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ORIGINAL RESEARCH ARTICLE

Impact of *Pediococcus parvulus* and *Saccharomyces cerevisiae* on *Brettanomyces bruxellensis* mousy compound production

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ABSTRACT

The presence of mousy off-flavours in wine, attributed to the production of *N*-heterocycles by lactic acid bacteria or *Brettanomyces bruxellensis*, poses a significant challenge in the winemaking industry. This study is the first one to investigate the impact of co-cultures focusing on *B. bruxellensis* with two common wine microorganisms, *Pediococcus parvulus* and *Saccharomyces cerevisiae* on the formation of mousy compounds. The screening was conducted in a synthetic medium under controlled conditions. It reveals the nuanced effects of the microorganism combinations. Depending on the *P. parvulus* or *S. cerevisiae* strain co-inoculated with *B. bruxellensis* a synergistic effect is observed on the 2-acetyltetrahydropyridine (ATHP) production, while an inhibitory effect on 2-acetylpyrroline (APY) production by *P. parvulus* is highlighted when co-inoculated with *B. bruxellensis*. These findings shed light on the potential impact of the alcoholic fermentation yeast (*S. cerevisiae*) on the production of mousy off-flavours. Finally, a correlation between ATHP and 2-ethyltetrahydropyridine (ETHP) appears to be confirmed with a ratio of 1:10 between ETHP and ATHP produced in the N-heterocycles assay medium.

KEYWORDS: *Brettanomyces bruxellensis*, *Pediococcus parvulus*, *Saccharomyces cerevisiae*, co-culture, wine off-flavours, mousiness

INTRODUCTION

Concerns about taints and unpleasant flavours are a major issue in the wine industry. Even if these problems are not harmful, they can still have a negative impact on the quality and the visual perception of the wine for consumers (Ridgway *et al.*, 2010). The mousiness is one of these defects. The presence of a mousy taint was initially reported in cider and was described as an unpleasant flavour closely resembling the smell of a place inhabited by mice (Thudichum, 1894) and can be due to the presence of three *N*-heterocycles or at least one of them (2-acetylpyrroline:APY; 2-acetyltetrahydropyridine:ATHP; 2-ethyltetrahydropyridine:ETHP) (Snowdon *et al.*, 2006). These compounds can be produced by different microorganisms. *Brettanomyces bruxellensis* were the first microorganisms known to form mousy taint in wine (Peynaud and Domercq, 1956). In the past, it was relatively straightforward to prevent this issue by protecting the wine from microbial spoilage using sulphur dioxide (SO_2) and maintaining high acidity levels (Bartowsky, 2009). However, in modern times, detecting wines with mousy off-flavours has become more common (Tempère *et al.*, 2019). Massini and Vuchot (2015) suggested that this could be due to a significant reduction in the use of SO_2, an increase in pH levels, and a growing trend towards spontaneous fermentations in winemaking. More recently, Pelonnier-Magimel *et al.* (2020) conducted a study on the quality of 52 Bordeaux wines. They found that 70.6 % of the wines without SO_2 exhibited off-flavours, with 6.2 % of them displaying a mousy off-aroma.

*Pediococcus sp*ecies have been isolated from wines around the world and these lactic acid bacteria (LAB) 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, they are linked to alteration involving an overproduction of diacetyl (Davis *et al.*, 1988) and the synthesis of biogenic amines (Landete *et al.*, 2005). *Pediococcus parvulus* is also involved in the production of extreme bitterness due to the production of 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 and Dols-Lafargue, 2021). The production of all these molecules has a detrimental effect on the quality of the wine. In 1998, Costello demonstrated that at least one strain of *P. parvulus* (P6b) could weakly produce the three mousy compounds and highlighted the general lack of information regarding the impact of *P. parvulus* on wine quality.

Several strains of *B. bruxellensis*, have been shown to produce a mousy taint associated with fermenting grape juice, synthetic media, or finished wine (Grbin and Henschke, 2000; Heresztyn, 1986; Moulis *et al.*, 2023; Peynaud and Domercq, 1956; Romano *et al.*, 2008). Their ability to produce ATHP and ETHP and their inability have been confirmed using different chemically defined media (Grbin, 1998; Moulis *et al.*, 2023).

Moulis *et al.* (2023) focussed on the ability of wine microorganisms to produce mousiness They isolated four species of yeast and three species of bacteria from mousy wines, although only *B. bruxellensis*, *Lentilactobacillus hilgardii*, and *Oenococcus oeni* were linked to *N*-heterocycle production. Screening extended to collection strains revealed significant variation in *N*-heterocycle levels and ratios within species. All the *B. bruxellensis* screened were able to produce ATHP and ETHP, however, no study has addressed potential interactions between this yeast and other microorganisms for mousiness.

Interestingly, Strickland conducted experiments to investigate the interaction between *P. parvulus* and *B. bruxellensis* and its potential effects on their 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). Although he demonstrated this interaction, he did not investigate its impact on mousy compounds produced.

The present study was first dedicated to investigating the ability of 3 strains from the *P. parvulus* species to produce compounds responsible for mousiness alteration and then to study a possible interaction between *P. parvulus* and *B. bruxellensis* about mousy compound production.

Possible interactions between *S. cerevisiae* and *B. bruxellensis* have also been studied. Acetaldehyde is produced in varying quantities depending on the strains of *S. cerevisiae* (Di Stefano and Ciolfi, 1982; Ochando *et al.*, 2020) and is identified as one of the precursors of mousy compounds (Snowdon *et al.*, 2006).

MATERIAL AND METHODS

1. Microbiology

1.1 Microbial strains

Three *B. bruxellensis* strains (CRBO L0417, CRBO L1751 and YJS 7816), three *P. parvulus* strains (CRBO 0202, CRBO 0601 and DSM 20332) and three *S. cerevisiae* strains [Zymaflore FX10®, GN, SB (Zimmer *et al.*, 2014)] were used. They have been delivered by the Centre de Ressources Biologiques Œnologique (CRBO, ISVV, Bordeaux University, Villenave d'Ornon, France), except for the YJS strain that was from the YJS collection (Laboratory for Molecular Genetics, Genomics and Microbiology, Strasbourg University, France).

1.2 Isolation from stock glycerol

For the different phenotyping experiments, single colonies were isolated from biomass stored at -80 °C, after streaking on YPD agar medium (yeast extract 10 g/L, peptone 10 g/L, glucose 20 g/L, and agar 25 g/L, pH 4.8; for yeast) or RGJ agar medium (yeast extract 5 g/L, red grape juice 250 mL/L, Tween 80 1 mL/L, and 0.1 g/L pimaricin, pH 4.8; for bacteria) and incubated for 4 to 7 days at 24 °C and then stored at 4 °C.

1.3 Cell preculture and culture

Each strain undergoes a pre-culture step in 3 mL of RGJ liquid medium. Precultures were incubated at 25 °C for 5 days for *B. bruxellensis* and 3 days for *P. parvulus* and *S. cerevisiae*. Next, the culture step was performed in 90 mL of RGJ medium inoculated at a rate of 2 % from the preculture, the incubation time was 4 to 7 days at 24 °C.

1.4 Screening of microbial strains for the ability to produce mousy *N*-heterocycles in *N*-heterocycles assay medium (*N*HAM) co-inoculated or not

The 90 mL of cultures were centrifuged (11,600 g for 9 minutes at 4 °C), then the pellets were washed twice with a buffer solution (KH_2PO_4 5.5 g/L, KCl 4.25 g/L, pH 4.5; 20 mL then 10 mL). The amount of population, inoculated in 22 mL of *N*HAM (ammonium citrate tribasic 2 g/L, calcium chloride 1 g/L, citric acid 2 g/L, D-fructose 50 g/L, acetaldehyde 100 mg/L, ethanol (96 %) 52 mL/L, iron sulphate 43 mg/L, L-lysine 5 g/L, L-ornithine 5 g/L, magnesium sulphate 12.5 mg/L, malic acid 5 g/L, manganese sulphate 25 mg/L, potassium chloride 4.25 mg/L, potassium phosphate monobasic 5.5 g/L, Tween 80 1 mL/L, pH 4.5, sterilised by filtration through 0.22 µm membranes), was adjusted to obtain 10^9 cells/mL for yeasts (using Thoma's chamber measurement) and an optical density (λ = 650 nm) index of 1 (corresponding to approximately 10^9 cells/mL) for the *P. parvulus* The medium was then incubated for 24 h at 25 °C. *B. bruxellensis* and *P. parvulus* strains have been tested for their ability to produce mousy compounds when inoculated together (nine couples) or separately. This screening has been done on two microbiological duplicates.

The same experimentation was performed with nine combinations of *B. bruxellensis* and *S. cerevisiae* strains. No replicate could be achieved due to technical issues; only preliminary results are discussed here.

For each batch of samples, a non-inoculated medium was incubated and analysed to control the absence of mousy compound production.

2. Chemical analysis

N-heterocycles (APY, ATHP, ETHP) were analysed using the method by SBSE-GC-MS optimised by Moulis *et al*. (2023) from Kiyomichi *et al.* (2023)

2.1 Sample preparation

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 as well as 20 µL of the deuterated internal standard (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 Analysis of ATHP, ETHP and APY by gas chromatography-mass spectrometry (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 oven 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: 230 °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-d3: 95/127/154.

2.4 Analysis of acetaldehyde by enzymatic kit

Acetaldehyde was quantified by enzymatic assay (K-ACHYD, Megazyme, Bray, Ireland) in *N*HAM after 24 h of incubation of *S. cerevisiae* strains.

3. Statistical analysis

Kruskal–Wallis statistical test (agricolae package, R, p-value < 0.05), ANOVA 1-way (p-value < 0.05), Student's t-test (p-value < 0.05) and Tukey's 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. Effect of co-inoculation of *Brettanomyces bruxellensis* and *Pediococcus parvulus* on mousy compound production

The results obtained for each strain inoculated alone are presented in Table 1. Depending on the specific strain, the three strains of *B. bruxellensis* exhibit varying levels of ATHP and ETHP production, as was shown previously by Moulis *et al*. (2023). It is observed that the three studied strains of *P. parvulus* can produce APY in large quantities and ETHP and ATHP to a lesser extent. These results highlight the ability of *P. parvulus* to produce mousy compounds.

This was already observed for one strain by Costello (Costello, 1998), but could not be confirmed until now.

Figure 1 represents the production of ATHP (A, B, C) and ETHP (D, E, F) when strains were separately or jointly inoculated. The cumulated amount is the sum of the production of the two microorganisms taken separately. For four out of the nine couples, the amount of ATHP (A, B, C) produced is significantly higher when the strains are inoculated together rather than separately. This synergy for ATHP production can be observed when *B. bruxellensis* CRBO L0417 or YJS 7816 is incubated with *P. parvulus* DSM 20332 or CRBO 0601. The level of the synergistic effect varies with the strains involved in the interaction. Concerning ETHP production (D, E, F), no interaction has been observed except for the couple *B. bruxellensis* CRBO L1751 and *P. parvulus* CRBO 0601 (E) for which a synergy is shown when strains are inoculated together.

The amounts of APY produced separately or jointly are shown in Figure 2. As a reminder, *B. bruxellensis* does not produce APY, which means that the cumulative quantities of APY are due to production by *P. parvulus* only. Results show that the presence of *B. bruxellensis*, whatever the strain, has an inhibitory effect on the production of APY by *P. parvulus*. Even if the difference is not significant for strain CRBO 0202 with its ropy character, APY was not produced when the strain was co-inoculated with *B. bruxellensis*.

TABLE 1. The concentration of the three *N*-heterocycles (APY, ETHP, and ATHP) produced independently by three *Brettanomyces bruxellensis* strains (CRBO L0417, CRBO L1751, YJS 7816) and three *Pediococcus parvulus* strains (CRBO 0202, CRNO 0601, DSM 20332) 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]	std[a]	av[a]	std[a]	av[a]	std[a]
Brettanomyces bruxellensis	CRBO L0417	< LOD[b]	-	61	55	123	70
	CRBO L1751	< LOD[b]	-	6	4	49	27
	YJS 7816	< 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, an average of the microbial duplicates; std, the standard deviation of the microbial duplicates;
[b]: LOD, the limit of detection; LOQ, the limit of quantification, according to Moulis *et al.*, 2023.

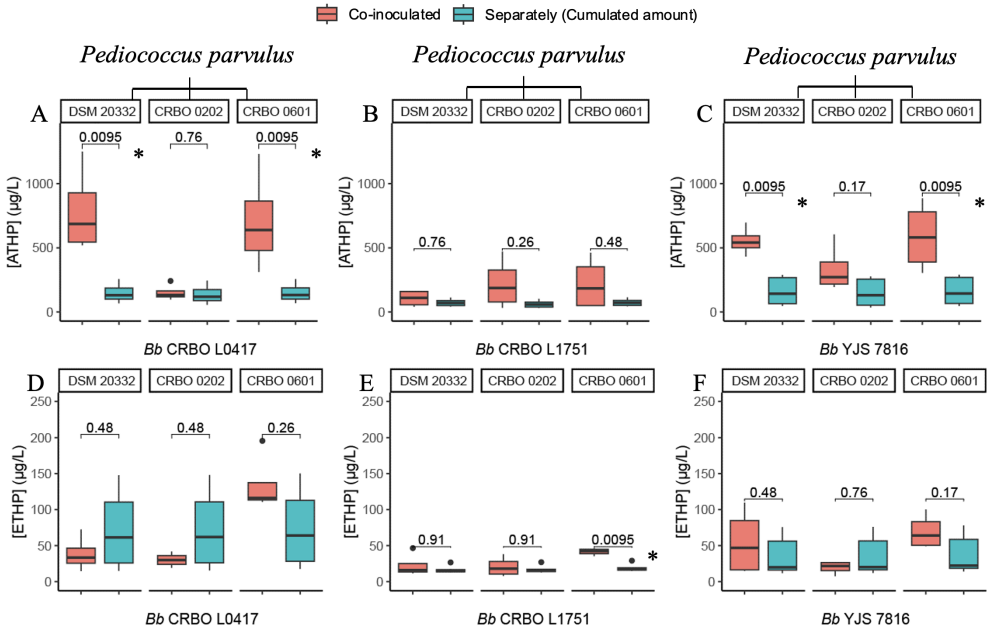


FIGURE 1. Comparison of the production of ATHP (A, B, C) and ETHP (D, E, F) by the three strains of *Brettanomyces bruxellensis* (CRBO L0417, CRBO L1751, YJS 7816) and three *Pediococcus parvulus* (DSM 20332, CRBO 0202, CRBO 0601) co-inoculated or separately in 24 h at 25 °C in *N*HAM; comparisons were made by a Wilcoxon test, the associated p-values are indicated (*: p-values < 0.05).

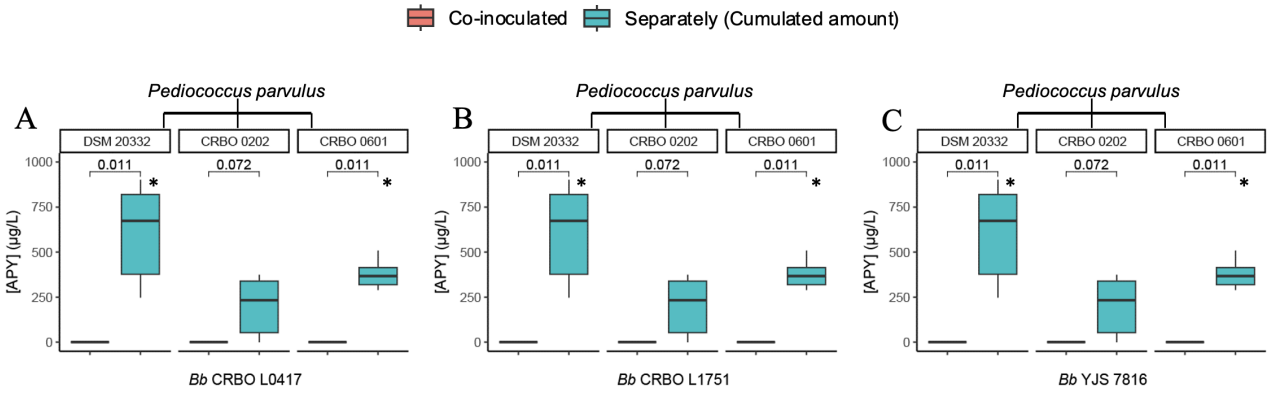


FIGURE 2. Comparison of the production of APY (A, B, C) by the three strains of *Brettanomyces bruxellensis* (CRBO L0417, CRBO L1751, YJS 7816) and three *Pediococcus parvulus* (DSM 20332, CRBO 0202, CRBO 0601) co-inoculated or separately in 24 h at 25 °C in *N*HAM; comparisons were made by a Wilcoxon test, the associated p-values are indicated (*: p-values < 0.05).

First, the joint inoculation of CRBO 0202 with any strain of *B. bruxellensis* did not appear to influence the production of both ATHP and ETHP (Figure 1). CRBO 0202 is the only strain of *P. parvulus* studied that was referenced as a ropy strain (Walling *et al.*, 2005). A possible explanation could be that the exopolysaccharide network produced by CRBO 0202 creates physical separation between yeasts and this strain preventing their interaction. This hypothesis would not explain the behaviour against APY (Figure 2).

Similarly, the ATHP production of the *B. bruxellensis* CRBO L1751 strain does not appear to be influenced by the co-inoculation of *P. parvulus* strains. The three *B. bruxellensis* strains tested belong to different genetic groups (Avramova *et al.*, 2018) and the production of *B. bruxellensis* species depends mainly on the strain and not on the genetic group (Moulis *et al.*, 2023). However, of the three strains tested, CRBO L1751 is the only diploid *Brettanomyces* strain, the other two are triploid (Avramova *et al.*, 2018; Harrouard *et al.*, 2022). The character of ploidy could have an impact on the production of *B. bruxellensis* when co-inoculated. Against that, the difference between strain CRBO L1751 and the two other strains of *B. bruxellensis* in the face of co-inoculation could not be explained.

Previous results (Strickland *et al.*, 2016) showed that less 4-ethylphenol (EP), a compound contributing to the "Brett character" in wine, was produced when *P. parvulus* and *B. bruxellensis* were jointly inoculated rather than separately. Here, *P. parvulus* and *B. bruxellensis* produce more ATHP and no APY at all when they are together. There are several hypotheses to explain that. First, Moulis (2023), proposed that ATHP production could correspond to a signal or a response to a signal between the different cells of *B. bruxellensis*, to respond to stress. Here, *B. bruxellensis* could reply to the stress of the presence of *P. parvulus* by ATHP over-production. *P. parvulus* could thus directly influence the metabolism of *B. bruxellensis*, resulting in the production of more ATHP than 4-ethylphenol in its presence. A second hypothesis is that *B. bruxellensis* could change the balance of acylation by *P. parvulus*. Costello and Henschke (2002) proposed a formation pathway for APY and ATHP by LAB from ornithine and lysine, respectively. This formation pathway, in both cases, ends up with acylation (of piperideine for ATHP and of pyrroline for APY). *B. bruxellensis* could inhibit APY production either by inhibiting the pathway or by preferentially inducing ATHP acylation by *P. parvulus*. Moreover, 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) and they can biosynthesise cadaverine to be metabolised by *B. bruxellensis*. It would be interesting to explore these hypotheses in a future study.

Moulis *et al.* (2023) highlighted an interesting correlation between the ETHP and ATHP produced by different microorganisms in the *N*HAM with a ratio of 1:10. Figure 3 represents the concentration of ETHP as a function of the concentration of ATHP. The same ratio as previously observed emerges as a tendency between ETHP and ATHP produced by *B. bruxellensis* in the presence or absence of *P. parvulus*.

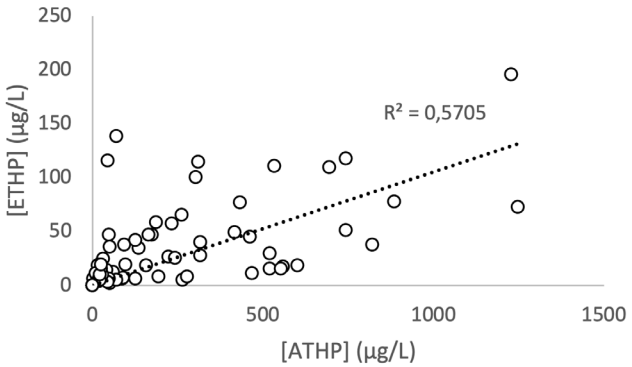


FIGURE 3. Representation of the concentration of ETHP as a function of the concentration of ATHP, with a Spearman ρ = 0.67 (p-value = 1.70×10^{-10}).

2. Exploration of the effect of co-inoculation of *Brettanomyces bruxellensis* and *Saccharomyces cerevisiae* on mousy compound production

The possible impact of the presence of *S. cerevisiae* on the production of mousy compounds by *B. bruxellensis* was studied. However, unfortunately, due to technical problems and a loss of sensitivity of the GC-MS instrument used, only one of the replicates could be considered. Despite this, some preliminary results can be presented.

Due to the technical issue, a calibration line associated with these experiments is not available and the results of the relative areas are presented. The strains of *S. cerevisiae* used in this experiment were also screened, none of the three *N*-heterocycles could be produced in the *N*HAM by these species when inoculated alone in the environment. Therefore, only the results of *B. bruxellensis*, with the presence or not of *Saccharomyces*, are shown here.

Figure 4 shows the relative areas of ATHP and ETHP for samples of three 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 (n = 2). However, it seems that more ATHP and ETHP were produced by the three strains of *B. bruxellensis* when they were in the presence of the SB strain. As described in the literature, *B. bruxellensis* does not produce APY (Grbin *et al.*, 2007; Moulis *et al.*, 2023), even in the presence of *S. cerevisiae*.

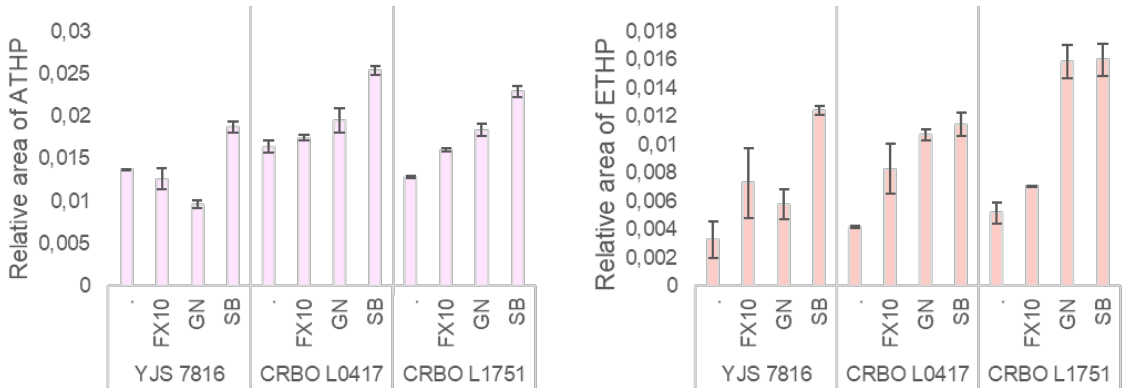


FIGURE 4. Relative areas of ATHP and ETHP produced by three strains of *Brettanomyces bruxellensis* (CRBO L0417, CRBO L1751, YJS 7816) co-inoculated (or not) with three strains of *Saccharomyces cerevisiae* (FX10, GN, SB); the standard deviation represents the deviation between the 2 chemical analyses.

TABLE 2. Summary table of the analysis of variance (ANOVA) of the relative area of ATHP and ETHP produced by three *Brettanomyces bruxellensis* and three *Saccharomyces cerevisiae* strains in 24 h at 25 °C in *N*HAM.

		ATHP		ETHP	
	Df[a]	Sum sq[a] (%)	P-value	Sum sq[a] (%)	P-value
Brettanomyces bruxellensis	3	87.71	8.41×10^{-7}	61.83	7.97×10^{-4}
Saccharomyces cerevisiae	3	7.65	1.70×10^{-2}	22.68	2.42×10^{-2}
Residuals	10	5.02		20.03	

[a]: Df, Degrees of freedom; Sum sq, Sum of squares.

In addition, the variability is mainly due to *B. bruxellensis* for ATHP (88 %) and ETHP (62 %) but the inoculation of *S. cerevisiae* also presents significant differences (Table 2). The variability due to *B. bruxellensis* has already been showed and discussed by Moulis *et al*. (2023). Figure 5 presents a boxplot of the relative area of ATHP and ETHP produced by the three strains of *B. bruxellensis* depending on the *S. cerevisiae* strain. The letters associated with each boxplot are the summary of the Tukey post-hoc tests for the two compounds corresponding to the significant difference shown. Concerning ATHP, the production was different between the strains of *S. cerevisiae* studied. *B. bruxellensis* strains were able to produce more when inoculated with SB than with FX10 (A) and the amount of ETHP produced by *B. bruxellensis* was increased when inoculated with SB and GN (B).

It is interesting to note that, a correlation exists between ETHP and ATHP produced by *B. bruxellensis* in the presence of *S. cerevisiae* (Spearman $\rho = 0.91$; Figure 6). However, the ratio cannot be compared to previous ones, because it was here calculated from the relative areas of ETHP and ATHP and not from their concentrations.

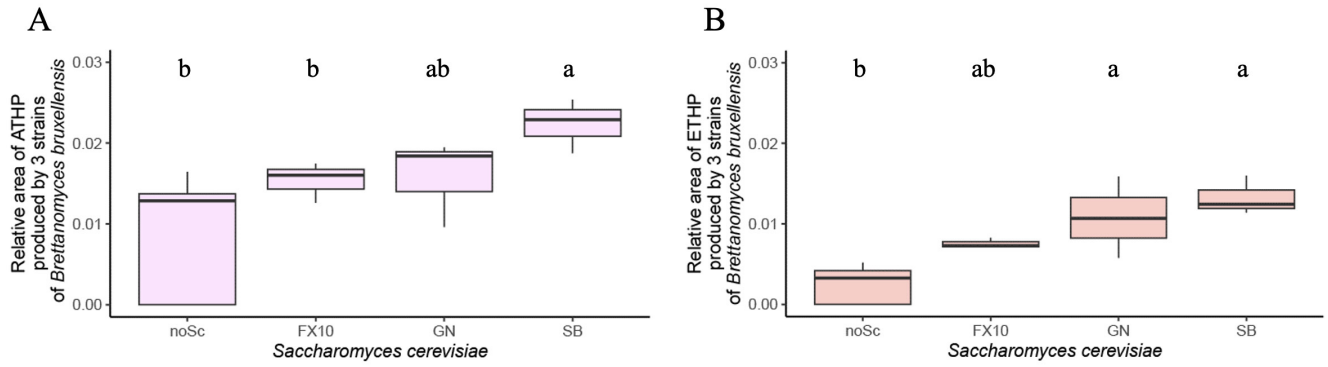


FIGURE 5. Comparison of the relative areas of ATHP (A) and ETHP (B) produced by three strains of *Brettanomyces bruxellensis* (CRBO L0417, CRBO L1751, YJS 7816) in the presence (or not) of three *Saccharomyces cerevisiae* strains (FX10, GN, SB) in 24 h at 25 °C in *N*HAM, depending on the strain of *S. cerevisiae*; comparisons were made by an ANOVA and Tukey post-hoc tests.

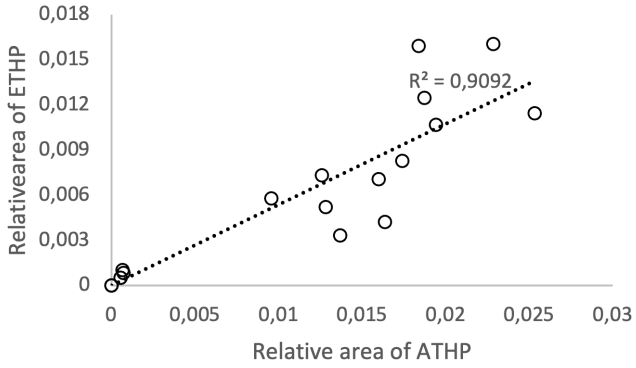


FIGURE 6. Representation of the relative area of ETHP as a function of the relative area of ATHP, with a Spearman ρ = 0.91 (p-value = 5.23×10^{-7}).

TABLE 3. Acetaldehyde levels after the incubation of three strains of *Saccharomyces cerevisiae* (FX10, GN, SB) for 24 h in *N*HAM.

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

[a]: av, average of the microbial duplicates; std, standard deviation of the microbial duplicates

Differences in the production of acetaldehyde have been observed among various strains of *S. cerevisiae* by Ochando *et al.* (2020). Acetaldehyde is identified as a precursor of mousy compounds (Costello and Henschke, 2002; Grbin *et al.*, 2007), prompting an investigation into its presence in *N*HAM after incubation with different *S. cerevisiae* strains.

Initially, the acetaldehyde is added to the *N*HAM at a level of 100 mg/L. The amounts of acetaldehyde after the incubation of *S. cerevisiae* are shown in Table 3. FX10 and GN strains would tend to consume acetaldehyde present in the medium while the SB strain would increase it. That may explain the synergistic effect of SB observed before, even though the behaviour of the *S. cerevisiae* strains in the presence of another microorganism could be totally different. However, this would not explain the situation for ETHP production in the presence of GN.

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, and 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 the production of these compounds.

In conclusion, there seems to be a real effect between different microorganisms when they are tested together in the same sample. It must also be kept in mind that in real conditions these microorganisms are never found alone in the environment, other species may be present in a minority way and could be impacted by synergistic or antagonistic effects of the production of mousy compounds. Moreover, APY production by *P. parvulus* strains appears 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. This work is the first to focus on the interaction between different microorganisms regarding the production of mousy compounds, until now, it was only tested in monoculture. These initial findings suggest that mousiness is not solely influenced by a single microorganism, but rather by a consortium of various microorganisms. Species previously considered as non-producers may thus contribute to a more complex consortium impact.

Further investigation on the role of acetaldehyde as a precursor for mousy compounds could provide additional insights into microbial interactions. Acetaldehyde is a key intermediate in many microbial metabolic pathways, and

its conversion into undesirable volatile compounds, such as those responsible for mousy off-flavours, needs particular attention. Future research should aim to better understand how fermentation conditions and microbial interactions influence concentrations of acetaldehyde, amino acids, biogenic amine (such as cadaverine), and other metabolites vary under different.

It is important to note that these studies were conducted in a model medium and that investigating these interactions in an oenological environment is necessary. These studies will not only deepen our understanding of the underlying mechanisms but also help develop strategies to mitigate the formation of unwanted compounds in microbial processes.

Moreover, the hypothesis advanced by Moulis *et al.* (2023) regarding the ratio of 1:10 found between ETHP and ATHP seems to be confirmed when *B. bruxellensis* is co-inoculated with *P. parvulus*. It would be interesting in the future to work on different media to see if this ratio would then change.

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