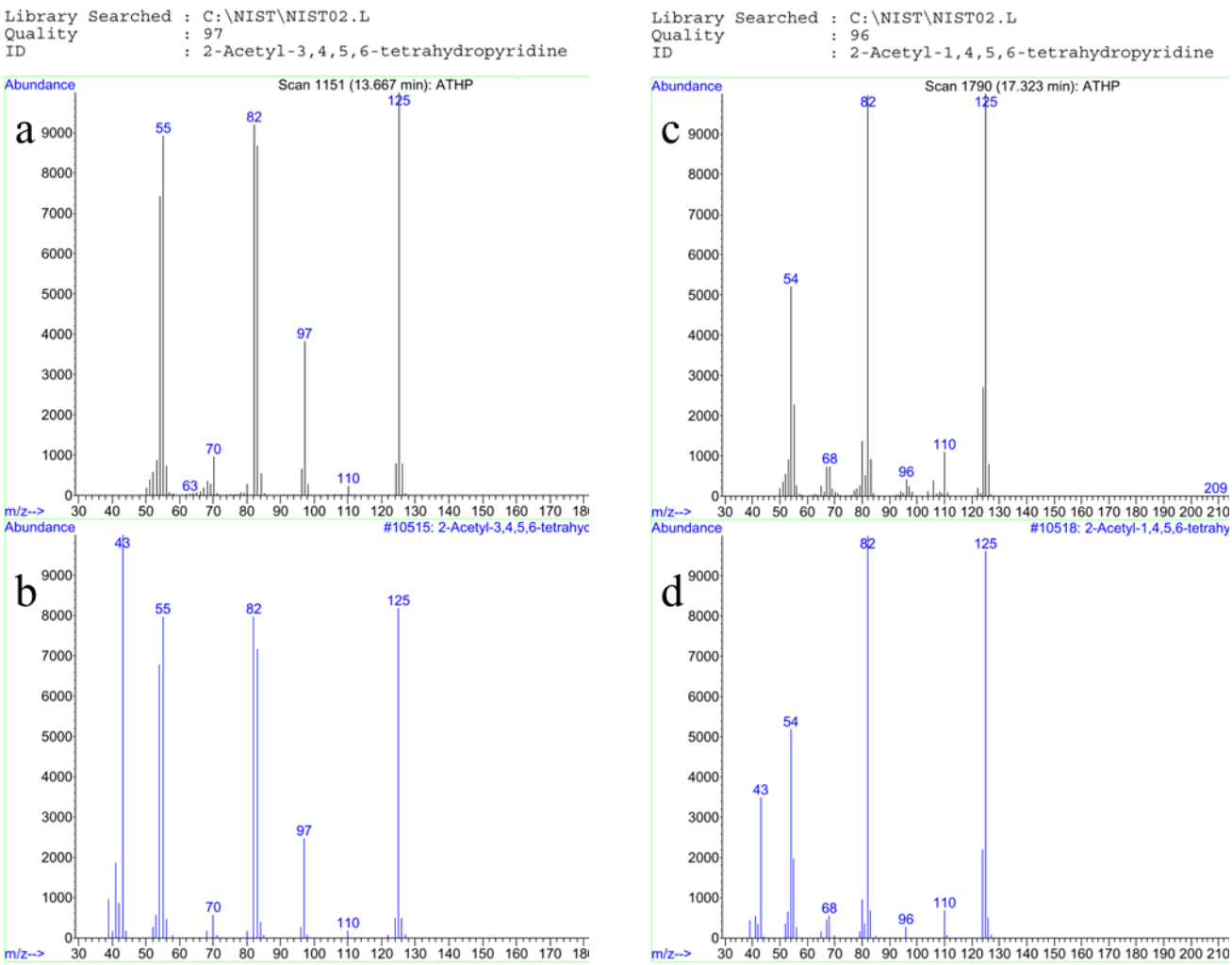
a LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation.

b Ftab was 5.41 for all compounds (Lack-of-fit F-test).

Appendix 4 : Extracted ion chromatogram of ATHP in scan mode (m/z 50 to 300).



Appendix 5 : MS spectra for 2-acetyl-1,4,5,6-tetrahydropyridine and 2-acetyl-3,4,5,6-tetrahydropyridine.



a MS spectra of 2-acetyl-3,4,5,6-tetrahydropyridine in Milli-Q water sample

b MS spectra of 2-acetyl-3,4,5,6-tetrahydropyridine from the NIST database

c MS spectra of 2-acetyl-1,4,5,6-tetrahydropyridine in Milli-Q water sample

d MS spectra of 2-acetyl-1,4,5,6-tetrahydropyridine from the NIST database

Appendix 6 : Concentration of APY, ETHP, ATHP and volatile phenols in the wines analysed. Each sample was analysed in triplicate.

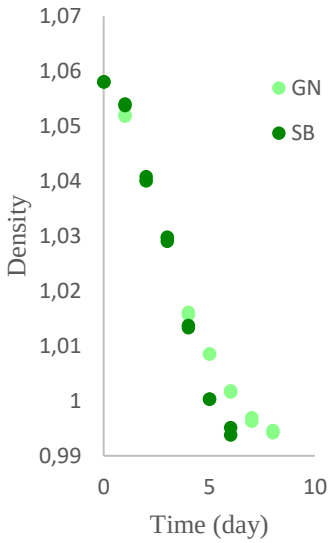
No.	Variety	Origin	Detail	Colour	Vintage	SO_2	Perception of mousy-off flavour	APY ($\mu g.L^{-1}$)	ETHP ($\mu g.L^{-1}$)	ATHP ($\mu g.L^{-1}$)	4-EP ($\mu g.L^{-1}$)	4-EG ($\mu g.L^{-1}$)	4-VP ($\mu g.L^{-1}$)	4-VG ($\mu g.L^{-1}$)
W_1-1	Grenache	France	Rhône	Red	2020	None	Yes	1.2 ± 0.3	15.4 ± 0.2	3.4 ± 1.6	0,5 ± 0,1	1,3 ± 0,1	< LOQ	< LOQ
W_1-2	Grenache	France	Rhône	Rose	2020	None	Yes	n. d.	17.4 ± 1.0	0.4 ±0.3	< LOQ	0,7 ± 0,1	12,8 ± 0,1	8,1 ± 0,2
W_1-3	Chardonnay	France	Rhône	White	2020	None	Yes	n. d.	21.2 ± 2.7	1.5 ± 1.2	3,3 ± 0,1	1,4 ± 0,1	15,7 ± 0,8	9,0 ± 0,2
W_1-4	Grenache	France	Rhône	Rose	2020	None	Yes	n. d.	17.8 ± 0.7	5.4 ± 1.9	n. d.	0,6 ± 0,1	20,7 ± 0,3	10,5 ± 0,2
W_1-5	Syrah	France	Rhône	Rose	2020	None	No	n. d.	17.0 ± 0.6	n. d.	n. d.	< LOQ	32,7 ± 0,2	3,3 ± 0,1
W_1-6	Grenache	France	Rhône	Red	2020	None	Yes	2.0 ± 0.6	22.3 ± 2.2	9.2 ± 3.6	0,4 ± 0,1	1,6 ± 0,1	< LOQ	2,3 ± 0,1
W_1-7	Grenache	France	Rhône	Rose	2020	None	Yes	n. d.	17.7 ± 1.3	n. d.	n. d.	1,6 ± 0,2	13,4 ± 0,3	12,5 ± 0,6
W_1-8	Grenache	France	Rhône	Rose	2020	None	No	n. d.	17.9 ± 0.9	n. d.	n. d.	0,4 ± 0,1	31,5 ± 0,6	14,0 ± 0,5
W_1-9	Grenache	France	Rhône	Rose	2020	None	Yes	1.8 ± 1.1	18.4 ± 0.6	8.7 ± 2.6	n. d.	0,3 ± 0,1	29,5 ± 2,0	10,7 ± 1,3
W_1-10	Syrah	France	Rhône	Rose	2020	None	Yes	n. d.	17.3 ± 0.1	5.6 ± 0.1	0,3 ± 0,1	< LOQ	< LOQ	n. d.
W_1-11	Grenache	France	Rhône	Rose	2020	None	Yes	n. d.	16.9 ± 1.3	2.4 ± 0.4	1,5 ± 0,1	0,3 ± 0,1	17,2 ± 1,0	2,7 ± 0,1
W_1-12	Syrah	France	Rhône	Red	2020	None	Yes	n. d.	16.7 ± 1.6	3.4 ± 1.5	n. d.	0,5 ± 0,1	25,3 ± 0,5	12,3 ± 0,2
W_1-13	Chardonnay	France	Rhône	White	2019	None	Yes	1.0 ± 0.5	18.9 ± 1.7	n. d.	n. d.	n. d.	19,3 ± 0,1	1,8 ± 0,1
W_1-14	Grenache	France	Rhône	Rose	2020	None	Yes	n. d.	18.1 ± 1.5	2.2 ± 0.7	n. d.	0,8 ± 0,1	27,2 ± 0,4	25,2 ± 2,5
W_1-15	Syrah	France	Rhône	Rose	2020	None	No	n. d.	20.7 ± 1.6	n. d.	0,3 ± 0,1	< LOQ	< LOQ	< LOQ
W_1-16	Grenache	France	Rhône	Rose	2020	None	Yes	n. d.	21.6 ± 0.7	11.9 ± 7.5	n. d.	0,6 ± 0,1	55,1 ± 1,3	16,8 ± 0,2
W_1-17	Syrah	France	Rhône	Red	2020	None	Yes	n. d.	84.9 ± 11.4	7.8 ± 1.9	n. d.	0,6 ± 0,1	26,3 ± 0,5	10,3 ± 0,1
W_1-18	Grenache	France	Rhône	Rose	2019	None	Yes	n. d.	19.0 ± 0.9	29.1 ± 6.4	n. d.	0,3 ± 0,3	28,6 ± 1,5	10,7 ± 1,0
W_1-19	Grenache	France	Rhône	Rose	2020	None	Yes	n. d.	17.2 ± 1.2	1.1 ± 0.6	< LOQ	0,7 ± 0,1	n. d.	7,7 ± 0,2
W_1-20	Chardonnay	France	Rhône	White	2018	None	Yes	n. d.	16.4 ± 4.1	0.8 ± 0.8	< LOQ	< LOQ	< LOQ	5,4 ± 0,1
W_1-21	Grenache	France	Rhône	Rose	2019	None	Yes	n. d.	32.2 ± 1.1	26.2 ± 4.5	n. d.	0,3 ± 0,1	51,2 ± 3,2	9,0 ± 0,4
W_1-22	Syrah	France	Rhône	Rose	2019	None	Yes	n. d.	18.4 ± 3.1	0.8 ± 0.4	n. d.	< LOQ	22,4 ± 0,4	4,4 ± 0,1
W_1-23	Chardonnay	France	Rhône	White	2018	None	Yes	n. d.	18.6 ± 2.1	10.1 ± 3.0	n. d.	< LOQ	38,0 ± 1,9	24,3 ± 0,9
W_1-24	Syrah	France	Rhône	Rose	2019	None	Yes	n. d.	18.8 ± 1.2	0.7 ± 0.5	n. d.	n. d.	16,5 ± 0,7	5,0 ± 0,4
W_1-25	Unknown	France	Rhône	Rose	Non	None	Yes	n. d.	n. d .	4.1 ± 0.8	3,6 ± 0,1	11,9 ± 0,3	46,1 ± 1,5	12,3 ± 0,4
W_1-26	Unknown	France	Rhône	Red	Non	None	Yes	n. d.	18.1 ± 1.4	2.4 ± 0.6	466,3 ± 7,4	107,4 ± 6,4	< LOQ	< LOQ
W_1-27	Unknown	France	Rhône	Rose	Non	None	No	n. d.	17.4 ± 0.7	n. d.	214,5 ± 6,1	135,0 ± 2,4	15,6 ± 0,3	5,4 ± 0,5
W_1-28	Unknown	France	Rhône	Red	Non	None	Yes	n. d.	120.5 ± 10.7	54.5 ± 4.9	138,4 ± 2,7	117,3 ± 8,5	< LOQ	n. d.
W_1-29	Blend	France	Rhône	Red	2019	None	No	n. d.	22.9 ± 1.4	2.6 ± 0.4	604,0 ± 3,1	109,0 ± 1,9	< LOQ	1,2 ± 0,1
W_1-30	Carignan	France	Rhône	Red	2020	None	No	n. d.	15.7 ± 1.9	0.5 ± 0.3	164,7 ± 0,2	29,2 ± 1,9	< LOQ	n. d.
W_1-31	Blend	France	Rhône	Rose	2020	None	No	n. d.	17.3 ± 1.5	n. d.	0,9 ± 0,1	0,5 ± 0,2	< LOQ	< LOQ
W_1-32	Unknow	France	Rhône	Red	2020	None	No	n. d.	16.7 ± 0.6	n. d.	1320,9 ± 19,0	285,0 ± 5,9	6,1 ± 0,4	< LOQ
W_1-33	Carignan	France	Rhône	Red	2020	None	No	n. d.	21.5 ± 3.4	n. d.	166,1 ± 2,1	29,7 ± 2,0	n. d.	n. d.
W_1-34	Unknown	France	Rhône	White	Non	None	Yes	n. d.	41.7 ± 3.6	n. d.	< LOQ	0,4 ± 0,1	65,4 ± 4,1	37,0 ± 2,1

W_1-35	Unknown	France	Rhône	Rose	Non	None	No	n. d.	17.1 ± 1.4	0.5 ± 0.1	< LOQ	0,3 ± 0,1	28,9 ± 0,9	13,4 ± 0,7
W_1-36	Unknown	France	Rhône	Red	Non	None	Yes	n. d.	19.8 ± 2.1	5.9 ± 0.9	2,3 ± 0,1	0,6 ± 0,1	< LOQ	< LOQ
W_1-37	Grenache	France	Rhône	Rose	2020	None	No	n. d.	18.6 ± 1.0	n. d.	0,3 ± 0,1	1,2 ± 0,1	36,2 ± 1,0	19,4 ± 0,3
W_2-1	Marsanne	France	Rhône	White	2020	None	No	n. d.	16.8 ± 1.3	n. d.	1,0 ± 0,2	0,4 ± 0,1	20,8 ± 2,6	29,7 ± 3,5
W_2-2	Marsanne	France	Rhône	White	2020	None	No	n. d.	17.6 ± 1.5	n. d.	1,7 ± 0,1	2,6 ± 0,2	38,3 ± 0,5	29,3 ± 1,9
W_2-3	Marsanne	France	Rhône	White	2020	None	No	n. d.	19.3 ± 1.1	n. d.	n. d.	0,3 ± 0,1	222,2 ± 2,7	147,2 ± 9,3
W_2-4	Marsanne	France	Rhône	White	2020	None	Yes	n. d.	19.4 ± 1.5	2.8 ± 0.1	n. d.	0,4 ± 0,1	58,2 ± 1,3	55,3 ± 0,7
W_2-5	Syrah	France	Rhône	Red	2020	None	No	n. d.	17.2 ± 1.6	n. d.	11,4 ± 0,2	0,9 ± 0,1	< LOQ	n. d.
W_2-6	Syrah	France	Rhône	Red	2020	None	No	n. d.	17.6 ± 0.9	n. d.	1,3 ± 0,1	0,6 ± 0,1	< LOQ	< LOQ
W_3-1	Gamay	France	Beaujolais	Red	2017	None	Yes	n. d.	27.4 ± 0.2	4.2 ± 1.0	n. d.	0,3 ± 0,1	6,9 ± 0,3	1,4 ± 0,1
W_3-2	Gamay	France	Beaujolais	Red	2017	None	Yes	n. d.	27.5 ± 5.8	1.8 ± 0.9	n. d.	0,3 ± 0,1	< LOQ	< LOQ
W_3-3	Gamay	France	Beaujolais	Red	2019	None	Yes	n. d.	21.4 ± 0.9	9.3 ± 1.8	< LOQ	0,6 ± 0,1	< LOQ	< LOQ
W_3-4	Gamay	France	Beaujolais	Red	2019	None	Yes	n. d.	15.7 ± 0.6	6.1 ± 1.6	< LOQ	0,6 ± 0,1	< LOQ	< LOQ
W_3-5	Gamay	France	Beaujolais	Red	2019	None	Yes	n. d.	18.8 ± 2.7	1.3 ± 0.5	n. d.	0,7 ± 0,1	< LOQ	< LOQ
W_3-6	Gamay	France	Beaujolais	Red	2019	None	No	n. d.	16.3 ± 1.0	0.8 ± 1.1	< LOQ	0,7 ± 0,1	< LOQ	< LOQ
W_3-7	Gamay	France	Beaujolais	Red	2020	None	Yes	4.0 ± 1.2	18.5 ± 2.9	0.1 ± 0.8	3,1 ± 0,1	0,8 ± 0,1	6,0 ± 0,3	1,2 ± 0,2
W_3-8	Gamay	France	Beaujolais	Red	2020	None	Yes	n. d.	18.8 ± 1.0	3.8 ± 2.0	166,2 ± 0,5	31,2 ± 1,8	< LOQ	< LOQ
W_4-1	Chardonnay	France	Sud Ouest	White	2020	None	Yes	n. d.	55.9 ± 2.0	8.0 ± 2.1	0,4 ± 0,1	0,7 ± 0,1	60,5 ± 1,0	40,4 ± 4,1
W_4-2	Merlot	France	Sud Ouest	rose	2020	None	Yes	n. d.	24.2 ± 1.7	0.6 ± 0.4	0,5 ± 0,2	0,7 ± 0,1	26,9 ± 1,0	15,0 ± 3,1
W_4-3	Syrah	France	Sud Ouest	rose	2020	None	Yes	n. d.	19.6 ± 1.2	3.2 ± 0.7	2,2 ± 0,8	2,3 ± 0,4	27,8 ± 1,4	11,5 ± 0,4
W_4-4	Syrah	France	Sud Ouest	rose	2020	None	Yes	n. d.	21.0 ± 2.0	2.9 ± 0.2	0,6 ± 0,2	2,0 ± 0,4	29,8 ± 1,1	18,6 ± 1,4
W_4-5	Merlot	France	Sud Ouest	Red	2020	None	Yes	4.2 ± 1.3	18.1 ± 1.0	7.6 ± 1.2	2,5 ± 0,1	1,0 ± 0,1	n. d.	< LOQ
W_4-6	Cabernet Sauvignon	France	Sud Ouest	Red	2020	None	Yes	4.2 ± 1.8	19.7 ± 2.1	5.1 ± 0.9	6,9 ± 0,1	1,5 ± 0,1	n. d.	n. d.
W_4-7	Syrah	France	Sud Ouest	Red	2020	None	Yes	5.9 ± 1.5	14.0 ± 0.7	12.1 ± 2.6	1,6 ± 0,1	0,9 ± 0,1	48,4 ± 4,1	1,3 ± 0,1
W_5-1	Merlot	France	Bordeaux	Red	2020	None	Yes	n. d.	20.0 ± 0.8	1.1 ± 0.3	6,5 ± 1,0	1,1 ± 0,2	< LOQ	n. d.
W_5-2	Merlot	France	Bordeaux	Red	2020	None	Yes	n. d.	16.0 ± 1.1	11.0 ± 2.8	0,6 ± 0,1	0,4 ± 0,1	n. d.	n. d.
W_5-3	Merlot	France	Bordeaux	Red	2020	None	Yes	n. d.	18.3 ± 3.2	0.3 ± 0.1	0,3 ± 0,1	< LOQ	29,6 ± 1,7	4,7 ± 0,2
W_5-4	Merlot	France	Bordeaux	Red	Non	None	Yes	n. d.	18.0 ± 1.6	24.6 ± 0.8	3,6 ± 0,1	0,7 ± 0,1	7,6 ± 0,4	n. d.
W_6-1	Koshu	Japan	Yamanashi	White	Non	None	Yes	n. d.	38.3 ± 1.6	10.8 ± 2.6				
W_6-2	Ryugan	Japan	Nagano	White	Non	None	Yes	5.1 ± 1.9	33.3 ± 3.6	35.2 ± 6.9				
W_6-3	Koshu	Japan	Yamanashi	White	Non	None	No	n. d.	27.4 ± 1.4	n. d.				
W_6-4	Muscat Bailey A	Japan	Yamanashi	Red	Non	None	Yes	7.7 ± 2.6	35.7 ± 5.0	17.7 ± 3.4				
W_6-5	Muscat Bailey A	Japan	Yamanashi	Red	Non	None	No	3.8 ± 1.7	28.0 ± 2.4	6.7 ± 1.0				
W_6-6	Muscat Bailey A	Japan	Nagano	Red	2020	None	No	n. d.	27.8 ± 2.3	n. d.				
W_7-1	Merlot	France	Bordeaux	Red	2016	SO_2	No	n. d.	6.3 ± 0.4	n. d.				
W_7-2	Merlot	France	Bordeaux	Red	2017	SO_2	No	n. d.	6.6 ± 0.9	n. d.				
W_7-3	Merlot	France	Bordeaux	Red	2018	SO_2	No	n. d.	n. d.	n. d.				

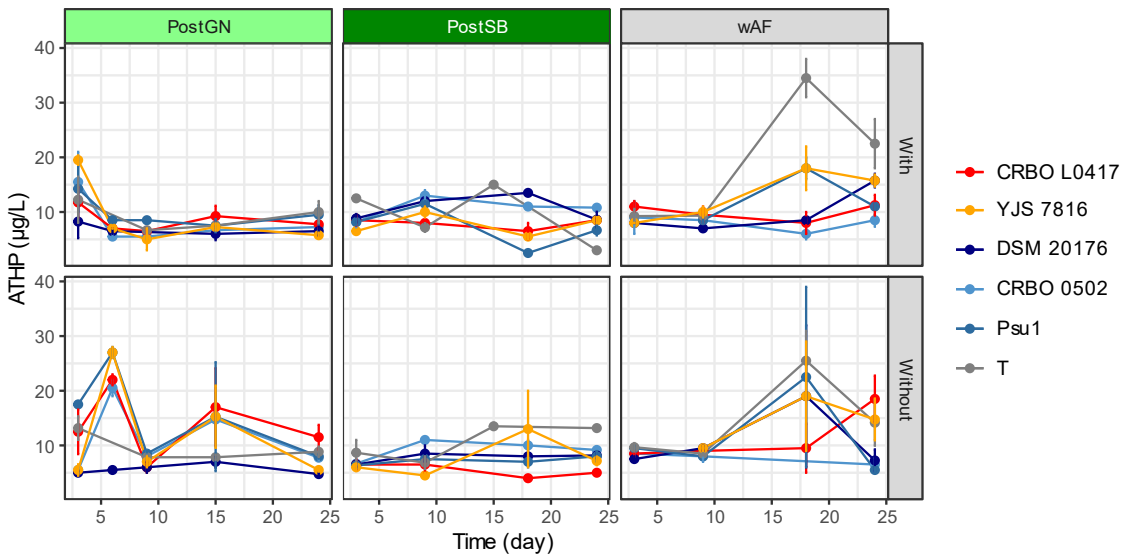
W_7-4	Merlot	France	Bordeaux	Red	2015	SO_2	No	n. d.	6.0 ± 0.1	n. d.
W_7-5	Sauvignon Blanc	France	Loire	White	2018	SO_2	No	n. d.	n. d.	n. d.
W_7-6	Chardonnay	France	Languedoc	White	2019	SO_2	No	n. d.	n. d.	n. d.

n. d. : not detected, LOQ : limit of quantification

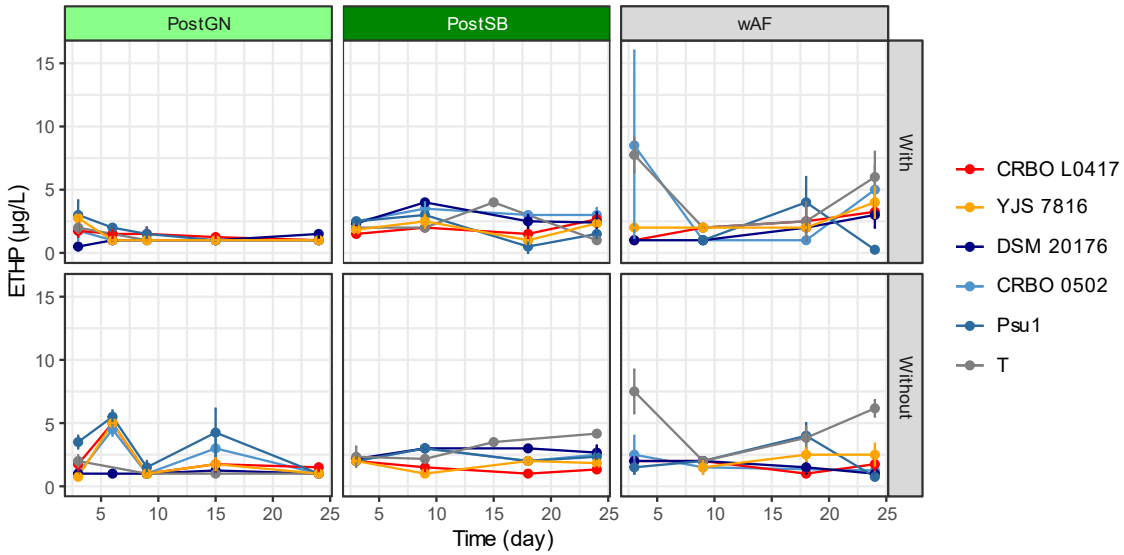
Appendix 7 : Alcoholic fermentation kinetics in Ugni blanc by 2 Saccharomyces cerevisiae *strains (GN and SB); density in function of time (day).*



Appendix 8 : ATHP levels in µg/L in function of the Saccharomyces cerevisiae *strains used for the alcoholic fermentation (GN, SB), the stirring action (with or without) and the time by 5 different microorganisms; (2* Brettanomyces bruxellensis*: CRBO L0417 and YJS 7816; 1* Lentilactobacillus hilgardii*: DSM 20176; 2* Oenococcus oeni*: CRBO 0502 and Psu1) and a blank only with the indigenous flora.*



Appendix 9 : ETHP levels in µg/L in function of the Saccharomyces cerevisiae *strains used for the alcoholic fermentation (GN, SB), the stirring action (with or without) and the time by 5 different microorganisms; (2* Brettanomyces bruxellensis*: CRBO L0417 and YJS 7816; 1* Lentilactobacillus hilgardii*: DSM 20176; 2* Oenococcus oeni*: CRBO 0502 and Psu1) and a blank only with the indigenous flora.*



Appendix 10 : Summary table of the analyse of variance (ANOVA) of the production of APY, ATHP, and ETHP in matrices of Ugni blanc depending on the different factors (AF : alcoholic fermentation; Stirring; Strain : producer microorganism add after fermentation) and their interaction (AF:Stirring; AF:Strain; Stirring:Strain ; AF:Stirring:Strain).

		APY							
		3		9		18		24	
	Df[a]	Sum sq[a]	P-value	Sum sq	P-value	Sum sq	P-value	Sum sq	P-value
AF	2	479852	3,95E-13	2641400	<2e-16	2156045	2,11E-13	16595358	<2e-16
Stirring	1	97821	1,25E-04	26836	5,19E-02	81938	9,61E-04	327971	6,73E-06
Strain	5	100425	8,80E-03	632185	2,50E-09	463509	1,34E-06	807465	1,04E-08
AF:Stirring	2	36710	9,57E-03	23803	1,81E-01	276054	1,21E-06	182662	2,83E-03
AF:Strain	8	142573	5,92E-03	96156	1,07E-01	363452	7,10E-06	1186328	7,07E-10
Stirring:Strain	5	98996	9,57E-03	58976	1,43E-01	160874	9,31E-04	87209	3,25E-01
AF:Stirring:Strain	8	64400	1,16E-01	178057	3,20E-03	86894	7,34E-03	87892	8,18E-01
Residuals	114	573932		246131		87775		1871332	

		ATHP							
		3		9		18		24	
	Df	Sum sq	P-value	Sum sq	P-value	Sum sq	P-value	Sum sq	P-value
AF	2	394,8	6,11E-06	59,31	7,77E-05	350,8	4,65E-02	564,6	1,45E-09
Stirring	1	252,7	6,43E-05	5,66	1,33E-01	94,9	2,80E-01	26,9	1,33E-01
Strain	5	114,4	1,72E-01	33,66	3,03E-02	251,1	5,27E-01	265,2	8,08E-04
AF:Stirring	2	74,2	8,18E-02	53,7	1,59E-04	12,9	6,86E-01	61,9	7,57E-02
AF:Strain	8	220,5	6,75E-02	64,88	5,25E-03	331,4	3,94E-01	563,7	7,83E-06
Stirring:Strain	5	117,8	1,59E-01	6,8	1,59E-04	43,9	9,63E-01	91,3	1,78E-01
AF:Stirring:Strain	8	288,7	5,07E-03	14,41	5,48E-01	119,5	6,72E-01	480,6	6,89E-05
Residuals	114	1356,7		88,81		1294,2		1480,6	

		ETHP							
		3		9		18		24	
	Df	Sum sq	P-value	Sum sq	P-value	Sum sq	P-value	Sum sq	P-value
AF	2	3,956	9,83E-02	19,868	8,98E-15	3,882	6,09E-02	41,48	2,35E-08
Stirring	1	6,525	5,06E-02	0,294	1,15E-01	0,129	7,19E-01	6,23	1,44E-02
Strain	5	2,684	1,64E-01	6,556	8,89E-07	7,722	1,40E-01	37,19	5,01E-06
AF:Stirring	2	6,87	1,33E-01	7,37	1,93E-03	1,61	2,13E-01	14,14	1,34E-03
AF:Strain	8	54,33	3,31E-04	1,683	2,83E-06	21,45	4,74E-03	26,3	2,20E-03
Stirring:Strain	5	6,87	1,60E-01	0,882	1,96E-01	3,501	4,81E-01	26,45	2,25E-04
AF:Stirring:Strain	8	29,21	1,16E-02	1,011	2,89E-01	0,331	9,50E-01	14,95	7,41E-02
Residuals	114	156,37		4,187		16,388		114,92	

[a]: Df, Degrees of freedom; Sum Sq, sum of squares

Appendix 11 : Supplementary data by chapter of the chemical quantification of the experimentation developed in III.2.; III.3.2.; IV.3.1.; IV.3.2.; IV3.3.; IV.4.

Other supplementary data are available, until de 31/05/2023 at :

https://filesender.renater.fr/?s=download&token=c23a9000-3ed4-4415-aaef-62b668c97cdb

Exploration of the phenomena associated with the production of pyrrole and pyridine derivatives responsible for mousy off-flavours in wine

In den letzten Jahren ist die Nachfrage der Verbraucher nach Bio-Lebensmitteln weiter gestiegen. Die Akteure des Weinsektors achten auf diese Erwartungen. In der Weinproduktion ist ein Rückgang in der Anwendung bestimmter Maßnahmen, die sich direkt auf die Weinqualität auswirken können, zu verzeichnen. So hat beispielsweise der „Mäuselton“ bei Weinen mit reduziertem Sulfitgehalt zugenommen. Derzeit scheinen drei Moleküle Verursacher dieses Defekts zu sein: 2-Acetylpyrrolin (APY), 2-Acetyltetrahydropyridin (ATHP) und 2-Ethyltetrahydropyridin (ETHP). Die durchgeführte Forschungsarbeit, die chemische, mikrobiologische und önologische Ansätze kombiniert, liefert Antworten auf die Mechanismen des Auftretens dieses Fehltons im Wein.

Um Antworten zu finden, wurden Methoden zur Quantifizierung der N-Heterozyklen, die für den „Mäuselton“ verantwortlich sind, mithilfe von SBSE-GC-MS-Techniken entwickelt. Die Bestimmungsmethode für Wein ermöglichte es, diese Substanzen in 74 verschiedenen Weinen zu quantifizieren. Die zweite Analysenmethode ist auf ein chemisch definiertes Medium abgestimmt, das alle bisher bekannten Vorstufen des „Mäuseltons“ enthält. Sie ermöglicht es, Mikroorganismen unter standardisierten Bedingungen auf ihre Fähigkeit zu testen, einen „Mäuselton“ zu erzeugen. Mit diesen Methoden konnte gezeigt werden, dass das Verhältnis der beiden Tautomere von 2-Acetyltetrahydropyridin nicht von den Unterschieden zwischen dem pH-Wert des Weines und der Mundhöhle beeinflusst wird.

Mikroorganismen wurden aus 25 Weinen mit „Mäuselton“ isoliert. Die am häufigsten anzutreffenden Arten sind Saccharomyces cerevisiae, Oenococcus oeni oder Brettanomyces bruxellensis. Von den 353 isolierten Mikroorganismen sind nur Stämme der Arten B. bruxellensis, Lentilactobacillus hilgardii und Oenococcus oeni in der Lage, Substanzen, die einen „Mäuselton“ verursachen, im Modellmedium zu produzieren. Anschließend wurden Stämme aus der Sammlung, die zu diesen drei Arten gehörten, unter denselben Bedingungen untersucht. Die Ergebnisse zeigen, dass die Konzentrationen und Verhältnisse der drei N-Heterozyklen signifikante Unterschiede zwischen den Arten aufweisen, dass jedoch alle untersuchten Stämme (22 Stämme von B. bruxellensis, 10 Stämme von L. hilgardii, 20 Stämme von O. oeni) die Fähigkeit haben, mindestens eine der drei Komponenten zu produzieren. Weitere Versuche in Modellmedien haben gezeigt, dass es bei der Produktion mäuselnder Substanzen zu einer Interaktion zwischen Mikroorganismen kommen kann.

Die Untersuchung dieser Wechselwirkungen wurde im Weinmilieu fortgesetzt. Ein Ugni blanc Most wurde mit zwei verschiedenen Saccharomyces cerevisiae Stämmen vinifiziert. Anschließend wurde die Produktion der mäuselnden Komponenten über 24 Tage verfolgt. Die Ergebnisse zeigten, dass unabhängig von den produzierenden Mikroorganismen einer der beiden S. cerevisiae Stämme zur Entwicklung von Bedingungen beiträgt, die eine höhere APY-Produktion im Wein ermöglichen.

Schließlich wurden Produktionskinetiken im Modellmedium durchgeführt, um festzustellen, in welchem physiologischen Stadium B. bruxellensis diese Verbindungen produzieren kann. Die Produktion von ATHP verläuft nicht linear. Die Verbindung tritt punktuell in hohen Konzentrationen auf, insbesondere zu Beginn der stationären Wachstumsphase, und verschwindet dann wieder. Es wurden Hypothesen über die physiologische Rolle dieser Verbindung aufgestellt.

Schlüsselwörter: Mäuselton; Weine; Brettanomyces bruxellensis; Milchsäurebakterien; Organoleptische Abweichungen

Exploration of the phenomena associated with the production of pyrrole and pyridine derivatives responsible for mousy off-flavours in wine

In recent years, consumer demand for organic food has continued to grow. The actors of the wine sector are attentive to these expectations. Wine production is evolving with decreases in the use of certain inputs that can directly impact the quality of wine. For example, there has been an upsurge in the "mousy off-flavour" defect in wines with reduced sulphite content. Today, three molecules seem to be at the origin of this defect: 2-acetylpyrroline (APY), 2-acetytetrahydropyridine (ATHP), and 2-ethyltetrahydropyridine (ETHP). Our work, combining chemical, microbiological and oenological approaches, provides answers to the mechanisms of appearance of this defect in wine.

In order to provide answers, methods for quantifying N-heterocyclics responsible for mousiness have been developed using SBSE-GC-MS techniques. The method of determination in wine made it possible to quantify the compounds in 74 different wines. The second method is adapted to a chemically defined medium, containing all the precursors known to date "mousy off-flavours". It makes it possible to test microorganisms for their ability to produce the mousy off-aroma under standardized conditions. These methods have been used to show that the proportion of the two tautomers of 2-acetyltetrahydropyridine is not impacted by the pH of the wine and buccal cavity.

Microorganisms were isolated from 25 wines with the defect. The most frequently encountered species are Saccharomyces cerevisiae, Oenococcus oeni, or Brettanomyces bruxellensis. Among the 353 microorganisms isolated, only strains of B. bruxellensis, Lentilactobacillus hilgardii, Oenococcus oeni are able to produce mousy compounds in model medium. Strains of collections belonging to these three species were then studied under the same conditions. The results show that the levels and ratios of the three N-heterocycles show significant variation between species but that all the strains evaluated (22 strains of B. bruxellensis, 10 strains of L. hilgardii, 20 strains of O. oeni) have the capacity to produce at least one of the three compounds. Further model media tests have shown that there may be interaction between microorganisms to produce mousy compounds.

The study of these interactions was continued in the wine environment. A white Ugni must was vinified using 2 different strains of Saccharomyces cerevisiae. And the production of the mousy compounds was then followed over 24 days. The results showed that, regardless of the microorganisms that produce, one of the two strains of S. cerevisiae contributes to the development of conditions for greater production of APY in wines.

Finally, production kinetics were undertaken in a model medium to determine at what physiological stage B. bruxellensis could produce these compounds. ATHP production is not linear. The compound appears promptly in significant concentrations, especially at the beginning of the stationary growth phase and then it’s disappearing. Hypotheses have been made about the physiological role of this compound.

Key words: Mousy off-flavours; Wines; Brettanomyces bruxellensis; lactic acid bacteria; Organoleptic deviations

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