International Journal of Food Microbiology 425 (2024) 110858

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Precursors consumption preferences and thiol release capacity of the wine yeasts *Saccharomyces cerevisiae, Torulaspora delbrueckii,* and *Lachancea thermotolerans*

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ARTICLE INFO

Keywords:
Thiols
Thiol precursors
Fermentation
Wine yeasts
Non-*Saccharomyces*

ABSTRACT

The aromatic profile of wine determines its overall final quality, and among the volatile molecules that define it, varietal thiols are responsible for shaping the distinctive character of certain wine varieties. In grape must, these thiols are conjugated to amino acids or small peptides in a non-volatile form. During wine fermentation, yeasts play a principal role in expressing these aromatic compounds as they internalise and cleavage these precursors, releasing the corresponding free and aroma-impacting fraction. Here, we investigate the impact of three wine yeasts (*Saccharomyces cerevisiae*, *Torulaspora delbrueckii* and *Lachancea thermotolerans*) on thiol releasing in synthetic grape must fermentations supplemented with different cysteinylated (Cys-4MSP and Cys-3SH) and glutathionylated (GSH-4MSP and GSH-3SH) precursors. We demonstrate higher consumption levels of cysteinylated precursors, and consequently, higher amounts of thiols are released from them compared to glutathionylated ones. We also report a significant impact of yeast inoculated on the final thiols released. Meanwhile *T. delkbrueckii* exhibits a great 3SHA releasing capacity, *L. thermotolerans* stands out because of its high 3SH release. We also highlight the synergic effect of the co-inoculation strategy, especially relevant in the case of *S. cerevisiae* and L. *thermotolerans* mixed fermentation, that has an outstanding release of 4MSP thiol. Although our results stem from a specific experimental approach that differs from real winemaking situations, these findings reveal the potential of unravelling the specific role of different yeast species, thiol precursors and their interaction, to improve wine production processes in the context of wine aroma enhancement.

1. Introduction

The wine sensory profile - determined by its chemical composition - is defined by taste, visual attributes, and aroma, being the latter the most influential factor in the overall sensory quality of wine (Polaskova et al., 2008). Several factors influence the aroma of wine, such as grape variety, terroir, geographical and climatic conditions, and the viticultural practices applied. In this context, the metabolites released by yeast during alcoholic fermentation play a fundamental role (Swiegers and Pretorius, 2007). Among these aromatic metabolites, varietal aromas (those derived from cleaving grape precursors) stand out because they serve as distinctive characteristics that define specific grape varieties (Ruiz et al., 2019). In grapes, varietal aroma compounds are found in the form of non-volatile precursors, conjugated with sugars or amino acids (and small peptides) as in the case of terpenes or thiols, respectively (Baumes, 2009). Yeasts, through the expression of specific hydrolytic enzymes, can release varietal aromas by acting on non-volatile precursors, releasing the corresponding volatile compounds that contribute to the aromatic profile of the resulting wine.

Thiols, mainly 4-methyl-4-sulfanylpentan-2-one (4MSP), 3-sulfanylhexan-1-ol (3SH), and its acetylated derived, 3-sulfanylhexyl acetate (3SHA), are responsible for the varietal character of white wine varieties

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https://doi.org/10.1016/j.ijfoodmicro.2024.110858
Received 30 May 2024; Received in revised form 5 August 2024; Accepted 6 August 2024
Available online 15 August 2024

such as *Sauvignon Blanc*, *Gewürztraminer, Verdejo, Trebbiano di Lugana* or *Grillo*. Despite their low concentration in wines (around 0.1 μg/L for 4MSP precursors, and between 40 and 600 μg/L for 3-SH), these molecules have an outstanding aromatic impact on these wines thanks to their low perception threshold (between 0.8 and 60 ng/L) (Ruiz et al., 2019). These compounds are usually related to the tropical and fruity character of wines, providing aromas of boxwood or currant in the case of 4MSP, grapefruit or passion fruit in the case of 3SH and 3SHA.

These aromas are not present in grape musts, as they are primarily found in the form of non-volatile conjugated precursors. The origin of these precursors is related to a detoxification mechanism in grapevines, whereby reduced glutathione (GSH) is bound to unsaturated compounds like alkenes or alkenols (Belda et al., 2017). Then, the glutathionyl fragment of thiol precursors can be hydrolysed to the cysteine-glycine dipeptide, to the γ-glutamine-cysteine dipeptide or directly to cysteine. Thus, in grape musts and fermenting wines, these three precursors from the original GSH tripeptide precursors can be found.

The final content of thiols in wine depends directly on both the concentration of the precursors in the must and the capacity of fermenting yeasts to metabolise them. Their concentration in grape must varies from 0.20 to 2770 μg/L for glutathionylated (Roland et al., 2010) and from 7.6 to 269.0 μg/L for cysteinylated ones (Waterhouse et al., 2016), depending on grape variety, ripeness, nitrogen treatments in the vineyard, and pre-fermentative steps in the winery (Fracassetti et al., 2018; Tirelli et al., 2022). During wine fermentation, yeasts internalise the thiol precursors by general amino acid transporters. In the cytoplasm, enzymes with β-lyase activity break the C—S bond of the precursors, releasing the corresponding volatile fraction of these conjugates (Howell et al., 2005; Santiago and Gardner, 2015). In the case of glutathionylated precursors, once inside the cell, they are transformed into cysteinylated precursors through a complex vacuolar route involving several enzymes, and then undergo hydrolytic breakdown mentioned above (Belda et al., 2017). Regarding the thiol 3SHA, it has been shown that the enzyme encoded by the *ATF1* gene, coding for an alcohol acetyltransferase, catalyses its transformation from 3SH (Kiene et al., 2021).

Thus, one of the most efficiently approaches to increasing of thiolic content in wines is to enhance the capability of yeast to consume and cleavage thiol precursors, which depends on its genetic potential (i.e. genes coding for the necessary β-lyase enzymes) and the fermentative conditions, especially nitrogen availability in grape musts since thiol precursors metabolisms is strongly regulated by Nitrogen Catabolite Repression (NCR) (Ruiz et al., 2019). Several strategies have been developed to find strains with a high capacity for thiol release in nature, focusing on the identification of allelic variants of the *IRC7* in *S. cerevisiae,* a gene encoding for a cystathionine-*S*-β-lyase enzyme (Roncoroni et al., 2011; Belda et al., 2016; Cordente et al., 2019). In fact, one of these alleles encodes a smaller enzyme with lower catalytic activity if compared to the full enzyme. Ruiz et al. (2021) demonstrated that this shorter allele was associated with a series of phenotypic and genomic singularities within *S. cerevisiae* strains that explain its high prevalence among wine strains. The low prevalence of strains carrying an efficient β-lyase enzyme for the cleavage of thiol precursors, together with the strong NCR acting on the genes involved in thiol metabolism, indicate that most *S. cerevisiae* strains have a limited capacity for varietal thiol release. Actually, it has been demonstrated that <10 % of the precursors in the must are transformed into volatile thiols during wine fermentations (Murat et al., 2001; Swiegers and Pretorius, 2007). In addition, although it is well-documented that *S. cerevisiae* is able to release thiols from conventional cysteine/glutathione (Cys/GSH) precursors, there is limited understanding of the preferences and mechanisms by which it processes oligopeptide-bound precursors.

Therefore, the search of non-*Saccharomyces* yeasts for oenological application is considered an interesting alternative for improving the release of thiol aromas during wine fermentation (Renault et al., 2016; Belda et al., 2016; Ruiz et al., 2018). Although its known that a high β-lyase activity is a common characteristic among non-*Saccharomyces* species, such as *Torulaspora delbrueckii*, *Kluyveromyces marxianus*, and *Lachancea thermotolerans* (Belda et al., 2016; Zott et al., 2011), little is known about the specific role of these species on the metabolism of diverse thiol precursors and volatile thiols releasing under fermentative conditions. The methodological challenges associated with synthetizing thiol precursors in vitro and later quantifying these precursors and the corresponding volatile thiols has limited the number of experimental works addressing remaining questions in the field. Thus, the primary objective of the present work is to provide new insights into these processes by focusing on the performance of both *S. cerevisiae* and two non-*Saccharomyces* yeasts (*T. delbrueckii* and L. *thermotolerans*) regarding varietal thiol production during alcoholic fermentation, measuring their capacity to consume a different precursor and their ability to release volatile thiols from them. To do this, we performed single and mixed fermentations under non-nitrogen-repressive conditions with these three species in synthetic grape must supplemented with thiol precursors. We analysed the final content of residual precursors, the release of the corresponding volatile thiols, as well as the overall volatile and non-volatile composition of the resulting wines.

2. Materials and methods

2.1. Yeast strains and precultures

S. cerevisiae AG023 (Agrovin S.A., Spain) and *T. delbrueckii* NSTD (Agrovin S.A., Spain) commercial strains were employed. Additionally, pre-commercial strain L. *thermotolerans* NS-G-32 (GenBank accession number KT222664; deposited and publicly available in the Complutense Yeast Collection) was employed. Yeasts were cryopreserved in cryogenic media (containing 25 % glycerol) at −80 °C, streaked on Sabouraud agar plates and maintained at 28 °C for routinely handling. Overnight precultures were carried out in 250 mL borosilicate bottles filled with 100 mL of Yeast Nitrogen Base (Difco, BD, USA) supplemented with 2.0 % of glucose under shaking at 150 rpm and 25 °C.

2.2. Cysteinylated and glutathionylated precursors of varietal thiols

Four precursors of varietal thiols 4-methyl-4-sulfanyl-pentan-2-one (4MSP) and 3-sulfanylhexan-1-ol (3SH) were used in the actual work. *S*-cysteine conjugate Cys-3SH was purchased from aromaLab GmbH, Martinsried, Germany. *S*-cysteine conjugate Cys-4MSP, *S*-glutathione conjugates GSH-3SH and GSH-4MSP were synthesised on the facilities of Taras Shevchenko National University of Kyiv (Ukraine) based on the described methods (Pardon et al., 2008; Shinkaruk et al., 2008; Grant-Preece et al., 2010). If necessary, the purification of the mentioned precursors was performed by means of preparative liquid chromatography.

2.3. Microvinifications and kinetics monitoring

All fermentation assays were carried out using synthetic grape must (SGM) prepared as described by (Henschke and Vladimir, 1993) with some modifications. Firstly, vitamins, trace elements and anaerobic factors (ergosterol and Tween 80) stock solutions were prepared separately, sterilised by 0.22 μm filtration and stored at 4 °C (maintaining the proportion of the original recipe). To prepare the SGM, the stocks solutions were dissolved in H_2O followed by glucose (100 g/L), fructose (100 g/L), malic acid (3.0 g/L), potassium *L*-tartrate (2.5 g/L), citric acid (0.2 g/L), KH_2PO_4 (1.14 g/L), $MgSO_4{\cdot}7H_20$ (1.23 g/L), and $CaCl_2{\cdot}2H_2O$ (0.44 g/L). Nitrogen content was adjusted to 67 mg/L of Yeast Assimilable Nitrogen (YAN) (by adding 0.316 g/L of $(NH_4)_2HPO_4$). Then, pH was adjusted to 3.5 with sodium hydroxide and SGM was sterilised by 0.45 μm filtration. All fermentations were run in triplicate using 200 mL of SGM in 250 mL borosilicate bottles at 25 °C with no agitation.

Two experimental conditions regarding thiols precursors were

assayed: must added with (i) cysteinylated precursors (Cys-3SH and Cys-4MSP), at a final concentration of 160 μg/L for each precursor (named as CP), and (ii) glutathionylated precursors (GSH-3SH and GSH-4MSP), at a final concentration of 360 μg/L for each precursor (named as GP). These concentrations were chosen in order to represent the concentrations found in natural must (Roland et al., 2011; Waterhouse et al., 2016) and ensuring enough quantity for the further quantifications. Thus, the final concentration of the different thiols precursors was: 727 nmol/L (Cys-4MSP), 720 nmol/L (Cys-3SH), 887 nmol/L (GSH-4MSP) and 883 nmol/L (GSH-3SH). For the single-species trials, a control condition without adding any thiol precursors was also included as a control for the non-detection of de novo synthesis of thiols. For each of these two experimental conditions (CP and GP), five inoculation strategies were carried out: single inoculation with *S. cerevisiae* (Sc), single inoculation with *T. delbrueckii* (Td), single inoculation with L. *thermotolerans* (Lt), mixed inoculation with *T. delbrueckii,* and *S. cerevisiae* (Td + Sc) and mixed inoculation with L. *thermotolerans* and *S. cerevisiae* (Lt + Sc).

Fermentations were inoculated to a final cell concentration of $2{\cdot}10^5$ CFU/mL ($1{\cdot}10^5$ cells/mL of each strain in the case of mixed fermentations), and at several timepoints 1.00 mL samples were taken, 10-fold serially diluted in saline solution and spread onto WL-agar (ThermoScientific, USA) to control yeasts populations based on the morphological characteristics of the grown colonies. For fermentation kinetics monitoring, fermentations were weighted every 24 h to determine the weight lost due to the CO_2 released. Fermentation was considered finished when weight loss was lower to 0.01 % during two consecutive days. Once fermentations were completed, 15-mL and 50-mL tubes were filled without headspace and stored at −80 °C for further analysis.

2.4. Basic oenological parameters analysis

Residual sugars, glycerol and ammonia concentration were enzymatically determined using a Y15 autoanalyzer (Biosystems, Spain). Ethanol, and non-volatile organic acids (tartaric, malic, lactic, acetic and citric acid) were quantified by HPLC (High Performance Liquid Chromatography) on a 1260 Infinity II LC system (Agilent Technologies, USA), equipped with an Allure Organic Acids column (250 mm × 4.6 mm I.D., 5 μm grain size, 60 Å pore size) (Restek GmbH, Germany), and preceded by a 4 mm × 3.0 mm I.D. precolumn (Security Guard C18™, Phenomenex, Germany). A refractive index detector (RID) and a multi wavelength detector (MWD) was used as the detection system. For these analyses, a modified method by Mecca et al. (2020) was used, which is based on the method by Schneider et al. (1987).

2.5. Determination of main volatile compounds concentration

Determination of fermentative aroma compounds (esters, fatty acids and higher alcohols) was carried out using the headspace solid phase microextraction gas chromatography mass spectrometry (HS-SPME-GC-MS) method described in Badura et al. (2023). Calibration of corresponding aroma compounds was performed in model wine solutions. Acquisition and quantitative analysis of data was performed using Agilent MassHunter Workstation software (GC-MS) and Agilent OpenLab CDS ChemStation Edition (HPLC).

2.6. Determination of thiol precursors concentration

The content of thiol precursors was assessed in wine samples after SPE-purification using Strata X-polymeric cartridges (200 mg, Phenomenex, Torrance, CA, USA). After SPE activation, 2 mL of sample, spiked with GSH-3SH-d_1 (500 μg/L; exact mass $[M + H^+]^+$: 409.159) internal standard (IS), were loaded onto the SPE column and eluted with 4 mL of methanol after a washing step with 2 mL water. The solvent was evaporated under nitrogen flow to 400 μL (Fracassetti et al., 2018). The detection and quantification of thiol precursors was carried out by UPLC/ESI-HRMS [Acquity UPLC separation module (Waters, Milford, MA, USA) coupled to a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer through an HESI-II probe for electrospray ionisation (Thermo Scientific, Waltham, MA, USA)] analysis following the separation and detection conditions reported by Fracassetti et al. (2018). For quantification, a deuterated IS was used. Peak area ratios were compared with a 8-points standard calibration curves obtained using synthetic thiol precursors (Cys-3SH, Cys-4MSP, GSH-3SH and GSH-4MSP).

2.7. Determination of varietal thiols concentration

Quantification of polyfunctional volatile thiols was performed according to the procedure described in Kiene et al. (2021), based on the methods of Herbst-Johnstone et al. (2013) and Coetzee et al. (2018). As an internal standard, 20 μL of an ethanolic solution containing 1.8 mg/L 3-sulfanylhexan-1-ol-d_5 (aromaLAB GmbH, Martinsried, Germany) was used. Calibration was performed in a fermented model with 6 concentration levels in duplicate: 4MSP: 75–4480 ng/L, 3SHA: 10–200 ng/L, 3SH: 50–750 ng/L.

2.8. Statistical analysis

Statistical analysis was performed with the package stats of R software, version 4.3.0 (R Development Core Team, 2019). Analysis of variance (ANOVA) and Tukey post-hoc tests were applied to compare the values of the metabolite concentration of the different fermentation assays. Precursors-thiols conversation rations were analysed using Student's *t*-test to compare the means between cysteinylated and glutathionylated precursors groups using the 'stats' R package (v4.3). To evaluate the influence of the two studied variables (thiols precursors added and inoculum strategy) on PCA metabolite ordination, a Permutational Analysis of Variance (PERMANOVA) was performed using the RRPP R package (v0.2.0).

3. Results

In this work, we have investigated the specific role of *S. cerevisiae, T. delbrueckii* and L. *thermotolerans* in the release of varietal thiols, one of the main aroma compounds that determine the varietal character of certain white wines, under both single inoculation and co-inoculation with *S. cerevisiae*. In order to simplify the studied system and minimise the impact of nitrogen content and amino acid uptake on thiol precursor metabolism, fermentations were conducted at the minimum required concentration of nitrogen for completing fermentation (67 mg N/L) (Barbosa et al., 2015; Ruiz et al., 2020), using a single inorganic nitrogen source (ammonium diphosphate salt), with no amino acids added to avoid interference with thiol precursors assimilation (Pinu et al., 2014). Despite the limitations of the assay in representing real-world industrial winemaking conditions, the aim of this study is to elucidate how different yeasts species metabolise different thiol precursors and release the corresponding volatile thiols.

3.1. Fermentation kinetics and metabolite profile of wines

Under the specific conditions of this experimental approach, all fermentations involving the *S. cerevisiae* strain (Sc; Td + Sc and Lt + Sc) achieved glucose depletion, although some residual fructose was observed at the end of fermentation (Table 1). Furthermore, the final ethanol and glycerol production (Table 1), as well as the weight loss kinetics - that indicate fermentation performance - were similar among these assays (Fig. S1) and yeasts populations during the fermentative process (Table S1, Fig. S2). As expected, fermentations with non-*Saccharomyces* strains as single inoculum (Td and Lt) exhibited slower fermentation kinetics (Fig. S1), although *T. delbrueckii* achieved a final ethanol and glycerol content similar to *S. cerevisiae* assays (Table 1,

Table 1
Main oenological parameters at the end of the fermentation assays involving *S. cerevisiae*.

Precursors	Yeast					
	Sc		Sc + Lt		Sc + Td	
	CP	GP	CP	GP	CP	GP
Ethanol (g/L)	93.60 ± 4.18 a	89.67 ± 4.46 a	90.27 ± 5.15 a	89.13 ± 10.02 a	89.00 ± 9.74 a	88.33 ± 6.67 a
Glucose (g/L)	0.00 ± 0.00 a	1.43 ± 2.48 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
Fructose (g/L)	4.97 ± 8.60 a	17.60 ± 16.35 a	4.00 ± 6.93 a	0.00 ± 0.00 a	4.57 ± 5.95 a	3.90 ± 5.75 a
Ammonia (mg/L)	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a	0.00 ± 0.00 a
Glycerol (g/L)	4.12 ± 0.10 a	3.59 ± 0.80 a	4.18 ± 0.12 a	4.09 ± 0.19 a	4.20 ± 0.17 a	4.06 ± 0.16 a
Malic acid (g/L)	2.20 ± 0.10 a	2.33 ± 0.15 a	2.10 ± 0.10 a	2.1 ± 0.17 a	2.10 ± 0.26 a	2.13 ± 0.12 a
Citric acid (g/L)	0.19 ± 0.01 a	0.14 ± 0.01 b	0.13 ± 0.01 b	0.14 ± 0.01 b	0.13 ± 0.02 b	0.13 ± 0.01 b
Lactic acid (g/L)	0.00 ± 0.00 c	0.00 ± 0.00 c	0.20 ± 0.03 a	0.13 ± 0.01 b	0.00 ± 0.00 c	0.00 ± 0.00 c
Acetic acid (g/L)	0.45 ± 0.03 a	0.41 ± 0.01 a	0.45 ± 0.06 a	0.44 ± 0.07 a	0.40 ± 0.05 a	0.38 ± 0.03 a
Σ Esters (mg/L)	29.12 ± 9.30 a	23.51 ± 9.06 a	25.14 ± 10.83 a	33.67 ± 19.44 a	31.71 ± 15.34 a	28.00 ± 6.64 a
Σ Higher alcohols (mg/L)	132.70 ± 55.15 a	78.03 ± 30.79 a	117.69 ± 61.08 a	133.69 ± 57.46 a	175.07 ± 125.19 a	124.03 ± 40.93 a
Σ Fatty acids (mg/L)	8.13 ± 0.01 a	8.96 ± 0.60 a	8.19 ± 0.02 a	8.52 ± 0.59 a	8.06 ± 0.16 a	8.19 ± 0.02 a

Data expressed the average value of 3 replicates along with the standard deviation. Different letters indicate the existence of statistical differences between the assays (p-value < 0.05). CP for assay with cysteinylated precursors and GP for assay with glutathionylated.

Table S2). It is noteworthy that the addition of cysteinylated precursors had a discernible negative impact on the fermentative kinetic compared to the addition of glutathionylated ones, particularly evident in L. *thermotolerans* assays (Table 1, Fig. S1).

The volatile and non-volatile metabolites were quantified at the end of the fermentation (Tables 1, S1, S2 and S3). Fig. S3 shows a Principal Component Analysis ordination based on them. We observe that the inoculation strategy exhibited a distinct pattern regarding the metabolite profile of the resulting wines, but the nature of the thiol precursors added had no significant impact on the metabolite profile, except in the case of L. *thermotolerans* single assays, where the negative effect of cysteinylated precursors was evident. Indeed, PERMANOVA analysis (Table S5) confirms that the strains used is the primary driver explaining the ordination of the samples based on their metabolite profile ($F = 89.77$, $p < 0.05$), with the effect of the thiol precursors used being residual although significant ($F = 3.40$, $p < 0.05$). We can also observe that, under single fermentation conditions, *S. cerevisiae* and *T. delbrueckii* exhibited quite similar metabolite profiles, whereas L. *thermotolerans* shows a distinctly different profile, mainly defined by the residual glucose and the production of lactic acid (Tables 1, S2), which was also detectable in the mixed fermentations Sc + Lt.

In addition, regarding the specific fermentative aromatic compounds produced during the different fermentation assays, no significant differences among each inoculation strategy on the three major families of volatile compounds detected (higher alcohols, esters and fatty acids) were observed (Tables S3 and S4). Despite that, some specific compounds showed an impact related to the use of different strains. Clear strain effects were shown in the final concentration of i-butyric acid ethyl ester, which increases by the presence of both non-*Saccharomyces* yeasts inoculated. Also, the lactic acid ethyl ester concentration showed the highest values when L. *thermotolerans* was present.

It is noticeable that the presence of different thiol precursors affects the concentration of certain specific compounds, but only under single fermentations. We observed again that the presence of specific thiol precursors at the concentrations assayed here seems to have a greater effect on the fermentation performance of non-*Saccharomyces* strains than on *S. cerevisiae* strain. For instance, the presence of glutathionylated precursors appears associated with a moderate increase in the fatty acid content (mainly in octanoic and decanoic acid concentration) in *T. delbrueckii* single assays.

3.2. Thiol precursor consumption and free thiols release at different experimental conditions

Synthetic grape must was supplemented with cysteine-conjugated (Cys-4MSP and Cys-3SH) and glutathione-conjugated (GSH-4MSP and GSH-3SH) thiol precursors, that are the main forms found in grapes. Although cysteinylated and glutathionylated precursors normally co-exist in a grape must, we decided to separately add and analyse the thiols released from the two main thiol precursors families. Thus, we can differentiate the thiols releasing capacity of yeasts from the different precursors being of particular importance for (i) the elucidation of thiol precursors metabolism, even because cysteinelated precursors can derive from glutathionylated precursors, and (ii) the estimation of the oenological impact of using *S. cerevisiae* and non-*Saccharomyces* yeasts on the aromatic profile of wines. The concentration of residual thiol precursors and volatile thiols released at the end of the fermentation assays are represented in Figs. 1 and 2, respectively. Although the low level of residual precursors relative to their initial concentration can also be explained by chemical transformation of the precursors in the medium, we assume that the consumption by yeasts plays the principal role.

We observe that yeasts exhibited an overall higher preference for cysteinylated precursors over glutathionylated ones (Fig. 1), especially remarkable in the case of 4MPS precursors, where >45 μg/L of GSH-4MSP were detected at the end of alcoholic fermentation (around 25 % of the initial concentration), compared with <5 μg/L of residual concentrations Cys-4MSP. Moreover, we report an overall greater production of free thiols from cysteinylated precursors than from glutathionylated precursors (Figs. 2, S4). Cysteinylated precursors are the preferential source of volatile thiols in our study, specially 4-MSP, although in the case of 3SH and 3SHA thiols we did not observe clear differences in the precursor sources of thiol production (Fig. S4; Table S6).

Despite these general observations, we can also describe specific behaviours regarding the different yeast strains assayed. Compared to non-*Saccharomyces* strains, the *S. cerevisiae* strain leaves lower levels of Cys-4MSP, Cys-3SH and GSH-3SH precursors, but not of GSH-4MSP. We observed a similar content of residual precursors under *S. cerevisiae* single and mixed assays (no significant differences), demonstrating the strong influence of *S. cerevisiae* under co-inoculation conditions. Besides, *S. cerevisiae* strain exhibited a higher releasing capacity of 4MSP, specifically from cysteinylated precursors, than the two non-*Saccharomyces* strains (Fig. 2; Table S6).

However, we do report other positive contributions to the thiolic content of wines from the non-*Saccharomyces* strains assayed here. *T. delbrueckii* strain showed a high 3SH release from glutathionylated precursors and its subsequent transformation into 3SHA thiol, the 3SH-derived acetylated thiol. *L. thermotolerans* strain exhibited the most different behaviour regarding thiol metabolism. We can highlight the capacity of L. *thermotolerans* strain to release 3SH thiol from both cysteinylated and glutathionylated precursors, significantly higher than in

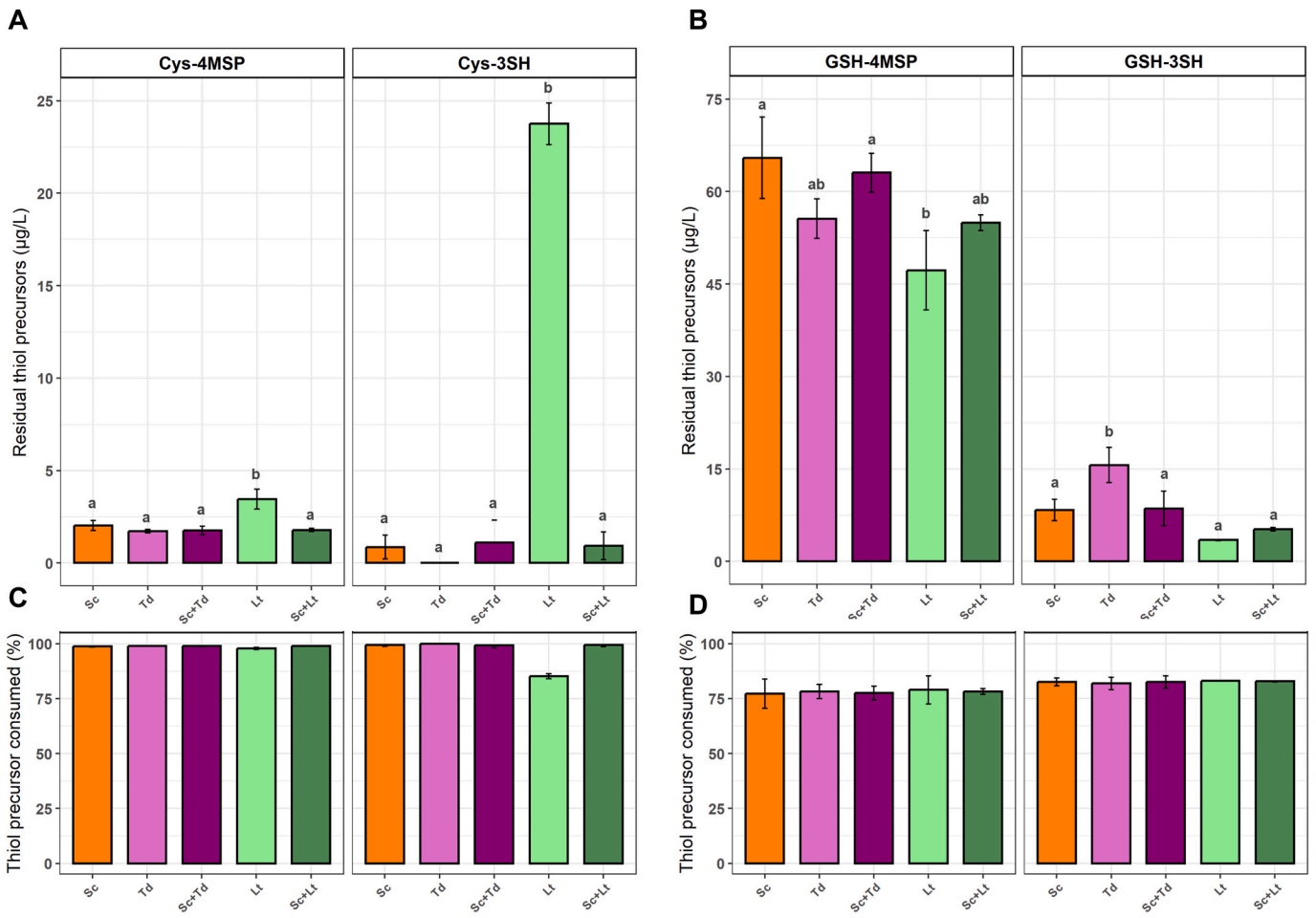

Fig. 1. Residual concentration of A cysteinylated thiol precursors and B glutathionylated thiol precursors measured at the end of the different fermentation trials. The percentages of C cysteinylated and D glutathionylated thiol precursors consumed are also represented. The different assays are represented by different colours: orange for *S. cerevisiae* single culture; purple for *T. delbrueckii* single culture; green for L. *thermotolerans* single culture; dark purple for *S. cerevisiae* + *T. delbrueckii* mixed culture; dark green for *S. cerevisiae* + *L. thermotolerans*. Different letters indicate the existence of statistical differences (ANOVA test, $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

any other assay (Fig. 2; Table S6). This was especially relevant in the case of 3SH releasing from cysteinylated precursors, as L. *thermotolerans* exhibits the lowest consumption capacity. Contrary, we report here the low 4MSP releasing capacity of L. *thermotolerans*, regardless of the precursor nature. Also, *L. thermotolerans* strain seems to be unable to produce 3SHA (Fig. 2), and that could partially explain the high 3SH final release in this species. Finally, we highlight the impact of L. *thermotolerans* and *S. cerevisiae* co-inoculation on the great increase in 4MSP release from Cys-4MSPs (Fig. 2A) and, to a lesser extent, from GSH-4MSP (Fig. 2B).

4. Discussion

The use of non-*Saccharomyces* strains to positively modulate the wine aromatic profile has been extensively studied in different studies using complex matrices, such as natural grape must. Additionally, it is common for *S. cerevisiae* and non-*Saccharomyces* strains to be intentionally co-inoculated with the indigenous yeast community of the grape must (Hu et al., 2016; Renault et al., 2016; Belda et al., 2017; Binati et al., 2020; Borren and Tian, 2020; Fan et al., 2023; Badura et al., 2023). Therefore, the specific contribution of these non-*Saccharomyces* strains remains mostly unknown.

Firstly, it is worth noting that the addition of cysteinylated precursors supposes a negative impact on fermentation kinetics, especially in L. *thermotolerans* fermentations. We hypothesised a potential toxicity of these thiol precursors at the conditions imposed by our experimental setup. The alternative pathways involved in thiol precursors break down have been linked to delayed fermentations. During this process, various compounds are produced that need to be detoxified through deacetylation pathways (Dournes et al., 2023). Likewise, studies testing various compounds (e.g., nutrients and glutathione-rich inactivated yeast) to improve the release of volatile thiols during fermentation showed increased concentrations of residual sugars at the end of the fermentation, along with an increase in total thiols (Alfonzo et al., 2021). We also can speculate that glutathionylated precursors, despite their low concentration in must, can serve a protective effect against oxidative stress, especially relevant during the final stages of alcoholic fermentation, causing different cellular damages. Among them, oxidative stress causes chemical alterations in the unsaturated fatty acids found in cell membranes. Glutathione from the glutathionylated precursors could act as a protective agent against oxidative stress, protecting fatty acids from damage and thereby influencing the volatile fatty acids composition of the resulting wines (Vázquez et al., 2019). Indeed, we observe that the addition of glutathionylated precursors supposes a moderate increase in some fatty acids such as octanoic and decanoic acid in the fermentation assays. Overall, given the impact of thiol-precursors on yeast fermentative performance in our specific conditions, further studies are necessary to thoroughly determine not only the toxic effects of the precursors but also the effects of intermediate compounds on the fermentative performance of specific strains.

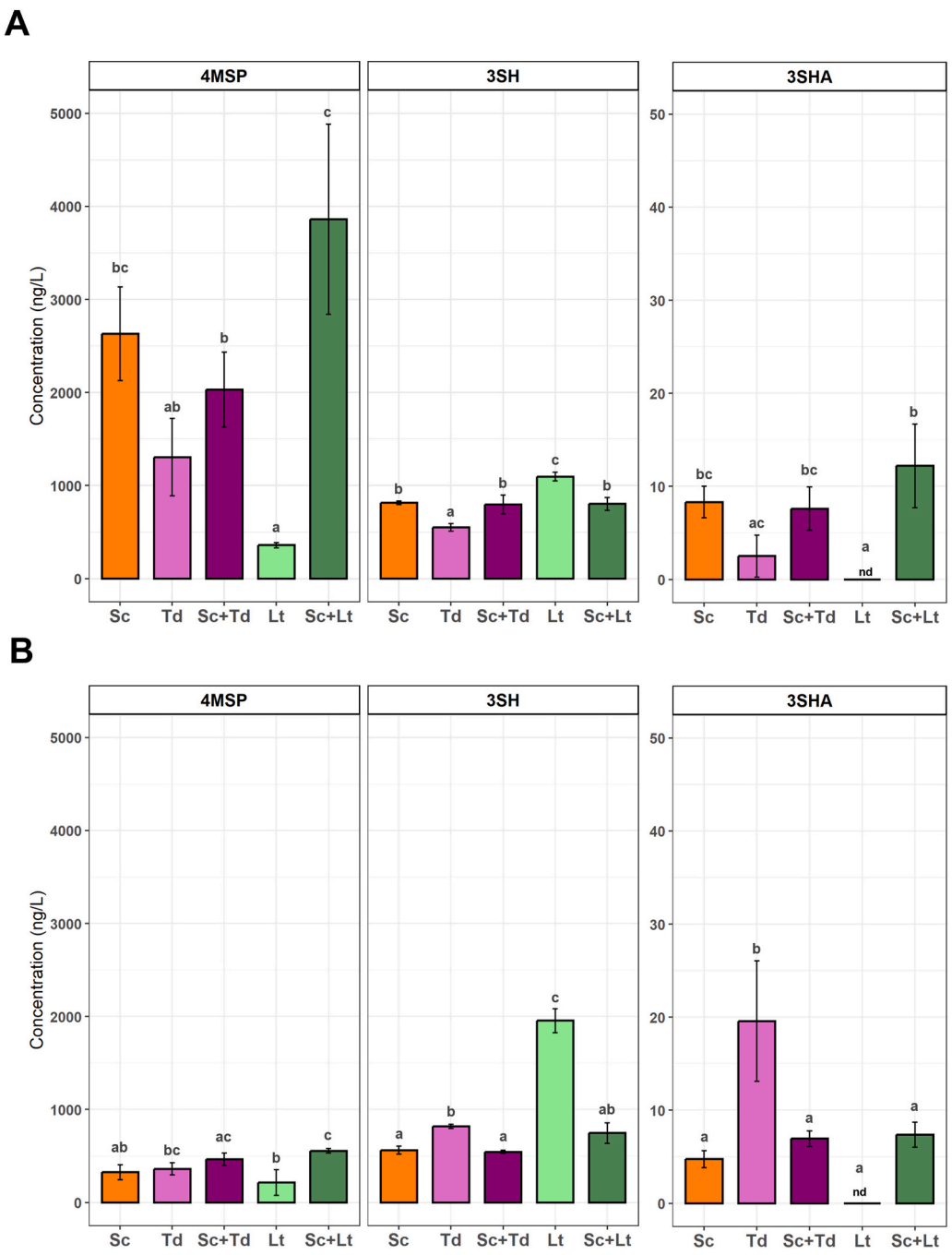

Fig. 2. Barplot representation of the concentration of the aromatic thiols measured at the end of the different fermentation trials. A panel shows the assays carried out by adding cysteinylated precursors. B panel shows the assays carried out by adding glutathionylated precursors. The different assays are represented by different colours: orange for *S. cerevisiae* single culture; purple for *T. delbrueckii* single culture; green for *L. thermotolerans* single culture; dark purple for *S. cerevisiae* + *T. delbrueckii* mixed culture; dark green for *S. cerevisiae* + *L. thermotolerans*. Different letters indicate the existence of statistical differences (ANOVA test, $p < 0.05$). nd indicates that the compound was not detected in the analytical determination. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Despite the differences on fermentation kinetics along the assays conducted in this work, we do not observe clear patterns regarding the volatile profiles of wines (Fig. S3). It has been reported that the presence of organic nitrogen sources is generally related to an increased concentration and diversity in the general volatile profile of the resulting wines (Pereira et al., 2021). In this study, synthetic grape musts were only supplied with a limited concentration of inorganic nitrogen. Thereby, nitrogen from organic sources remains limited along all the fermentation, despite the limited amount of amino acids released from the cell lysis. Furthermore, the impact of the fermentative matrix is of great importance as well. While synthetic matrices offer numerous advantages, particularly in terms of more precise unravelling of specific contributions and biological mechanisms, they have been shown to significantly reduce the production of volatile molecules (Vicente et al., 2023). Thus, the combination of both factors (i.e. limited organic nitrogen and synthetic matrix) can explain the homogeneity regarding the volatile profile of the resulting wines.

The main goal of our study is to unravel the contribution of different thiol precursors and different yeast species to the volatile thiols production during wine fermentation. Firstly, we observed a low capacity of

wine yeasts for releasing thiols from the corresponding thiol precursors, despite the high consumption ability. Thiol precursors were added at an initial concentration on the order of micrograms per litre (see methods section), and even though we observed almost no residual precursors (Fig. 1), we reported a final concentration of volatile thiols on the order of nanograms per litre (Fig. 2), yielding molar conversion rates, from 0.6 to 4.5 %, depending on the species combination, precursors nature and the released volatile thiol (Table S6). Low yields (around 1 %) on thiols releasing from their non-volatile precursors have been frequently reported (Roland et al., 2010; Subileau et al., 2008; Bonnaffoux et al., 2018). Also, nitrogen sources, both nature and concentration, have been described to have an impact on the release of volatile thiols (Cordente et al., 2019). Thus, adjustments in the content of organic nitrogen content in musts from warm areas affected by climate change could be necessary to secure an adequate thiols release (Gutiérrez-Gamboa et al., 2020).

Despite the low thiol releasing capacity, we observe a high consumption level of cysteinylated precursors. These precursors are internalised and subsequently broken down in a simpler pathway than glutathionylated precursors (Ruiz et al., 2019). The breakdown of glutathionylated precursors results in the production of two intermediate peptides, specifically CysGly- and γ-GluCys-before the formation of cysteinylated precursors, and the release of volatile thiols. Thus, due to the complexity of the molecular processes involved in their release, a less efficient metabolism of glutathionylated precursors is expected (Bonnaffoux et al., 2018).

Regarding the impact of different yeast inoculated on thiol production, previous studies carried out in natural grape musts have reported a higher ability of non-*Saccharomyces* for releasing thiols, especially in the case of 4MSP released by *T. delbrueckii* co-inoculated with *S. cerevisiae* (Zott et al., 2011; Renault et al., 2016;Belda et al., 2017). Indeed, it has been demonstrated that *T. delbrueckii* harbours the full-length isoform of the orthologous gene of *IRC7*, that is widespread among the *T. delbrueckii* strains (including the NSTD strain, used in our study) (Belda et al., 2017). However, *T. delbrueckii* did not show higher 4MSP production than *S. cerevisiae*, either under single or mixed fermentation. This discordance, in addition to confirming the complexity of thiol metabolism, highlights that the chemical context—particularly the nature and availability of the nitrogen source and the type of must (natural or synthetic)—significantly influences thiol release (Pinu et al., 2014). These new findings can enhance the management of volatile thiol production during grape must fermentation by providing more precise control over fermentation conditions to optimize thiol release.

Additionally, the results concerning the release of 3SHA in *T. delbrueckii* suggest high relevant activity of alcohol acetyltransferase enzyme in this yeast under the experimental condition adopted. Nevertheless, a BLAST preliminary analysis showed that there is not an orthologous to *S. cerevisiae ATF1* gene identified in the *T. delbrueckii* genome, therefore, other genetic mechanisms should be involved in the production of 3SHA in this species.

Finally, we highlight the outstanding 4MPS production exhibited by the co-inoculation of L. *thermotolerans* and *S. cerevisiae*, which is not explained by the sum of the production of the two strains separately. Several studies have described that non-*Saccharomyces* presence affects the metabolite and transcriptomic profile of *S. cerevisiae* (Barbosa et al., 2015; Curiel et al., 2017; Tronchoni et al., 2017; Ruiz et al., 2020). Specifically, Shekhawat et al. (2019) suggested that the presence of L. *thermotolerans* involves competitive interaction affecting the *S. cerevisiae* transcriptome as well as certain specific metabolic pathways, such as cysteine metabolism. Thus, we suggest that the effect observed for the co-inoculum of both species on the thiol production could be explained by a possible interaction and synergic effect. We hypothesise that L. *thermotolerans* impacts the metabolism of *S. cerevisiae* by enhancing the release of volatile thiols in the co-culture by competing for the amino acid forming the thiol precursors. This observation, along with the highly efficient cleavage of Cys-3SH by L. *thermotolerans*, highlights the necessity for studies on the genetic basis of thiol metabolism in L. *thermotolerans* to fully understand the effect on thiol release reported here.

5. Conclusions

This study provides evidence of the specific contribution of yeast to the development of aromatic thiols in wine. By using a simplified fermentative condition (in absence of an organic source of nitrogen), we can conclude about the potential of *Saccharomyces* and non-*Saccharomyces* yeast strains assayed in this work (*S. cerevisiae*, *T. delbrueckii*, *L. thermotolerans*) in releasing volatile thiols from the different conjugated precursors available in grape musts. Both the nature of thiols precursors added at the beginning of fermentations, and the inoculation strategy followed, exhibited an impact on final thiols content of wines. The strains used in this study showed a higher preference for consuming cysteinylated precursors over glutathionylated ones, with the former being the main source of thiols, especially in the case of 4-MSP production. Additionally, we can highlight a positive contribution of the non-*Saccharomyces* strains on the final content of thiol. For instance, the capacity of *T. delbrueckii* to further transform 3-SH into its acetylated derivative, or the capacity of L. *thermotolerans* producing 3-SH and its enhancing the production of 4-MSP when co-inoculated with *S. cerevisiae*. Nevertheless, despite the advances presented here, we encourage further work under different fermentative conditions and sources of organic nitrogen to fully address the specific influence of non-*Saccharomyces* in thiol precursors metabolism.

CRediT authorship contribution statement

J. Vicente: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **F. Kiene:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **D. Fracassetti:** Validation, Methodology, Formal analysis, Conceptualization. **I. De Noni:** Methodology, Formal analysis. **R. Shemehen:** Methodology, Formal analysis. **A. Tarasov:** Writing – original draft, Funding acquisition, Formal analysis. **A.V. Dobrydnev:** Methodology, Formal analysis. **D. Marquina:** Supervision, Funding acquisition. **A. Santos:** Validation, Supervision, Funding acquisition. **D. Rauhut:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Funding acquisition, Conceptualization. **I. Belda:** Writing – review & editing, Writing – original draft, Validation, Supervision, Investigation, Funding acquisition, Conceptualization. **J. Ruiz:** Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

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

Data availability

All the data supporting the results are included in the main text and the appendix

Acknowledgments

This work has been supported by grant PID2019-105834GA-I00 (acronym Wineteractions) funded by the Spanish State Research Agency/Science and Research Ministry (10.13039/501100011033). We acknowledge support of DAAD organisation within Leonhard Euler Program 2017/2018, project “Aroma compounds in wine” (57376344),

which included the synthesis of thiol precursors. We thank Prof. Dr. Rainer Jung (supervisor from Hochschule Geisenheim University, Germany) and Prof. Dr. Yulian Volovenko (supervisor from Taras Shevchenko National University of Kyiv, Ukraine) for their support within the DAAD project. We thank Dr. Olesia Volovenko and Enamine Ltd. (Kyiv, Ukraine) for the purification of thiol precursors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2024.110858.

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