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ORIGINAL RESEARCH ARTICLE

# Modulation of the 'flinty' aroma compound phenylmethanethiol during fermentation: impacts of yeast starter culture and nitrogen supplementation

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## ABSTRACT

The sulfur compound phenylmethanethiol (PMT) has been associated with 'flinty' and 'struck-match' aromas in wine, and it is considered to be an important contributor to the flavour of certain white wine styles. Little is known about the factors driving the formation of this potent sulfur aroma compound during winemaking conditions, as well as the precursors/pathways that might be involved in its formation. In this study, we demonstrate that a range of practical winemaking strategies may have significant implications on the final concentration of PMT in wine. Specifically, the choice of yeast strain used to perform alcoholic fermentation was shown to modulate the formation of this 'flinty' compound under laboratory-scale conditions, and in the absence of oak. The nutritional level of the fermentation media was also found to significantly impact PMT formation by yeast, as nitrogen additions in the form of inorganic nitrogen promoted the formation of PMT. Finally, the potential role of both benzaldehyde and hydrogen sulfide as precursors to PMT formation was also explored, as well as the contribution of other alternative pathways.

**KEYWORDS:** wine, sulfur, aroma, flint, thiols, nitrogen

## INTRODUCTION

Volatile sulfur compounds (VSCs) in wine can be considered a 'double-edged sword'. While some of these compounds, particularly hydrogen sulfide ($H_2S$) and methanethiol (MeSH), contribute negatively to wine sensory properties and are associated with 'reductive' off-flavours (Smith *et al*., 2015), other VSCs make a positive and pleasant contribution to wine and are associated with important varietal characters and stylistic aroma expression of wine (Capone *et al*., 2017; Tominaga *et al*., 2003b). Prominent examples of positive VSCs include the polyfunctional thiols 4-methyl-4-sulfanylpentan-2-one (4-MSP), 3-sulfanylhexan-1-ol (3-SH) and its acetate ester, 3-sulfanylhexyl acetate (3-SHA), which impart 'fruity' aromas in white wines (Darriet *et al*., 1995; Tominaga *et al*., 1998). Similarly, the aryl thiols 2-furanmethanethiol (2-FMT) and phenylmethanethiol (PMT) are particularly important odorants and have been associated with 'roasted coffee' and 'flinty/struck flint' aromas, respectively (Espinase Nandorfy *et al*., 2023; Tominaga *et al*., 2000; Tominaga *et al*., 2003a, 2003b). This group of positive VSCs have very low perception thresholds, in the low nanogram-per-litre range, and are important contributors to the flavour of white wines, particularly in Sauvignon blanc and Chardonnay (Capone *et al*., 2017; Espinase Nandorfy *et al*., 2023; Guth, 1997), and also in sparkling wines (Tominaga *et al*., 2003b).

The different precursors and pathways leading to the formation of the polyfunctional thiols 3-SH, 3-SHA and 4-MSP, as well as their contributions to wine aroma and flavour, have been extensively studied (reviewed in Roland *et al*. (2011); Coetzee and du Toit (2012)). In contrast, little is known about the formation and impact of 2-FMT in wine (Blanchard *et al*., 2001; Tominaga *et al*., 2000), and particularly of the 'flinty' compound PMT (Espinase Nandorfy *et al*., 2023).

The formation and fate of polyfunctional thiols in wine are mediated by several factors (Smith *et al*., 2015). A prominent role is played by the *Saccharomyces cerevisiae* yeasts used to perform wine fermentation, as the choice of yeast strain modulates the concentration of 4-MSP, 3-SH and 3-SHA in both white and red wines (Cordente *et al*., 2022; Dubourdieu *et al*., 2006; King *et al*., 2008). It is known that precursors of 4-MSP and 3-SH are present in grape must as non-volatile odourless compounds, mainly as conjugates of glutathione and cysteine (Capone *et al*., 2010; Fedrizzi *et al*., 2009), as well as other breakdown dipeptide intermediates (Bonnaffoux *et al*., 2017; Cordente *et al*., 2015). Once the cysteinylated precursors are formed through degradation of their glutathionylated or dipeptide precursors, or assimilated from grape must, these are cleaved by yeast enzymes with cysteine-*S*-conjugate β-lyase activity to release the free thiols 3-SH and 4-MSP, in both white and red wines (Cordente *et al*., 2022; Roncoroni *et al*., 2011). Even though, and to the best of our knowledge, no amino acid/peptide precursors of either PMT or 2-FMT have been identified in grape must and/or wine, *S. cerevisiae* has been shown to catalyse the bioconversion of cysteine-aldehyde conjugates of both aryl thiols *in vitro* (Huynh-Ba *et al*., 2003; Zha *et al*., 2018) to release the free thiols, through a reaction dependent on yeast β-lyase activity (Zha *et al*., 2018).

A second mechanism is involved in the synthesis of both 3-SH and 2-FMT (Araujo *et al*., 2017; Blanchard *et al*., 2001; Harsch *et al*., 2013), which requires the replacement of the carbonyl group of an aldehyde with the sulfhydryl group of a suitable sulfur donor, such as $H_2S$ or cysteine. During alcoholic fermentation, $H_2S$ is actively produced by the wine yeast from the metabolism of both inorganic (sulfite, sulfate, elemental sulfur), as well as organic sulfur (cysteine, glutathione) sources (Araujo *et al*., 2017; Cordente *et al*., 2009). Depending on the thiol, the nature and the origin of the aldehyde precursor will be different. The C6-compound (*E*)-2-hexenal, which can be found in high concentrations in the grape must, has been shown to react with $H_2S$ to produce 3-SH during grape must fermentations (Harsch *et al*., 2013). The 'roasted coffee' aroma compound 2-FMT is believed to be formed primarily by the reaction between $H_2S$ and furfural, the latter released by toasted wood/staves when oak materials come in contact with wine during oak barrel fermentation or ageing (Blanchard *et al*., 2001; Tominaga *et al*., 2000). Given the similarities between these thiol-containing compounds, benzaldehyde has been considered to be the aldehyde precursor to PMT formation (Tominaga *et al*., 2003a). While oak barrels are believed to be the main source of benzaldehyde in wine (Herrera *et al*., 2020), small amounts of benzaldehyde (in the low $\mu gL^{-1}$ range) are produced by yeast *de novo* during fermentation (Delfini, 1991; Dumitriu *et al*., 2019) and can be found naturally in white grape musts (García *et al*., 2003; Rosillo *et al*., 1999). These low concentrations of benzaldehyde might be enough for some PMT formation (in the low $ngL^{-1}$ range), as this 'flinty' compound can be found in unoaked white wines (Piano *et al*., 2014). Given the extremely low aroma detection threshold of PMT (0.3 $ngL^{-1}$ in aqueous ethanol) (Tominaga *et al*., 2003a), this compound can be present at concentrations of sensory significance in wines, particularly for Chardonnay and sparkling wines (Capone *et al*., 2017; Espinase Nandorfy *et al*., 2023; Tominaga *et al*., 2003a).

In this study, the different mechanisms and potential precursors that might give rise to the formation of PMT have been studied in detail, as well as some of the winemaking conditions that potentially can promote the formation of PMT. The choice of yeast strain was assessed regarding its role in the modulation of the formation of PMT, in laboratory-scale fermentations in both natural white and synthetic grape musts (SGM). Similarly, the nitrogen content of the fermentation media and its role in PMT formation was also studied.

## MATERIALS AND METHODS

### 1. Chemicals

Analytical reagents were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) unless otherwise indicated. The following compounds were synthesised in-house as per the cited methods: 4-MSP (Howell *et al*., 2004); [$^2H_{10}$]-4MSP, 3-SH, [$^2H_5$]-3-SHA and 3SHA (Kotseridis *et al*., 2000);

[$^2H_{10}$]-3-SH (Pardon *et al*., 2008). [$^2H_6$]-Benzaldehyde and [$^2H_5$]-benzoic acid were supplied by Cambridge Isotope Laboratories (Novachem, Collingwood, Vic, Australia), and [$^2H_5$]-benzyl acetate and [$^2H_5$]-benzyl alcohol by CDN Isotopes (SciVac, Hornsby, NSW).

## 2. Microorganisms and culture conditions

All yeast strains were obtained from The Australian Wine Research Institute (AWRI) culture collection. Yeast cultures were maintained on solid yeast extract peptone dextrose (YPD) agar plates (2 % glucose, 2 % peptone, 1 % yeast extract, and 2 % agar). The strains used in this study are summarised in Table S1. Strain AWRI3442 was isolated from AWRI1616 using chemical mutagenesis (Cordente *et al*., 2009), and contains a potentially inactivating mutation in the *MET17* gene (unpublished results), which might lead to an impairment of the ability of yeast to sequester $H_2S$ and result in the subsequent accumulation of this off-flavour in wine (Linderholm *et al*., 2008).

## 3. Laboratory-scale fermentations

Laboratory-scale fermentations were performed in triplicate in different fermentation media such as synthetic grape must (SGM), and Chardonnay and Pinot noir musts.

The composition of the SGM is described in Schmidt *et al*. (2011) with minor modifications. The sugar content of the SGM was adjusted to 100 $gL^{-1}$ of glucose, and 100 $gL^{-1}$ of fructose, and the concentration of some trace minerals were modified to: $MnSO_4 \cdot H_2O$ 3.5 $mgL^{-1}$, $ZnCl_2$ 1 $mgL^{-1}$, $FeSO_4 \cdot 7H_2O$ 6 $mgL^{-1}$, $CuSO_4 \cdot 5H_2O$ 1.5 $mgL^{-1}$. The contribution of the organic nitrogen, in the form of α-amino nitrogen (from proteinogenic amino acids, including citrulline, ornithine and gamma-aminobutyrate) to the yeast assimilable nitrogen (YAN) was adjusted to 80 mg N $L^{-1}$. Then different concentrations of inorganic nitrogen, in the form of ammonia, were added to the juice to give a total YAN to either 100, 150, 175, 250 or 350 mg N $L^{-1}$, depending on the specific experiment. For both the yeast screening and benzaldehyde spiking experiments in SGM, a concentration of YAN of 175 mg N $L^{-1}$ was used. The pH of the media was adjusted to pH 3.5 with concentrated HCl before filtering the media.

A filtered-sterilized Chardonnay must (must MV) from the McLaren Vale region in South Australia (Australia) was used for the screening of different strains in laboratory-scale fermentation with the following basic physicochemical composition: 212 mg N $L^{-1}$ YAN (85 $mgL^{-1}$ ammonia and 142 $mgL^{-1}$ α-amino nitrogen), 216 $gL^{-1}$ sugars, titratable acidity (pH 8.2) 3.7 $gL^{-1}$ and pH 3.55. The total $SO_2$ was below the detection limit of 3 $mgL^{-1}$.

To assess the effect of inorganic nitrogen in PMT formation, a low-YAN filtered-sterilised Chardonnay must (must AH) from the Adelaide Hills region in South Australia (Australia) was used with the following composition: 147 mg N $L^{-1}$ YAN (64 $mgL^{-1}$ ammonia and 94 $mgL^{-1}$ α-amino nitrogen), 209 $gL^{-1}$ sugars, titratable acidity (pH 8.2) 4.7 $gL^{-1}$, pH 3.25 and 19 $mgL^{-1}$ total $SO_2$. Inorganic ammonia was added to the juice to achieve a total YAN of 250 and 350 mg N $L^{-1}$, and the pH was adjusted back to 3.25 with concentrated HCl. A concentration of at least 140 mg N $L^{-1}$ is considered necessary for most yeast to complete fermentation under anaerobic conditions and moderate sugar concentrations (Torrea *et al*., 2011).

Finally, an unfiltered Pinot noir must (2020 vintage) from the McLaren Vale wine region in South Australia (Australia) was also used. The basic physicochemical composition of the Pinot noir must was 197 mg N $L^{-1}$ YAN (58 $mgL^{-1}$ ammonia and 149 $mgL^{-1}$ α-amino nitrogen), 233 $gL^{-1}$ sugars, 1640 nephelometric turbidity unit (NTU) turbidity, and pH 3.44. Total $SO_2$ was below the detection limit of 3 $mgL^{-1}$. Pinot noir wines were fermented without the skins (rosé style wine fermentation).

Yeast starter cultures were prepared by growing cells in YPD medium aerobically for 24 h to stationary phase at 28 °C. Then $1 \times 10^6$ cells $mL^{-1}$ were inoculated into 50 % diluted must, which had been previously filtered through 0.22 µm Stericup filters (Millipore), and grown for another 48 h at 22 °C. The acclimatised cells were inoculated into 90 mL of must at a density of $1 \times 10^6$ cells $mL^{-1}$. Fermentations were conducted in 100 mL glass bottles (Schott Duran) fitted with stir bars and stirred at 120 rpm using a magnetic stirrer. Fermentations were conducted at 17 °C except for the Pinot noir ferments which were fermented at 22 °C. The lids of the Schott bottles were fitted with $H_2S$ detector tubes (Komyo, Kitagawa, Japan). Fermentation progress was followed by $CO_2$ weight loss, measured every 24 h, which was completed (< 2 $gL^{-1}$ sugars). After fermentation, potassium metabisulfite was added to achieve a final concentration of 50 $mgL^{-1}$ total $SO_2$, and ferments were cold-settled at 4 °C until sampled for different volatile and non-volatile compound analyses.

## 4. Analysis of aroma compounds

### 4.1. Analysis of volatile sulfur compounds

Polyfunctional thiols 3-SH, 3-SHA and 4-MSP, and aryl thiols 2-FMT and PMT were analysed once ferments achieved dryness on a Sciex QTRAP 6500 coupled to an Exion UHPLC system using the method described by Capone *et al*. (2015), with some modifications (Cordente *et al*., 2022).

The low molecular weight sulfur compounds (LMWSCs) hydrogen sulfide ($H_2S$), methanethiol (MeSH), dimethyl sulfide, diethyl sulfide, dimethyl disulfide, diethyl disulfide, ethanethiol (EtSH), carbon disulfide, methyl thioacetate (MeSAc) and ethyl thioacetate (EtSAc), were determined using an Agilent 8355 sulfur chemiluminescence detector coupled to an Agilent 7890B gas chromatograph (Forest Hill, VIC, Australia). LMWSCs analysis was carried out as described previously (Siebert *et al*., 2010), with slight modifications, as described in Cordente *et al*. (2022). The $H_2S$ that was liberated during fermentation ($H_2S_f$) was detected in the headspace using silver nitrate selective gas detector tubes (Komyo, Kitagawa, Japan) (Winter *et al*., 2011).

### 4.2. Benzenoid compounds

Benzenoid compounds (benzyl acetate, benzyl alcohol, benzoic acid and benzaldehyde) were quantified by HS-SPME-GC/MS using an Agilent 7890A GC coupled to an Agilent 5975C MS and equipped with a Gerstel MPS2-XL, as described in Espinase Nandorfy *et al*., 2023 and included benzoic acid, using [$^2H_5$]-benzoic acid as its internal standard.

## 5. Statistical analysis

Minitab 21 statistical software (Minitab Inc., Pennsylvania, USA) was used for statistical analysis. The chemical data were analysed by one-way ANOVA, using Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$), and P values were determined by a two-tailed Student's t-test. GraphPad 10 for Windows (GraphPad Software, San Diego, USA) was also used for regression (statistical) analysis and graphing.

# RESULTS

## 1. Modulation of phenylmethanethiol formation by yeast in laboratory-scale fermentations

Commercial yeast manufacturers offer a wide range of yeast strains, with different aromatic profiles and fermentation properties, but little is known about their ability to modulate the formation of the 'flinty' compound PMT during wine fermentation conditions. To improve our understanding of the role of yeast on PMT production, a group of 13 *Saccharomyces* strains were screened in different fermentation media such as synthetic grape must (SGM), a filtered Chardonnay must, and an unfiltered Pinot noir must. The strains, which are summarised in Table S1, were chosen based on their diverse ability to produce two of the potential precursors for PMT formation: the sulfur donor $H_2S$, and the benzenoid precursor compound benzaldehyde. Additionally, strains with a diverse ability to release the 'fruity' thiols 3-SH and 4-MSP were also selected to assess whether yeast β-lyase activity might also be involved in PMT formation (Cordente *et al*., 2019).

In the SGM ferments the choice of wine yeast strain had a statistically significant effect on PMT formation ($P < 0.001$, $F = 5.3$), and a 6-fold difference was found between the highest and the lowest PMT-producing strains (5.3 vs. 0.9 $ngL^{-1}$, respectively) (Figure 1a, Table S2). PMT was the only thiol detected of the five analysed in this medium, suggesting *de novo* formation from fermentation-derived metabolites. A statistically significant correlation was observed between PMT and benzaldehyde ($R = 0.693$, $P < 0.01$) (Figure S1 and Table S3), with the highest PMT-producing yeast, the *Saccharomyces uvarum* strain AWRI1375, also producing high levels of benzaldehyde (21.3 $\mu gL^{-1}$) (Table S2). On the other hand, no correlation was observed between PMT formation and $H_2S$, either when this potential sulfur donor was measured at the end of fermentation or released during the fermentation process ($H_2S_f$) (Table S3).

The Chardonnay ferments (must MV) were characterised by an even greater variability between yeast strains in their ability to produce PMT ($P < 0.0001$, $F = 15.2$), with the highest producing strain, AWRI2865, yielding 6.4 $ngL^{-1}$ of PMT (Figure 1b, Table S4). Unlike the SGM ferments, no correlation was found between PMT and benzaldehyde in Chardonnay (Table S5); the two highest benzaldehyde-producing strains, AWRI1375 and AWRI4234, did not produce any detectable PMT, despite producing moderate levels of $H_2S$ (Table S4). Even though no correlation was found between PMT and $H_2S$, a significant positive correlation ($R = 0.620$, $P < 0.05$) was observed with MeSH (Figure S2). While the different strains assessed showed some variability in 3-SH and 3-SHA release, no correlation between these 'fruity' thiols and PMT levels was observed in the Chardonnay wines (Table S5).

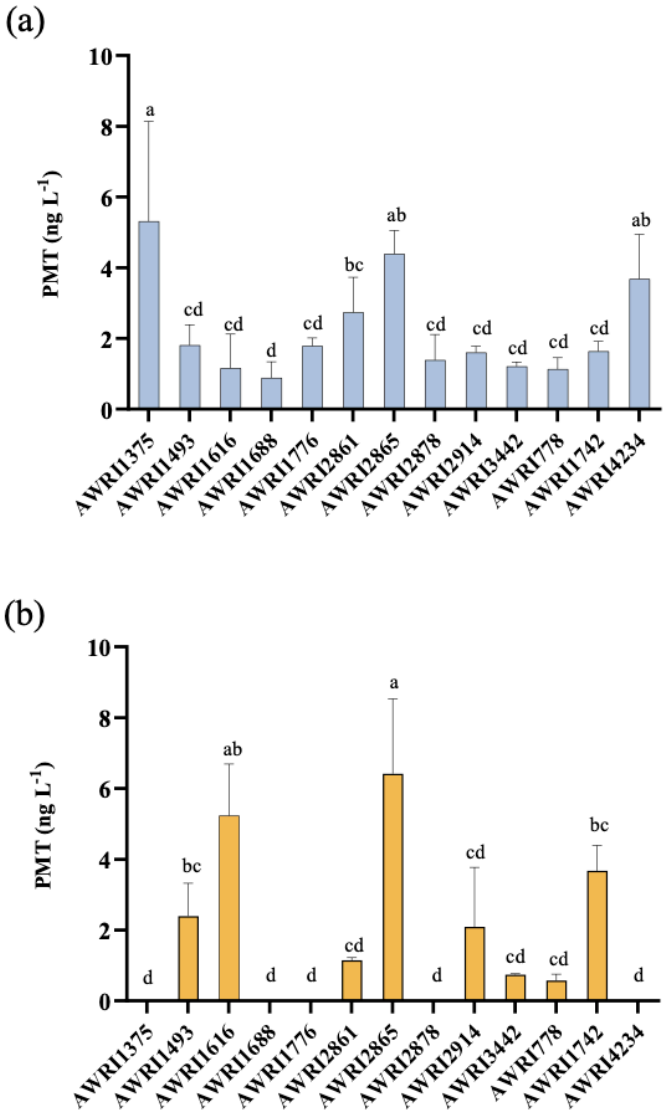



**FIGURE 1.** Production of phenylmethanethiol (PMT, $ngL^{-1}$) by 13 wine yeast strains after laboratory-scale fermentation of a synthetic grape must (SGM) (a, blue) or a Chardonnay (must MV) (b, yellow).

Means with the same letter are not significantly different from each other [Tukey's HSD test, $P < 0.05$].

Overall, the lowest PMT concentrations were observed in the Pinot noir wines, with only one of the strains assessed, namely the high-$H_2S$-producing strain AWRI3442, producing levels of PMT (1.3 $ngL^{-1}$) just above its limit of quantification (1.1 $ngL^{-1}$) (Figure S3).

## 2. Potential precursors involved in phenylmethanethiol formation: role of hydrogen sulfide and benzaldehyde

The fact that relatively high concentrations of PMT were observed in the synthetic media (~ 6 $ngL^{-1}$), similar to PMT concentrations measured in a group of commercial white wines from around the world (Espinase Nandorfy *et al*., 2023; Mateo-Vivaracho *et al*., 2010), indicates that PMT can be generated *de novo* during the fermentation process from yeast-derived metabolites. While previous research has suggested that benzaldehyde might act as a precursor to PMT formation (Tominaga *et al*., 2003a), this has not been conclusively demonstrated. In the case of the potential sulfur donor precursor, while the formation of the related aryl thiol 2-FMT has been associated with excess production of $H_2S$ by yeast (Blanchard *et al*., 2001), this has not yet been proven for PMT formation.

To confirm the role of $H_2S$ and benzaldehyde as precursors to PMT formation, a series of laboratory-scale fermentations were conducted in SGM. Four strains with different $H_2S$-producing abilities, as assessed in the previous screening experiment, were selected, and the media was spiked with different concentrations of benzaldehyde when the first signs of $H_2S$ being produced by yeast were observed in the gas detector tubes ($H_2S_f$) (around 30 h after inoculation for AWRI3442, 42 h for AWRI1375 and 46 h for both AWRI2865 and AWRI1616).

For the two low-$H_2S$-producing strains, AWRI2865 and 1616, the addition of external benzaldehyde did not have any impact on PMT formation when compared to the control or non-spiked ferments (Figure 2). Overall, at any of the different benzaldehyde spiked levels, these two strains produced the lowest concentrations of PMT of all the yeasts assessed. Conversely, for the moderate (AWRI1375) and high (AWRI3442) $H_2S$-producing strains, the formation of PMT was significantly enhanced by the addition of external benzaldehyde (Figure 2). The largest effect was observed in wines fermented with strain AWRI3442, in which a two-fold increase in PMT formation was observed between the largest addition of benzaldehyde (2000 $\mu gL^{-1}$) and the non-spiked ferments (18.4 vs. 8.2 $ngL^{-1}$, respectively) (Figure 2, Table 1).

Interestingly, the spiking of external benzaldehyde only resulted in a small accumulation of this compound in the final wines (Table 1). On average, after the addition of 2000 $\mu gL^{-1}$ of benzaldehyde, only 4.2 $\mu gL^{-1}$ of additional benzaldehyde could be detected when compared to the non-spiked wines. Analysis of potential metabolites derived from benzaldehyde showed that it was mainly converted by yeast to benzyl alcohol and benzoic acid, with small amounts of benzyl acetate also being produced (Table 1). The three *S. cerevisiae* strains showed a slight preference for the reduction of benzaldehyde to benzyl alcohol, while *S. uvarum* AWRI1375 showed a preference for its oxidation to benzoic acid (Table 1). On average, in the model ferments with the addition of 500 or 2000 $\mu gL^{-1}$ of benzaldehyde, 87.9 % and 77.1 % of the initial

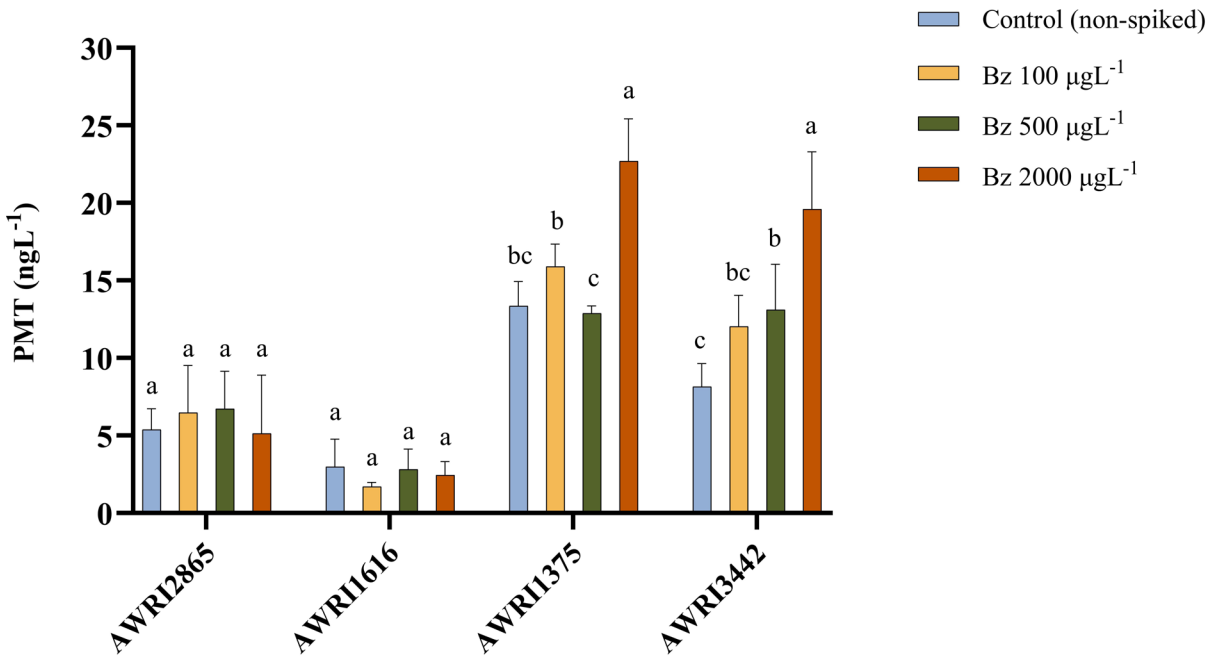



**FIGURE 2.** Formation of phenylmethanethiol (PMT, $ngL^{-1}$) by four yeast strains in a synthetic grape must (SGM).

Strains are shown in ascending order in their ability to produce hydrogen sulfide ($H_2S$). Different amounts of benzaldehyde (Bz) (100, 500 and 2000 $\mu gL^{-1}$) were spiked in the ferments at the first signs of $H_2S$ production. Means with the same letter are not significantly different from each other (Tukey's HSD test, $P < 0.05$) for each of the yeast strains assessed.

**TABLE 1.** Effect of benzaldehyde spiking in volatile compound formation after fermentation of a synthetic grape must (SGM) with four yeast strains with different $H_2S$-producing phenotypes.

| Yeast strain ($H_2S$ production phenotype) | Must[1] | PMT ($ngL^{-1}$) | Benzenoid compounds ($\mu gL^{-1}$) | | | | | LMWSCs ($\mu gL^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Benzaldehyde | Benzyl alcohol | Benzyl acetate | Benzoic acid | ΣBenzenoids[2] | $H_2S_f$ (µg) | $H_2S$ | MeSH | MeSAc |
| AWRI2865 (low) | SGM | 5.4 ± 1.3$^{a}$ | 5.4 ± 1.9$^{b}$ | 7.1 ± 4.1$^{c}$ | < LOD | 30 ± 17$^{c}$ | 36 ± 18$^{c}$ | 7.2 ± 0.8$^{a}$ | 1.4 ± 0.3$^{a}$ | 6.1 ± 1.3$^{a}$ | < LOD |
| | SGM + Bz 100 $\mu gL^{-1}$ | 6.5 ± 3.0$^{a}$ | 5.5 ± 1.5$^{b}$ | 70 ± 2.4$^{c}$ | 0.7 ± 0.1$^{c}$ | 8.1 ± 7.7$^{c}$ | 82 ± 9$^{c}$ | 7.5 ± 0.6$^{a}$ | 1.4 ± 0.5$^{a}$ | 5.6 ± 2.6$^{a}$ | < LOD |
| | SGM + Bz 500 $\mu gL^{-1}$ | 6.7 ± 2.4$^{a}$ | 5.9 ± 0.7$^{b}$ | 349 ± 17$^{b}$ | 3.3 ± 0.2$^{b}$ | 171 ± 23$^{b}$ | 499 ± 29$^{b}$ | 7.8 ± 1.1$^{a}$ | 1.4 ± 0.3$^{a}$ | 5.0 ± 0.5$^{a}$ | < LOD |
| | SGM + Bz 2000 $\mu gL^{-1}$ | 5.1 ± 2.7$^{a}$ | 10.1 ± 2.4$^{a}$ | 1433 ± 59$^{a}$ | 13.2 ± 1.0$^{a}$ | 489 ± 42$^{a}$ | 1861 ± 93$^{a}$ | 7.6 ± 0.9$^{a}$ | 2.1 ± 1.1$^{a}$ | 5.6 ± 1.2$^{a}$ | < LOD |
| AWRI1616 (low) | SGM | 3.0 ± 1.6$^{a}$ | 9.9 ± 0.5$^{a}$ | 3.7 ± 0.8$^{c}$ | <LOD | 136 ± 38$^{b}$ | 131 ± 34$^{c}$ | 11.2 ± 0.9$^{a}$ | 1.3 ± 0.6$^{a}$ | 6.7 ± 1.1$^{a}$ | 4.8 ± 1.0$^{a}$ |
| | SGM + Bz 100 $\mu gL^{-1}$ | 1.7 ± 0.3$^{a}$ | 11.7 ± 2.6$^{a}$ | 48 ± 1.9$^{bc}$ | < LOD | 79 ± 19$^{b}$ | 117 ± 16$^{c}$ | 10.2 ± 0.6$^{a}$ | 0.8 ± 0.5$^{a}$ | 6.1 ± 0.7$^{a}$ | 4.9 ± 1.1$^{a}$ |
| | SGM + Bz 500 $\mu gL^{-1}$ | 2.8 ± 1.2$^{a}$ | 13.2 ± 2.7$^{a}$ | 219 ± 24$^{b}$ | 0.6 ± 0.1$^{b}$ | 168 ± 37$^{b}$ | 388 ± 30$^{b}$ | 10.8 ± 1.9$^{a}$ | 1.0 ± 0.3$^{a}$ | 6.2 ± 1.8$^{a}$ | 5.9 ± 0.4$^{a}$ |
| | SGM + Bz 2000 $\mu gL^{-1}$ | 2.5 ± 0.9$^{a}$ | 15.4 ± 3.3$^{a}$ | 1068 ± 169$^{a}$ | 3.8 ± 0.3$^{a}$ | 555 ± 57$^{a}$ | 1475 ± 37$^{a}$ | 9.6 ± 1.5$^{a}$ | 1.2 ± 0.1$^{a}$ | 6.4 ± 0.8$^{a}$ | 5.4 ± 0.2$^{a}$ |
| AWRI1375 (moderate-high) | SGM | 13.4 ± 1.6$^{bc}$ | 20.4 ± 2.0$^{a}$ | 6.5 ± 1.0$^{c}$ | < LOD | 86 ± 29$^{c}$ | 101 ± 23$^{c}$ | 124 ± 3.1$^{a}$ | 3.8 ± 0.6$^{a}$ | 2.1 ± 0.7$^{a}$ | 5.0 ± 0.1$^{a}$ |
| | SGM + Bz 100 $\mu gL^{-1}$ | 15.9 ± 1.5$^{b}$ | 20.7 ± 3.0$^{a}$ | 17 ± 1.5$^{c}$ | < LOD | 94 ± 35$^{c}$ | 119 ± 27$^{c}$ | 125 ± 12$^{a}$ | 4.2 ± 0.5$^{a}$ | 1.7 ± 0.6$^{a}$ | 4.5 ± 0.2$^{a}$ |
| | SGM + Bz 500 $\mu gL^{-1}$ | 12.9 ± 0.5$^{c}$ | 20.0 ± 3.6$^{a}$ | 74 ± 2.6$^{b}$ | < LOD | 334 ± 69$^{b}$ | 408 ± 65$^{b}$ | 125 ± 19$^{a}$ | 3.1 ± 2.1$^{a}$ | 2.7 ± 0.4$^{a}$ | 5.2 ± 0.5$^{a}$ |
| | SGM + Bz 2000 $\mu gL^{-1}$ | 22.7 ± 2.7$^{a}$ | 22.9 ± 2.5$^{a}$ | 382 ± 42$^{a}$ | 2.2 ± 0.3$^{a}$ | 1033 ± 73$^{a}$ | 1347 ± 74$^{a}$ | 114 ± 12$^{a}$ | 6.7 ± 5.0$^{a}$ | 2.5 ± 0.4$^{a}$ | 5.2 ± 1.4$^{a}$ |
| AWRI3442 (high) | SGM | 8.2 ± 1.5$^{c}$ | 6.6 ± 0.5$^{b}$ | 3.9 ± 0.4$^{c}$ | < LOD | 101 ± 14$^{c}$ | 98 ± 13$^{c}$ | 98.4 ± 3.6$^{a}$ | 2.1 ± 1.5$^{a}$ | 3.7 ± 1.0$^{ab}$ | 16.8 ± 0.7$^{a}$ |
| | SGM + Bz 100 $\mu gL^{-1}$ | 12.0 ± 2.0$^{bc}$ | 9.2 ± 1.2$^{ab}$ | 44 ± 1.7$^{c}$ | < LOD | 61 ± 36$^{c}$ | 105 ± 19$^{c}$ | 91.9 ± 3.6$^{b}$ | 2.4 ± 1.8$^{a}$ | 2.0 ± 0.2$^{b}$ | 14.5 ± 0.8$^{a}$ |
| | SGM + Bz 500 $\mu gL^{-1}$ | 13.1 ± 2.9$^{b}$ | 11.2 ± 3.5$^{a}$ | 221 ± 23$^{b}$ | 0.7 ± 0.1$^{b}$ | 287 ± 112$^{b}$ | 461 ± 107$^{b}$ | 101 ± 4.1$^{a}$ | 1.2 ± 0.5$^{a}$ | 4.0 ± 0.2$^{a}$ | 17.6 ± 1.4$^{a}$ |
| | SGM + Bz 2000 $\mu gL^{-1}$ | 18.4 ± 4.2$^{a}$ | 10.9 ± 0.7$^{ab}$ | 1122 ± 164$^{a}$ | 4.4 ± 0.9$^{a}$ | 551 ± 15$^{a}$ | 1475 ± 20$^{a}$ | 96.9 ± 3.0$^{ab}$ | 3.3 ± 0.1$^{a}$ | 3.3 ± 0.8$^{ab}$ | 16.2 ± 1.6$^{a}$ |

[1]Benzaldehyde (Bz) was spiked when the first signs of $H_2S$ being produced by yeast were observed in the gas detector tubes ($H_2S_f$). [2]Expressed as benzaldehyde equivalents. Means with the same letter are not significantly different from each other (Tukey's HSD test, $P < 0.05$) for each of the yeast strains assessed. LOD (limit of detection).

benzaldehyde, respectively, could be recovered as one of these benzenoid-derived metabolites (Table 1).

Overall, a statistically significant correlation was observed between the levels of benzaldehyde in the final wines and PMT formation ($R = 0.554$, $P < 0.05$) (Figure 3a, Table S6). An even stronger correlation was observed between the concentrations of PMT and those of the sulfur donor $H_2S$, either as $H_2S$ in the final wine ($R = 0.871$, $P < 0.0001$) or with the $H_2S$ released during fermentation ($H_2S_f$) ($R = 0.852$, $P < 0.0001$) (Figure 3b and c, respectively).

## 3. Biochemical pathways leading to the formation of the potential PMT precursor benzaldehyde: role of *ARO10*

The biochemical pathways leading to the formation of benzaldehyde by yeast are poorly understood. It is believed that the branch of the Ehrlich pathway responsible for the catabolism of the aromatic amino acid phenylalanine, linked with the mandelate pathway, might play a major role in benzaldehyde formation in yeast (Martin *et al*., 2016; Valera *et al*., 2020). In particular, the yeast enzyme phenylpyruvate decarboxylase, encoded by *ARO10*, has been suggested to catalyse two of the steps that might potentially lead to the formation of benzaldehyde and benzyl alcohol (Valera *et al*., 2020).

The role of *ARO10* in the modulation of the formation of both benzaldehyde and PMT was investigated in SGM through its deletion and overexpression in the haploid wine yeast strain AWRI1631. The overexpression of *ARO10* resulted in an 8-fold increase in the levels of benzaldehyde at the end of fermentation when compared to the control strain (Figure 4a), however, this was not translated into statistically significant higher PMT levels in the final wine (Figure 4b). The deletion of *ARO10* resulted in a small, although not statistically significant, decrease in the levels of both PMT

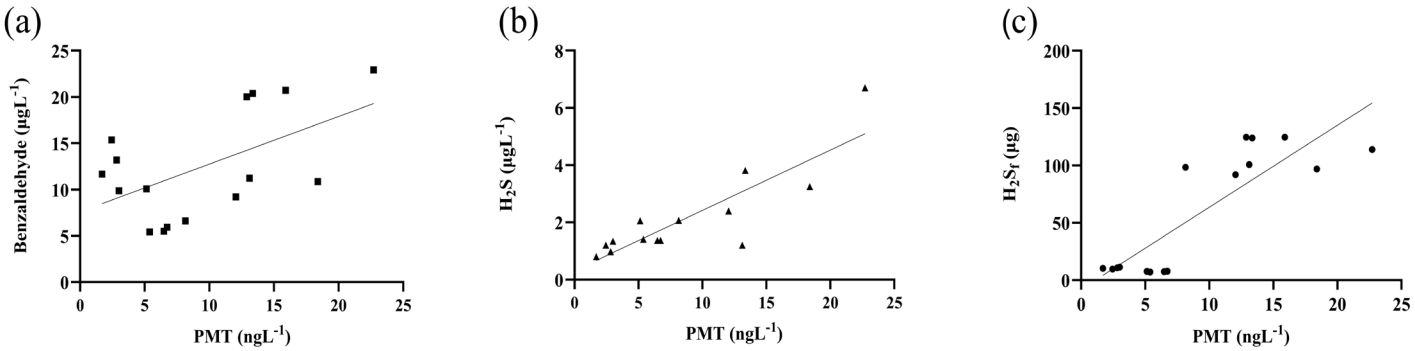



**FIGURE 3.** Relationship between phenylmethanethiol (PMT) and its potential precursors in a synthetic grape must (SGM) spiked with different amounts of benzaldehyde and fermented with four strains with different hydrogen sulfide (H2S)-producing abilities.

Benzaldehyde (a) and $H_2S$ (b) were measured in the finished wines, while $H_2S_f$ (c) represents the total $H_2S$ released during the fermentation process. The correlation coefficients (R) were found to be 0.554 ($P < 0.05$) for benzaldehyde (a), 0.871 ($P < 0.0001$) for $H_2S$ (b) and 0.852 ($P < 0.0001$) for $H_2S_f$ (c).

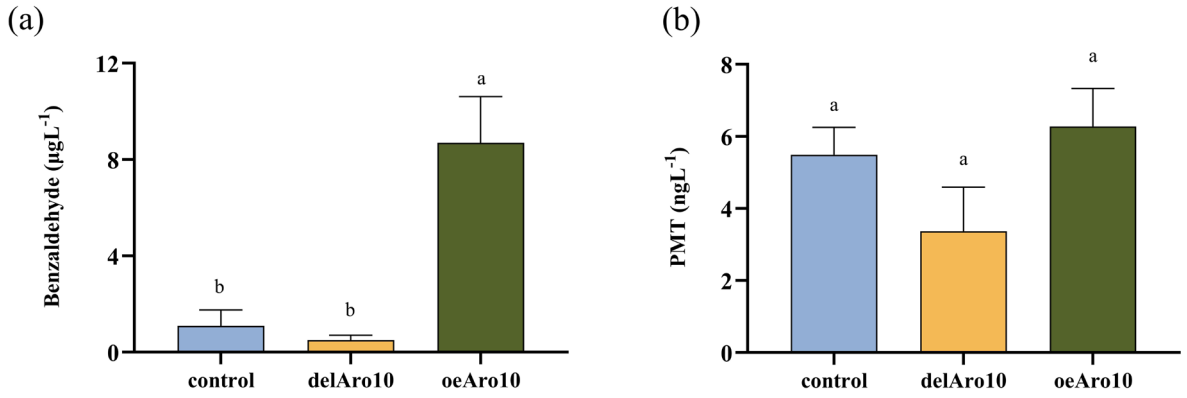



**FIGURE 4.** Production of (a) benzaldehyde and (b) phenylmethanethiol (PMT) after fermentation of a synthetic grape must (SGM) by the haploid yeast strain AWRI1631 (blue), in which the ARO10 gene was deleted (delAro10, yellow) or overexpressed (oeAro10, green).

Means with the same letter are not significantly different from each other (Tukey's HSD test, $P < 0.05$).

and benzaldehyde when compared to the control strain (Figure 4b).

## 4. The role of the nitrogen content of the grape must on phenylmethanethiol formation

Nitrogen is a critical grape nutrient for yeast growth and fermentation activity, which affects not only the rate and completion of fermentation but also wine's composition and sensory attributes (Bell and Henschke, 2008). In particular, it is well-known that the nitrogen content of the grape must plays an important role in the formation of VSCs by yeast during fermentation (Bekker *et al*., 2023), and low nitrogen juices are generally associated with enhanced $H_2S$ formation and 'reductive' characters in the final wine (Vos and Gray, 1979).

To investigate whether PMT formation is also affected by the nitrogen content of the fermentation media, laboratory-scale fermentations were carried out in both an SGM and a low YAN Chardonnay (must AH), to which increasing concentrations of nitrogen in the form of inorganic ammonia were added prior to fermentation. The same four yeast strains used in the benzaldehyde spiking experiment were used here. In the control low YAN (147 mg N $L^{-1}$) Chardonnay must, the addition of two levels of ammonia to yield a final YAN concentration of 250 and 350 mg N $L^{-1}$ resulted in a general increase in the levels of PMT produced by the four yeast strains assessed (Figure 5). The lowest $H_2S$-producing strain in these experimental conditions, AWRI1616, showed the strongest response to YAN additions when compared to the other strains, with the highest YAN addition (YAN350) resulting in an 8-fold increase in PMT formation when compared to the control must (6.2 vs. 0.8 $ngL^{-1}$, respectively) (Table S7). This increase was more modest, around 3-fold, for strains AWRI2865 and AWRI1375 (Figure 5). Conversely, the high $H_2S$-producing strain AWRI3442 only showed a small increase of 0.4 $ngL^{-1}$, between the YAN350 and control treatments (Table S7).

ANOVA analysis showed that both the choice of strain ($P < 0.0001$, $F = 13.0$) and nitrogen additions ($P < 0.0001$, $F = 16.5$) had a similar effect on PMT formation, while the interaction was also statistically significant ($P < 0.0001$, $F = 21.7$). Similar, to the results obtained in the Chardonnay must MV, PMT formation did not correlate with the levels of any of its potential precursors (benzaldehyde or $H_2S$) (Table S8). In fact, the levels of both benzaldehyde and $H_2S$ released during fermentation ($H_2S_f$) decreased with nitrogen additions, while the levels of $H_2S$ in the finished wine were not generally affected by nitrogen (Table S7).

Similar results were obtained after fermentation in SGM, as the addition of nitrogen resulted in a dramatic increase in the levels of PMT produced by three of the four yeast strains assessed (Figure 6, Table S9). ANOVA analysis showed that YAN additions had a strong effect on PMT formation ($P < 0.0001$ $F = 18.8$), while the choice of yeast strain was not relevant ($P = 0.712$, $F = 0.46$). The interaction strain: YAN was also statistically significant ($P < 0.01$, $F = 3.1$). Similar to the Chardonnay nitrogen addition experiment, the low-$H_2S$-producing strains AWRI1616 and AWRI2865, also showed the strongest response to nitrogen additions, with a 5 and 9-fold increase in PMT formation, respectively, between the lowest and highest nitrogen concentrations (Figure 6). Again, PMT formation did not correlate with either benzaldehyde or

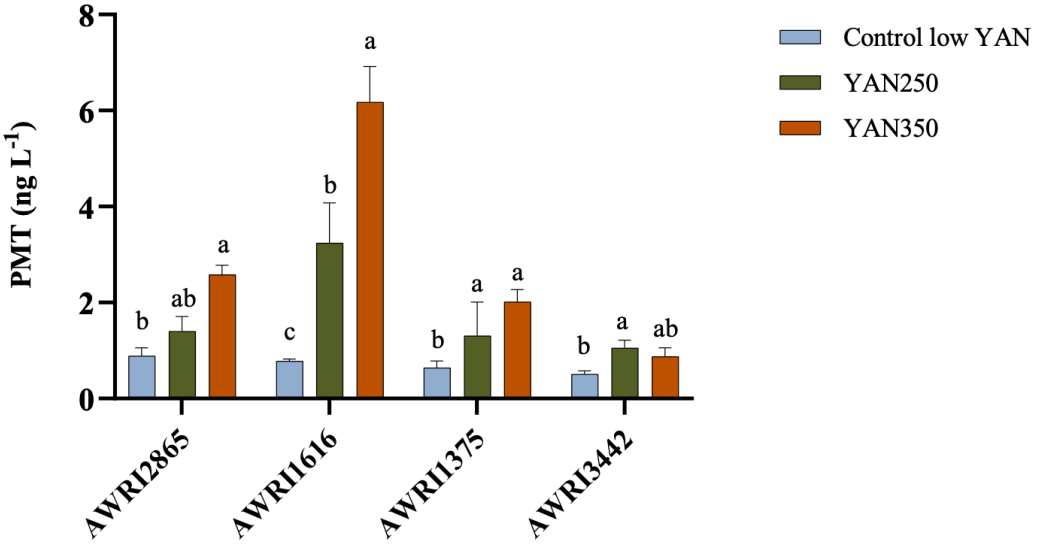



**FIGURE 5.** Production of phenylmethanethiol (PMT, $ngL^{-1}$) by four yeast strains after fermentation of a Chardonnay must (must AH) with different YAN concentrations. The original low YAN must (147 mg N $L^{-1}$) (blue), was supplemented with inorganic nitrogen to achieve a final concentration of either 250 or 350 mg N $L^{-1}$ (green and brown, respectively).

Means with the same letter are not significantly different from each other (Tukey's HSD test, $P < 0.05$) for each of the yeast strains assessed.

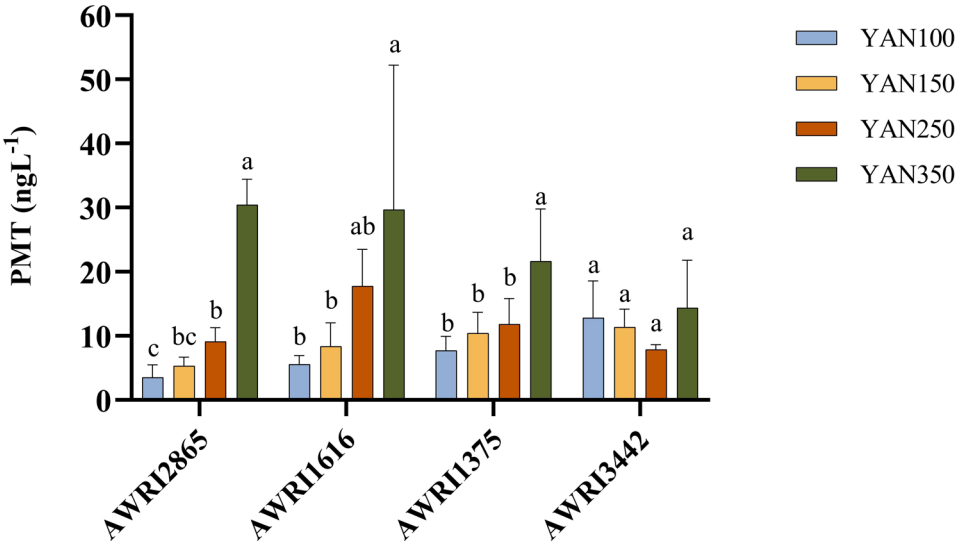



**FIGURE 6.** Production of phenylmethanethiol (PMT, $ngL^{-1}$) by four yeast strains after fermentation of a synthetic grape must (SGM) with different YAN concentrations. SGM with a YAN concentration of 100 mg N $L^{-1}$ (blue), was supplemented with inorganic nitrogen to achieve a final concentration of either 150, 250 or 350 mg N $L^{-1}$ (yellow, brown and green).

Means with the same letter are not significantly different from each other (Tukey's HSD test, $P < 0.05$) for each of the yeast strains assessed.

$H_2S$ (Table S10); in particular, benzaldehyde concentrations tended to decrease with nitrogen additions for all the yeast strains assessed.

A significant positive correlation was observed between PMT and MeSH ($R = 0.499$, $P < 0.05$) (Figure S4); with both strains AWRI1616 and 2865 producing the highest concentrations of PMT also accumulating high concentrations of MeSH, particularly for the YAN350 treatment (Table S9).

## DISCUSSION

This is the first study that has shown that the choice of yeast strain for alcoholic fermentation may be used as a tool for winemakers to modulate the levels of phenylmethanethiol (PMT) in wine. Furthermore, a group of commercial strains that showed a consistently enhanced ability to produce PMT in different fermentation conditions was also identified. The concentrations of PMT found in our laboratory-scale fermentations in Chardonnay were in the same range as those found for a large sample of commercial Chardonnay wines from Australia, New Zealand and France (Espinase Nandorfy *et al*., 2023). The fact that PMT can be generated during alcoholic fermentation, and without oak contact, is in agreement with a previous study by Piano *et al*. (2014), who found a substantial amount of PMT (6.4 $ngL^{-1}$) after fermentation of the Italian white grape cultivar Arneis in stainless steel tanks. Furthermore, the relatively lower levels of PMT observed in our Pinot noir ferments compared to Chardonnay are in agreement with Tominaga *et al*. (2003a), who found concentrations of this compound to be lower in Bordeaux red wines compared to white wine varieties such as Sauvignon blanc and Chardonnay. On the other hand, Mateo-Vivaracho *et al*. (2010) found that the levels of PMT in Grenache rosé wines were slightly higher than those in Chardonnay, but still lower than in Verdejo and Sauvignon blanc. Some studies have also pointed out that for Chardonnay and Sauvignon blanc, the geographic origin of the grapes has a significant influence on the contents of PMT in the finished wine (Capone *et al*., 2017; Mateo-Vivaracho *et al*., 2010; Tominaga *et al*., 2003a). These differences might reflect the specific viticultural and oenological practices in each region, as well as climate differences (Gambetta *et al*., 2014; Mateo-Vivaracho *et al*., 2010; Ugliano, 2009).

PMT was also detected after fermentation of an SGM and in similar concentrations to those found in Chardonnay, which suggests that one of the possible, and relevant, mechanisms for the formation of this compound might involve yeast fermentation-derived metabolites. Previous research on the related thiols 2-FMT and 3-SH (Blanchard *et al*., 2001; Harsch *et al*., 2013) pointed out the involvement of a sulfur donor ($H_2S$ or cysteine) and an aldehydic precursor in the formation of these compounds, which was further investigated in laboratory-scale fermentations in both SGM and Chardonnay.

The results obtained in SGM supported the potential role of benzaldehyde and $H_2S$ as precursors to PMT formation; however, this was not necessarily the case in the Chardonnay ferments. This indicates that other precursors might also be involved in PMT formation during grape must fermentations, which will be discussed later on. Firstly, in SGM a statistically significant positive correlation was observed between the levels of benzaldehyde produced by yeast and PMT in wines made with 13 yeast strains of the *Saccharomyces* genus. This was later confirmed in a spiking experiment where SGM was

fermented using four strains with different $H_2S$-producing abilities, and to which benzaldehyde was added when the first signs of $H_2S$ formation by yeast were observed. This experiment was performed to increase the probability that both precursors were present at the same time in the fermentation media. The fact that the addition of external benzaldehyde to wines fermented with the two high-$H_2S$-producing strains promoted PMT formation, while the two low-$H_2S$-producing strains were not impacted by these additions, indicates that under these circumstances $H_2S$ might be the limiting factor for the formation of PMT. These results are in agreement with Harsch *et al*. (2013), who observed that for the formation of 3-SH from the precursors *(E)*-2-hexenal and $H_2S$, the latter was the limiting factor in some Sauvignon blanc juices. These findings also agree with anecdotal evidence that the struck match/flinty characters associated with some styles of commercial Chardonnay winemaking are the result of reductive fermentation and barrel-aged or barrel-fermented wines, which will lead to the formation of LMWSCs such as $H_2S$ and the release of benzaldehyde from the oak into the wine.

Interestingly, the addition of external benzaldehyde did not result in a substantial accumulation of this compound in our experimental wines at the end of fermentation. Instead, the concentrations of the related metabolites benzoic acid and benzyl alcohol, and to a lesser extent benzyl acetate, were increased considerably. This observation is consistent with the Cannizzaro reaction, in which the chemical disproportionation of benzaldehyde yields benzoic acid and benzyl alcohol (Mazzoni *et al*., 2019). Our experimental results also confirm the high reactivity of aldehydic compounds during winemaking conditions (Harsch *et al*., 2013; Valera *et al*., 2020). For example, the C6-compounds *(E)*-2-hexenal and *(E)*-2-hexen-1-ol are almost completely metabolised from the grape must by yeast during the first 24 h of inoculation (Harsch *et al*., 2013), which greatly limits the time window for both $H_2S$ and C6-compounds to act as precursors to 3-SH formation during the fermentation process. Although the kinetics of benzaldehyde metabolism and PMT formation were not studied, the limited coexistence of the pair benzaldehyde-$H_2S$ might explain their low efficiency in acting as precursors for PMT formation under winemaking conditions. This would result in the production of extremely low levels of PMT ($ngL^{-1}$) from precursors that are produced in concentrations of several orders of magnitude ($\mu gL^{-1}$).

Regarding the fate of benzaldehyde during fermentation conditions, it has been shown that the related fusel aldehydes which are derived from yeast catabolism of amino acids through the Ehrlich pathway tend to be converted to either the corresponding fusel alcohol or acid and the balance between these compounds depends on the redox status of the cell (Hazelwood *et al*., 2008). Under anaerobic conditions, such as those found in wine fermentation, the reduction of the fusel aldehyde to the corresponding fusel alcohol by yeast alcohol dehydrogenases is favoured (Vuralhan *et al*., 2003). This agrees with the results obtained with the three *S. cerevisiae* strains assessed, which tended to accumulate benzyl alcohol preferentially over benzoic acid. The opposite was observed for the *S. uvarum* strain AWRI1375, which also concurs with another study in a synthetic media spiked with benzyl alcohol in which *S. uvarum* accumulated higher concentrations of benzoic acid when compared to *S. cerevisiae* (Delfini, 1991). Therefore, it can be hypothesised that differences in the overall cellular redox metabolism might explain some of the variations in PMT formation observed between yeast strains, which in turn supports the idea that yeast dehydrogenases might play an important role in PMT formation.

While the results in SGM point towards a relevant role of both benzaldehyde and $H_2S$ as PMT precursors, this is not the case for the laboratory-scale fermentation experiments conducted in two Chardonnay musts. Contrary to the results obtained in SGM, no correlation was observed between PMT formation and the concentrations of both benzaldehyde and $H_2S$ in the finished wines. These results are in agreement with a previous study which also found no correlation between PMT and benzaldehyde concentrations for a group of nine commercial Chardonnay wines (Tominaga *et al*., 2003a); while in the same study, only a weak linear correlation was observed for a group of 20 Sauvignon blanc wines.

Interestingly, no correlation was found between PMT and $H_2S$ production, irrespective of whether $H_2S$ was measured as "free $H_2S$", liberated during fermentation using gas detection tubes, or measured as "total $H_2S$", where both the free and the bound portion of $H_2S$ were measured using GC-SCD analysis (Franco-Luesma and Ferreira, 2014; Siebert *et al*., 2010). $H_2S$ is a reactive nucleophile that readily reacts with many wine matrix compounds (Bekker *et al*., 2016), including various metals leading to the formation of metal-bound $H_2S$ species (Franco-Luesma and Ferreira, 2014). This may explain why no correlation between $H_2S$ liberation and PMT production was observed. It is possible that $H_2S$ was liberated and immediately reacted with benzaldehyde to produce PMT, and the remaining $H_2S$ was bound to other wine matrix compounds. Similarly, the rapid conversion of benzaldehyde to benzoic acid (Delfini, 1991) makes it challenging to evaluate the correlation between benzaldehyde production and PMT liberation.

It is also possible that other precursors might be involved in PMT formation under grape must fermentation conditions. A weak but statistically significant correlation was observed between PMT and the levels of the sulfur-containing compound MeSH in both Chardonnay juices assessed, as well as in the fermentation of an SGM spiked with different concentrations of inorganic nitrogen. Whether MeSH might play a direct role as a sulfur donor for PMT formation, or potentially represents a compound whose liberation may correlate with increased $H_2S$ and PMT production remains unknown, and further experiments would have to be conducted to confirm these assumptions. While $SO_2$ could also be considered as a potential sulfur donor for PMT formation (*via* its reduction to $H_2S$ by yeast metabolic activity), $SO_2$ is also well known for its propensity to bind aldehydic compounds, which in turn might reduce the availability of free benzaldehyde to act as a precursor for PMT formation (Bueno *et al*., 2016).

Unfortunately, $SO_2$ was not analysed in this study so no further assumptions can be made on the potential dual role of $SO_2$ on PMT formation.

The potential role of yeast β-lyase activity on PMT formation was also characterised in this study, as this enzymatic activity has been shown to be responsible for the release of the 'fruity' thiols 3-SH and 4-MSP during wine fermentation from odourless cysteine *S*-conjugates naturally present in the grape must (Cordente *et al*., 2022; Roncoroni *et al*., 2011). There is also evidence that *S. cerevisiae* possesses the capacity to generate both 2-FMT and PMT from cysteine-aldehyde conjugates *in vitro* (Huynh-Ba *et al*., 2003), which might involve the same β-lyases responsible for 'fruity' thiol release (Zha *et al*., 2018). However, in the Chardonnay wines, no correlation was observed between β-lyase activity (measured as 3-SH formation) and PMT levels in the final wines, and strains previously selected by yeast manufacturers to produce high concentrations of 'fruity' thiols in whites did not perform well in releasing PMT. On the other hand, the low thiol releaser AWRI2865 produced the highest levels of PMT of all strains in Chardonnay. These results are not surprising since, to the best of our knowledge, no cysteinylated or related amino acid precursors of PMT have been reported in grape juice. Alternatively, a second mechanism also involving yeast β-lyases might be relevant as these enzymes can also release $H_2S$ from the sulfur-containing amino acid cysteine (Santiago and Gardner, 2015), which could then react with an aldehydic precursor to form the corresponding thiol. Even though the formation of 2-FMT by yeast has been observed during fermentation conditions in the presence of high concentrations of both furfural and cysteine (Blanchard *et al*., 2001; Zha *et al*., 2017), this mechanism is also unlikely to play a role in PMT formation during real wine fermentation conditions given that in white musts the concentrations of cysteine are usually very small (Bell & Henschke, 2008).

The initial nitrogen content of the fermentation media was confirmed to play a pivotal role in modulating the formation of PMT during fermentation. In both SGM and Chardonnay musts, and for three of the four yeast strains assessed, the formation of PMT was enhanced with the supplementation of nitrogen in the form of inorganic ammonia to the must before inoculation. These surprising results cannot be explained by an increase in the levels of the potential precursors benzaldehyde and $H_2S$, which in fact showed the opposite trend, and decreased with increasing additions of inorganic nitrogen.

Even though this enhanced formation of PMT with increasing nitrogen content of the must was contrary to what was expected initially, Blanchard *et al*. (2001) also observed a similar trend for the formation of 2-FMT, which increased slightly with increasing concentrations of inorganic nitrogen in the fermentation media. On the other hand, when the media was supplemented with organic nitrogen in the form of the amino acid asparagine, 2-FMT concentrations decreased dramatically.

Unlike the 'fruity' thiols 3-SH and 4-MSP, our results also suggest that the formation of PMT is not negatively regulated by nitrogen catabolite repression (NCR). This is a physiological response in which the presence of a rich nitrogen source such as inorganic ammonia represses the expression of genes involved in the use of other poor nitrogen sources by yeast, such as β-lyases, as well as the general amino acid transporter Gap1p involved in cysteinylated precursor uptake (Subileau *et al*., 2008; Thibon *et al*., 2008). Together, these results reinforce the idea that yeast β-lyases and/or cysteinylated precursors might not be involved in PMT formation.

Therefore, the observed changes in PMT formation with ammonia supplementation might not be explained as a direct consequence of the role that ammonia plays in regulating nitrogen metabolism in yeast (*via* NCR) but rather due to an indirect effect of this metabolite on yeast physiology. Supporting this, Torrea *et al*. (2011) found that supplementation of a low-nitrogen Chardonnay grape juice with ammonium led to changes in some physicochemical properties of the finished wine. In particular, it was found that increasing the YAN of the control juice from 160 to 320 $mgL^{-1}$, which resembles the levels of nitrogen supplementation achieved in our study, resulted in a statistically significant decrease in the pH of the finished wine, which was even further decreased with additional ammonium supplementations. This observation can be explained as ammonium ion uptake by yeast is associated with the excretion of proton ions into the extracellular medium, which will result in a lower wine pH (Torija *et al*., 2003; Torrea *et al*., 2011). Although the specific role that wine pH might play on PMT formation will have to be determined in future experiments, these observations suggest that chemical transformations in the extracellular matrix might be relevant for PMT formation, which might be promoted at lower pHs. The chemical formation pathway of PMT from its putative precursors, benzaldehyde and $H_2S$, would involve a more complex pathway than the formation of 2-FMT from furfural and $H_2S$, where a reduction step that converts the thioaldehyde intermediate to the final thiol product could occur if the ferment is still active (Waterhouse *et al*., 2016). However, when fermentation has been completed, the pathway for the reduction of the thioaldehyde intermediate generated from the reaction between $H_2S$ and benzaldehyde remains unclear.

Finally, the potential links between yeast aromatic amino acid catabolism and the formation of benzaldehyde, and potentially PMT, were also explored in this study. The yeast phenylpyruvate decarboxylase *ARO10* not only plays a pivotal role in the degradation of the aromatic amino acid phenylalanine through the Ehrlich pathway (Vuralhan *et al*., 2003) but is also involved in the formation of different benzenoid metabolites such as benzaldehyde and benzyl alcohol (Martin *et al*., 2016; Valera *et al*., 2020). The pivotal role of the *S. cerevisiae ARO10* gene in the production of benzaldehyde was confirmed in this study; although it cannot be discarded that other yeast decarboxylases might also play a minor role, as the inactivation of *ARO10* did not completely

eliminate benzaldehyde formation by yeast. *ARO10* is known to be under the control of NCR (Dai *et al*., 2021; Vuralhan *et al*., 2003) and, as a consequence, *ARO10* expression is low or negligible in the presence of preferred nitrogen sources such as ammonium salts, which might explain our experimental observation that benzaldehyde concentrations tended to decrease with increasing ammonia additions in both SGM and Chardonnay. The fact that increasing the formation of benzaldehyde by yeast during fermentation by overexpressing *ARO10* did not translate into a statistically significant increase in PMT formation in SGM, might indicate that both $H_2S$ and benzaldehyde need to coexist in time, or that the relatively low amounts of benzaldehyde produced by yeast are not enough for a substantial increase in PMT formation.

## CONCLUSION

In this study, we have demonstrated the relevant role that yeast strain selection, as well as the nitrogen content of the grape must, plays in the formation of the 'flinty' compound PMT during alcoholic fermentation of both natural and synthetic grape musts. While these are two easy and affordable strategies to implement under winemaking conditions to modulate the formation of this elusive compound, and potentially the 'flinty' characters in wine, their effectiveness will need to be confirmed under pilot-scale winemaking conditions.

Our results in synthetic grape juice indicate that in the absence of oak, PMT can be generated *de novo* from yeast fermentation-derived metabolites, and in particular, both $H_2S$ and benzaldehyde represent potential precursors to PMT during alcoholic fermentation. However, results obtained in natural Chardonnay grape must fermentations suggest that other precursors might also be involved in the formation of this elusive compound. Further studies will need to be carried out to determine the possible involvement of other putative precursors such as the sulfur donors MeSH and/or $SO_2$, as well as to elucidate the exact mechanism behind PMT formation during fermentation conditions. Whether the formation of PMT occurs solely as a result of yeast metabolism and/or it also relies on chemical transformations in the extracellular fermentation matrix remains to be determined.

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