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ORIGINAL RESEARCH ARTICLE

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

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ABSTRACT

In recent years, the frequency of occurrence of mousy off-flavours in wines has increased. This could be caused by the significant decrease in sulphur dioxide addition during processing, the increase in 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. Thus 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. In total, 252 bacteria with 90.5 % of *Oenococcus oeni* and 101 yeast 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 standardised 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 with N-heterocycles production: *B. bruxellensis*, *Lentilactobacillus hilgardii* and *Oenococcus oeni*. The 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 variations according to the 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.

KEYWORDS: 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 the visual perception of the consumer (Ridgway *et al.*, 2010). The mousy taint was first reported in cider. It was described as a "Peculiarly disagreeable flavour in wine, which is closely resembling 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-flavours (Tempère *et al.*, 2019). Massini and 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 a 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.

1. Mousy off-flavour

Mousiness is a consistent underlying problem for the wine industry (Fugelsang and 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 makes 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 and Henschke, 1995). However, when the wine comes into contact with saliva, the neutral pH of the mouth (Larsen *et al.*, 1999) permits the 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 at 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 the other hand, microbial mechanisms leading to the development of this alteration are still unclear. It has been suggested that the presence of 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 and Henschke, 2002).

2. *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 in tolerance regarding the grape variety. It may be explained by the phenolic composition of wines. *B. bruxellensis* is really well known for its negative contribution to wine odour (Chatonnet *et al.*, 1992; Harrouard *et al.*, 2022). *B. bruxellensis* can produce volatile phenols, such as 4-ethylphenol and 4-ethylguaicol from cinnamic acid derivatives (Romano *et al.*, 2008). These compounds are undesirable and associated with 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 and Domercq, 1956; Schanderl, 1951). They can also induce turbidity in wine (Van Der Walt and Van Kerken, 1958; Van Der Walt and Van Kerken, 1959; Van Zyl, 1962) and are sometimes associated with spoilage of other beverages such as soft drinks (Kolfschoten and 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 and 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).

3. 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 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.

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, such as acidic pH, relatively low temperatures and the presence of ethanol (Torriani *et al.*, 2011). At that time, the ability to produce the mousy off-flavour is not verified before commercialisation.

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 and 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 standardised 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. The 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 defects in wines. All of them were 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 and 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 YPD-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 pimaricin 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 pimaricin 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 on 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 MicroflexTM LT/SH MALDI-MS System using Flex Control, MTB Compass (Version 3.1) and a MALDI-BiotyperTM 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 was performed to differentiate clones of *Saccharomyces cerevisiae* and *O. oeni*.

The Whatman® FTA Clone Saver card technology (Cat. No. WB120028; Whatman, Sigma-Aldrich, France) has been used for 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 and Lonvaud-Funel, 2012; Claisse and Lonvaud-Funel, 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 and 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 a 15 mL tube for 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 (11,600 *g* for 9 min at 4 °C) and 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 of 1.10^9 cells 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 in three solutions: an amino acid, an ethanal and an *N*HAM base; these solutions were sterilised by filtration through 0.22 µm membranes).

TABLE 1. Composition of 1L for N-Heterocycles Assay Medium (NHAM) (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

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

2. Chemical analysis

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

TABLE 2. Performance parameters of the analytical method in the 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.

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 an 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 a constant flow rate (1.1 mL/min). The GC temperature was programmed from 40 °C to 80 °C at a rate of 3 °C/min, then up to 150 °C at a rate of 6 °C/min and finally up to 240 °C (held for 5 min) at a rate of 10 °C/min.

An Agilent 5975 mass selective detector (Agilent Technologies, Les Ulis, France) operating in electron ionisation (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 one-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 and Dufour, 2023), ggplot2 (Wickham et al., 2023).

RESULTS AND DISCUSSION

1. 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, four different species, i.e., *B. bruxellensis*, *Pichia manshurica*, *Priceomyces carsonii* and *Saccharomyces cerevisiae* were identified among the 101 isolates. Concerning LAB, three 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 1A (for yeasts) and Figure 1B (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 and so in its absence or at low levels, microorganisms can grow more freely in 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 bottling. One of our samples did not present any microorganisms, we can assume that it was filtered for sterilisation 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.

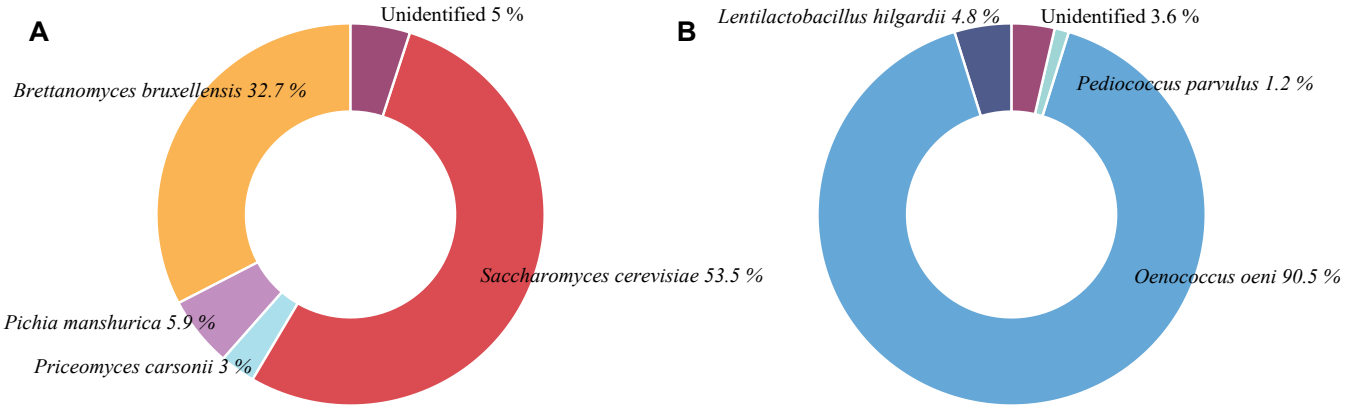


FIGURE 1. Frequency of species of yeast (A) and lactic acid bacteria (LAB) (B) in all the isolates from 25 mousy wines.

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	

On the other hand, it is difficult to identify the origin of the contamination, indeed it is possible to find *B. bruxellensis* in the cellars in bioadhered forms, but also a winemaking environment (Connell *et al.*, 2002; Fugelsang and 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 a 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.

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, which were present in 32 % of the spoiled wines, their capacity to produce mousy off-flavours in a model medium was also tested. However, none of them were able to produce mousy compounds. Therefore, the screening method may not be suitable for such bacteria. AAB were forming clusters of cells but 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 colour, 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 us 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 on the strain. Although *Saccharomyces cerevisiae* was present in more than half of the wine samples, no clones were producers.

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*.

2. Screening of the collection strains.

Production of mousy *N*-heterocycles was characterised 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.

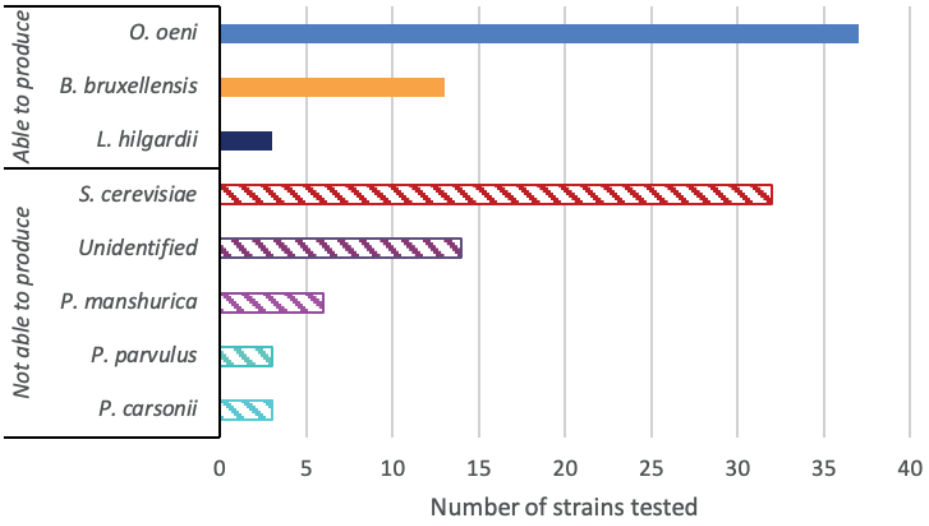


FIGURE 2. Production ability of at least one mousy compound in an NHAM for all the screened strains depending on their species.

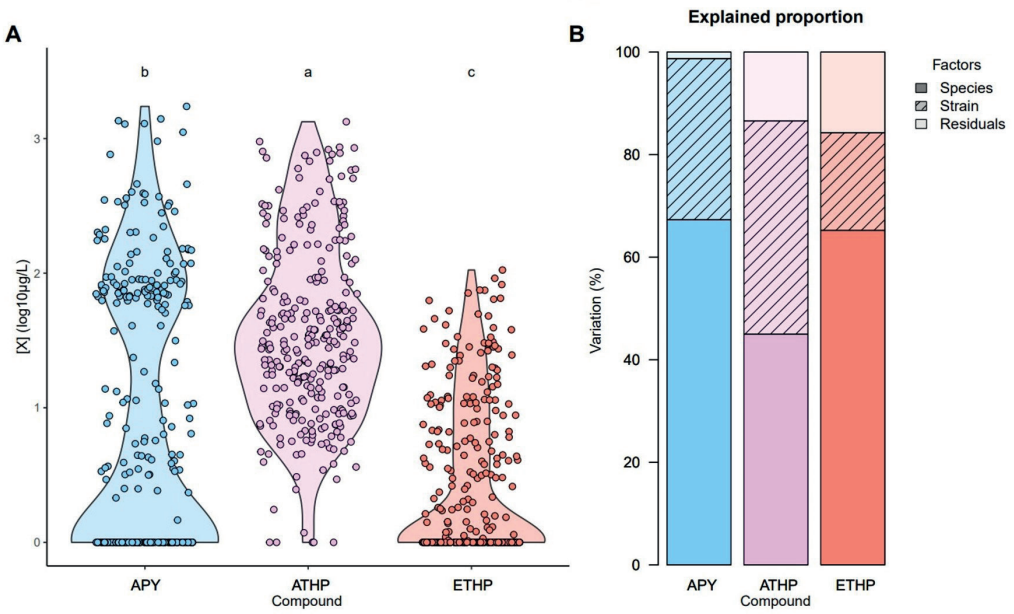


FIGURE 3. (A) Concentration of log10 µg/L produced by 52 different microorganisms in an NHAM medium (all concentrations below the limit of quantification replaced by 0); and (B) variability explained (with a p-value < 0.05) according to species and strain.

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.

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 3B, Table 4) followed by the strain factors. The concentrations of APY produced are explained for more than 67 % by the species studied and almost 31 % by the strains. Therefore, almost 100 % of its variation is explained while the explained variances of ATHP and ETHP are below 90 %, leaving a share of residual variation unexplained. Pushing the analysis further, no variability can be explained in *B. bruxellensis* by genetic groups nor in *O. oeni* by phylogenetic groups. Therefore, these problems are not dealt with here.

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

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 theoretically identical population levels for *B. bruxellensis* (measured 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.*

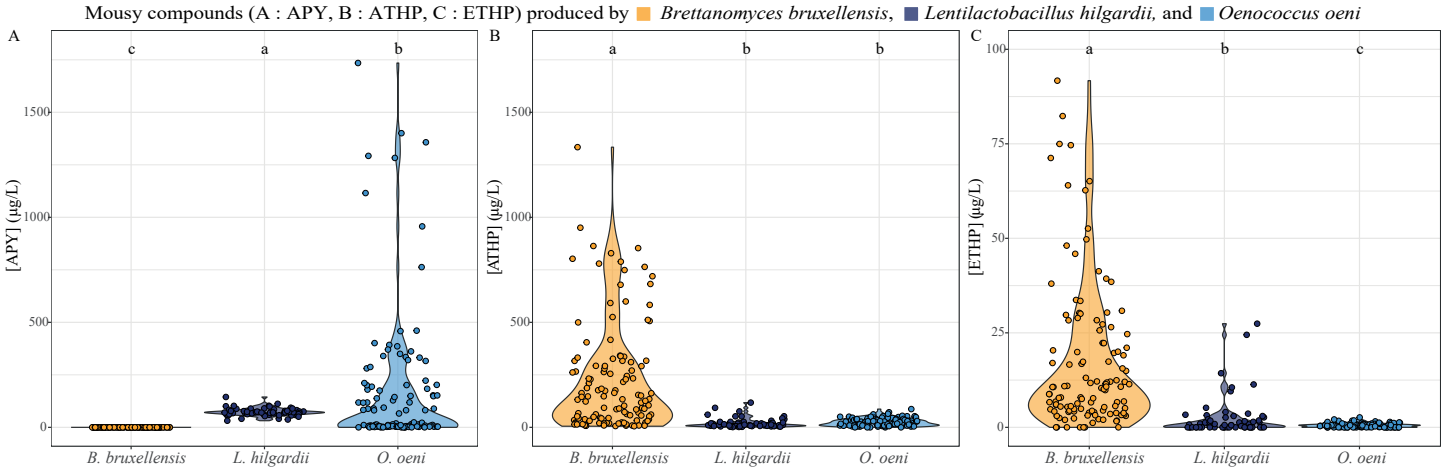


FIGURE 4. Mousy compounds (A: APY, B: ATHP, C: ETHP) in µg/L produced by *Brettanomyces bruxellensis*, *Lentilactobacillus hilgardii* and *Oenococcus oeni* in an NHAM.

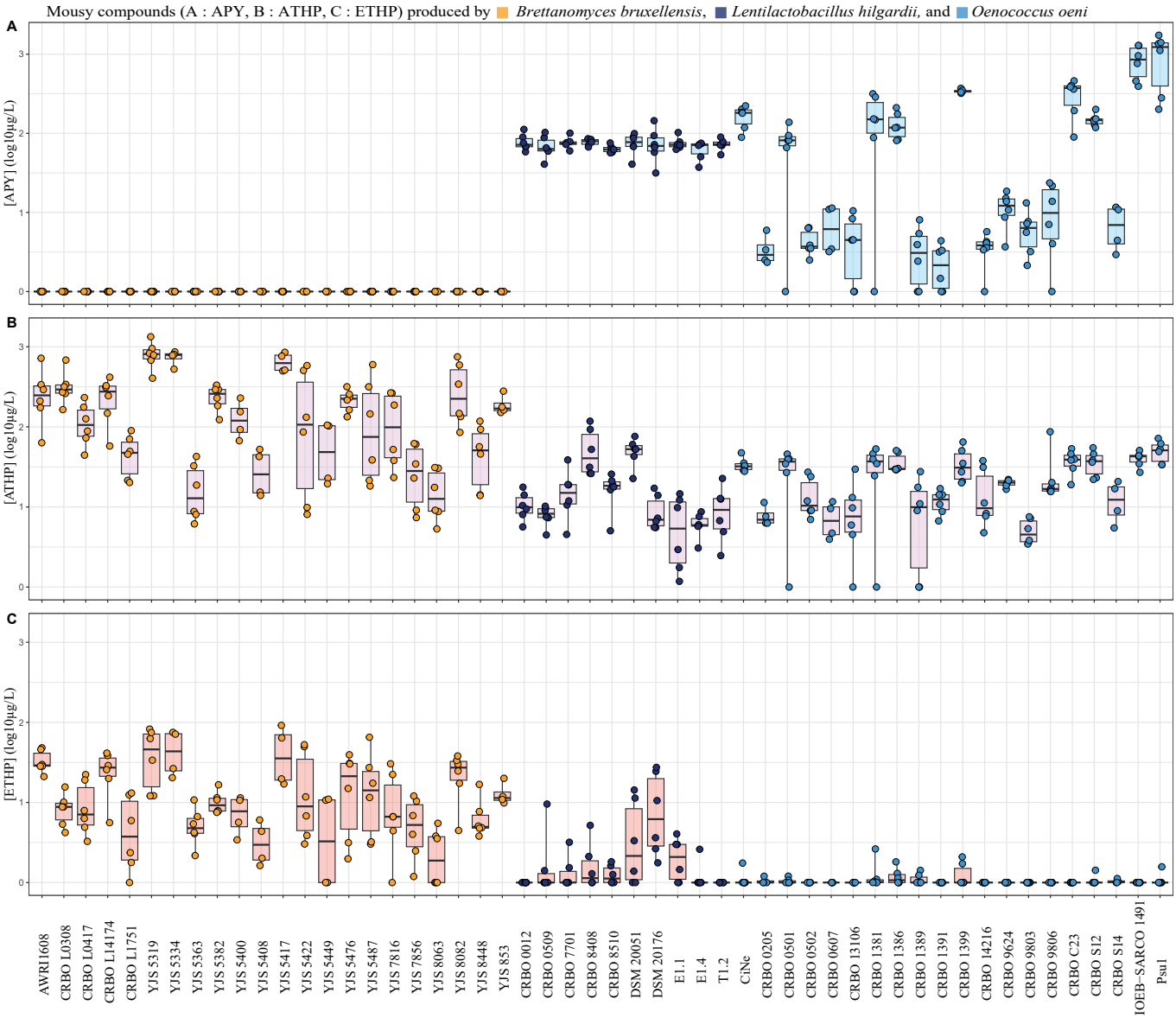


FIGURE 5. Mousy compound (A: APY, B: ATHP, C: ETHP) produced by 22 strains of *Brettanomyces bruxellensis* (in orange), 10 strains of *Lentilactobacillus hilgardii* (in dark blue) and 20 strains of *Oenococcus oeni* (in sky blue) in 24 h in NHAM expressed in log10 µg/L.

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 3B), 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 and Lucas, 2019; Table S2), PSU 1 produces APY at concentrations one hundred times higher than CRBO S14. Similarly, in *B. bruxellensis*, the two strains producing the most ATHP, YJS 5319 and YJS 5334, come from two different genetic groups (Avramova, 2018; Table S2). The production of the three compounds from the different genetic groups is 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 and 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.

Recall that the synthetic medium used is a medium extremely far from the 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)

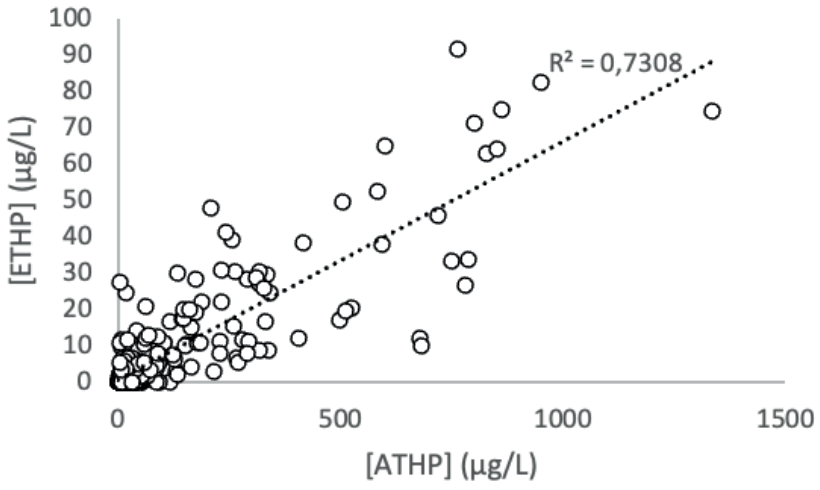
It remains interesting to note that here, the proportion of the different compounds produced was in favour of the two acetylated compounds even if the two compounds are produced by *B. bruxellensis* concerning ATHP and by *O. oeni* concerning APY. A correlation even could exist between ETHP and ATHP, with a Spearman ρ = 0.64 (Figure 6). This correlation is more obviously 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 and Kunz, 2021).

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 two 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 and 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 the 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.

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



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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