



ENOLOGY ORIGINAL RESEARCH ARTICLES

Detoxification capabilities of copper and pesticides by winemaking yeasts

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Article number: 9438



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Associate editor:
Hervé Alexandre



Received:
27 May 2025

Accepted:
18 September 2025

Published:
28 October 2025



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ABSTRACT

Viticulture stands out as an agricultural sector most reliant on xenobiotic substances for plant protection from fungal diseases and pest attacks, employing pesticides in conventional farming and copper in organic practices. These compounds are then found in grape juice and wine, causing a major health concern, as well as negatively affecting the fermentation process. Current methods for addressing this issue, relying on adsorbents, metal-chelating polymers, or thermovinification, often lack efficiency and present environmental drawbacks associated with energy consumption or chemical inputs. Based on the reported sorption capacities of yeasts, treatment of the harvest with non-*Saccharomyces* (NS) strains emerges as a biological alternative for the elimination of xenobiotics from grape musts. In this context, this study aims to provide a global perspective on the variability of relationships and interactions occurring between yeasts from the winemaking environment and xenobiotic compounds during the early stages of the process. To this end, the incidence of xenobiotics (a mix of twenty-three pesticides commonly used in the vineyard or copper) on the fermentative activity of thirteen strains of *Saccharomyces* and NS yeasts in synthetic must, and, conversely, the capacity of xenobiotic detoxification of these yeasts was investigated. A comparative study based on conducting fermentations in the presence or absence of xenobiotics first demonstrated that the presence of copper did not significantly influence the growth and fermentation dynamics of the various yeast strains examined, with the notable exception of an NS strain. In contrast, the growth and fermentative capacities of all yeast strains were negatively affected by pesticide exposure, exhibiting strain-specific responses. Most strains could efficiently remove copper initially present in the medium, with a rate depending on the growth capability of the strains and retained copper even after losing viability. The studied yeasts were also capable of removing pesticides from the medium, but with an efficiency that differed according to the strain and the pesticide type. Some pesticides resisted detoxification (folpel), while others (zoxamide or cyazofamid) were eliminated by all strains. Furthermore, NS yeast demonstrated stronger detoxification capabilities than *Saccharomyces cerevisiae*, indicating its potential for the pre-fermentation detoxification of grape musts.

KEYWORDS: non-*Saccharomyces* yeasts, copper, pesticides, detoxification, wine fermentation

INTRODUCTION

Despite increasing recognition of their environmental and health impacts and strong recommendations for reduced application, conventional viticulture continues to exhibit a significant reliance on synthetic fertilisers and chemical plant protection products (Grimalt & Dehouck, 2016). Organic viticulture, while adhering to principles of reduced synthetic inputs, faces its own challenges. Within this system, copper-based compounds represent one of the few permitted plant protection treatments, leading to their potentially excessive and widespread application, consequently resulting in elevated copper concentrations within vineyard environments. As a result, xenobiotics and metals can be recovered in grape juice and wine (Chiarello *et al.*, 2023; Corrias *et al.*, 2021; Eissa *et al.*, 2024). While the majority of detected pesticide residues are often found at levels below their Maximum Residue Limits (MRLs) (Eissa *et al.*, 2024; Soleas & Goldberg, 2010), certain studies have reported instances of concentrations exceeding these regulatory thresholds in specific samples, as mandipropamid and mepanipyrim in Vernaccia di Oristano grape juice (Corrias *et al.*, 2021). Furthermore, a wide array of different compounds is frequently detected (Chiarello *et al.*, 2023; Eissa *et al.*, 2024), raising concerns about potential synergistic effects that could pose risks even when individual pesticide levels are below established limits. Concerning copper concentrations, values are generally below 1 mg/L in musts from conventional agriculture (Calin *et al.*, 2012; Donici *et al.*, 2019; Sun *et al.*, 2018), but can be as high as 15 mg/L in must from organically farmed vineyards (García-Esparza *et al.*, 2006).

These viticultural practices may generate issues extending beyond consumer acceptance due to health considerations (Liviz *et al.*, 2025), potentially altering alcoholic fermentation (Kosel *et al.*, 2019; Sun *et al.*, 2016). The structural heterogeneity of pesticides applied in vinification leads to a range of effects, varying in intensity, on yeast development, which can be attributed to their diverse modes of action. First, the presence of certain pesticides during fermentation has been shown to limit *S. cerevisiae* cell number or viability, thus slowing down the process (Cabras & Angioni, 2000; Calhelha *et al.*, 2006). These effects have been explained by alterations in membrane integrity, impairment of redox balance maintenance, or modifications in metabolic activity (Becerra *et al.*, 2023; Gava *et al.*, 2021). For instance, tetraconazole has been shown to substantially modify methionine and ergosterol biosynthetic pathways in *S. cerevisiae* (Sieiro-Sampedro *et al.*, 2020). Pesticides can also directly impede yeast cell activity (Jawich *et al.*, 2005). The impact of copper on *S. cerevisiae* is concentration-dependent. At low concentrations, copper can have a positive effect by binding to amino acids, acting as an enzyme cofactor, or stabilising the conformation of certain proteins (Festa & Thiele, 2011). However, excessively high concentrations of copper negatively affect the development of *S. cerevisiae* (Capece *et al.*, 2017) through the generation of reactive oxygen species (ROS), altering

cell membrane integrity and disrupting protein conformation (Avery *et al.*, 1996; Farrés *et al.*, 2016). Furthermore, an excess of copper negatively influences polyphenol levels in wines (Sun *et al.*, 2017) and contributes to oxidation problems (González-Álvarez *et al.*, 2012; Noguerol-Pato *et al.*, 2014). However, limited information is available regarding the generality of these effects and underlying mechanisms, both within the *S. cerevisiae* species and, more broadly, across other yeast species present in the wine environment.

In this context, solutions have been sought to limit the content of pesticides and heavy metals in wines. Effective approaches include the use of adsorbents, such as activated carbon, zeolite (an aluminosilicate crystal), or plant fibres, which have been shown to reduce residues of certain pesticides by approximately 50 to 90 % (Grinbaum *et al.*, 2017; Lempereur *et al.*, 2014). Similarly, techniques for detoxifying heavy metals in grape juice and wine use metal-chelating polymers. A limitation of these methods, however, is that their effectiveness is dependent on the specific nature of the xenobiotic (Grinbaum *et al.*, 2017). Furthermore, they rely on the use of chemical additives, which contradicts the current trend in winemaking practices towards more sustainable, input-free processes. Finally, the potential for reducing xenobiotic content by modifying the vinification process itself is very limited; only thermovinification with cold settling has shown a modest decrease, up to 30 %, in some pesticides (Grinbaum *et al.*, 2017). Conversely, recent studies have demonstrated that certain microorganisms, mainly bacteria but also some fungi, possess pesticide-degrading activities, often using these compounds as carbon sources (Borowik *et al.*, 2023; Książek-Trela & Szyrka, 2022; Nandhini *et al.*, 2021). However, the majority of bacteria exhibiting these detoxification effects are unsuitable for food production due to their pathogenic nature.

Given the limitations of existing detoxification methods, exploring the potential of wine-associated yeasts for pesticide removal in musts presents a compelling alternative. These yeasts are increasingly valued in winemaking for their contribution to aromatic complexity and their role as bioprotective agents before fermentation (Borren & Tian, 2021; Padilla *et al.*, 2016). Moreover, the observed reduction in pesticide levels between must and wine in some studies hints at potential degradation (chemical or biological) or adsorption by yeast cell wall constituents like chitin and glucan (Cabras *et al.*, 2003; Caridi, 2007; Navarro *et al.*, 1999).

In this context, the objective of this study is to assess the ability of wine-associated yeasts to remove pesticides and copper, focusing on the early phases of fermentation. The ultimate aim is to explore the feasibility of employing yeasts for the pre-fermentative detoxification of musts. Our investigation will specifically address the impact of copper and pesticides on yeast growth and fermentative performance, considering the variability based on xenobiotic nature and yeast species/strain. Conversely, we will also explore the detoxification abilities of these yeasts towards these compounds.

MATERIALS AND METHODS

1. Strains

A total of 13 yeast strains commonly found in wine production were studied. They include 3 commercial *Saccharomyces cerevisiae* (S) strains from the Lallemand yeast collection (LALLEMAND SAS) and 10 non-*Saccharomyces* (NS) yeast strains from the species *Metschnikowia pulcherrima*, *Metschnikowia fructicola*, *Metschnikowia chrysopterae*, *Metschnikowia zizyphicola*, *Starmerella bacillaris*, *Torulaspora delbrueckii*, *Lachancea thermotolerans*, *Pichia* sp., commercialised by LALLEMAND SAS (six strains) or isolated from vineyards or cellars. Four of the non-*Saccharomyces* strains (NS1) are of the same species. Strains were stored at -80°C in a mixture of YEPD medium/glycerol (80:20). Precultures were carried out in sterile YEPD medium (bactopeptone 20 g/L; bacto yeast extract 10 g/L; glucose 20 g/L; ultra-pure water). Agar (20 g/L) and FeCl_3 (0.1 mM) were added to prepare the solid medium. Strains from freeze stock solution were plated in solid YEPD medium for 72 hours at 28°C . One pure colony was inoculated in liquid YEPD medium and shaken (280 rpm) at 28°C for 24 hours. One mL of the preculture was transferred to YEPD medium and incubated under agitation at 28°C for 24 hours. The preculture was then centrifuged (4,500 rpm, 5 minutes, 4°C) and the supernatant removed. The pellet was resuspended in sterile water to wash the cells. The population of the pre-culture was measured using a flow cytometer.

2. Solutions and media

2.1. Pesticide solution

A total of 23 pesticides were selected for this study based on their prevalence of use and/or detection in vineyards and wines (information provided by Laboratoires Dubernet, Narbonne, France; Table 1). Each compound (except phosphorous acid) was added to the synthetic medium at a final concentration equivalent to 10 times its limit of detection by the quantification method used at Dubernet laboratories, with the total pesticide concentration not exceeding 1 mg/L. Phosphorous acid is added independently at a concentration of 5 mg/L.

Each pesticide was initially solubilised independently in acetone to create a stock solution at a concentration of 25 mg/mL. Subsequently, all stock solutions (with the exception of phosphorous acid, which was handled separately as needed) were diluted and combined in pure ethanol to achieve a concentration 1,000 times the desired final concentration in the synthetic medium (Table 1). A stock solution containing all the tested pesticides (excluding phosphorous acid) was prepared and is hereafter referred to as the “pesticide mix”. Phosphorous acid was prepared separately using the same treatment. Both the 1,000 \times concentrated pesticide mix and the phosphorous acid solution were stored at 4°C in tinted glass bottles. To prevent potential reactions between certain pesticides and plastic, only glass vessels were used, and contact with plastic surfaces was minimised throughout the experimental procedures.

2.2. Fermentation synthetic medium

The synthetic medium used in this study mimics the composition of a grape juice, containing 200 mg/L of assimilable nitrogen and 200 g/L of sugars (Rollero *et al.*, 2015). The quantities of each compound used are listed in Table S1 to Table S5. To test the impact of copper, this metal was added in the form of copper sulphate (CuSO_4) at a concentration of 7.5 mg_{Cu}/L. This copper content corresponds to 7.5 times the limit authorised in Europe (Commission Regulation (EC) N° 606/2009, 2009). However, the medium always contains 0.25 mg/L of Cu due to the quantity of CuSO_4 in the trace elements stock solution (Table S3). Pesticides were added independently to synthetic grape juice (1 mL pesticide mix/L). Phytosterols are added just before use to prevent foaming during preparation.

3. Fermentation conditions

Fermentations were conducted in 300 mL glass fermenters. For each experiment, the appropriate concentrations of metals or pesticides were added to a suitable volume of SM medium. Following thorough homogenisation, the medium was distributed into the fermenters and then pasteurised at 100°C for 15 minutes under atmospheric pressure. Subsequently, the medium in each fermenter was saturated with oxygen by bubbling air under stirring for 30 minutes. The fermenters were stored at 4°C for a maximum of two days before inoculation.

Each fermenter was inoculated with an initial cell concentration of 10^6 cells/mL, supplemented with phytosterol (5 mg/L), and incubated at 20°C with continuous magnetic stirring. The initial mass of each fermenter was recorded immediately after inoculation by weighing.

3.1. Fermentations with copper

For each strain, fermentations were performed in triplicate using synthetic must (SM) without metals as a control. Fermentations were conducted at 20°C with continuous magnetic stirring. To monitor fermentation activity, the fermenters were weighed at various time points to determine the cumulative CO_2 released. After seven days of fermentation, ethanol was added to each fermenter to reach a final concentration of 12 % (v/v), accounting for the ethanol already produced during fermentation. Fermentations were then stopped after a further five days (12 days total).

3.2. Fermentations with pesticides

For each strain, fermentations were performed in triplicate under two conditions: in synthetic must (SM) supplemented with a pesticide mix and phosphorous acid, and in SM alone as a control. All fermentations were carried out simultaneously at 20°C with continuous magnetic stirring using the robotic system PhenoFerm. This system enabled the automatic hourly weighing of fermenters to monitor mass loss. The ALFIS software (a data processing software developed internally by INRAe SPO) simultaneously recorded the mass data and directly calculated the fermentation progress based on CO_2 release.

TABLE 1. List of pesticides studied with associated concentrations.

Molecules	Levels to be tested (mg/L)	Limit of quantification (mg/L)	Limit of detection (mg/L)
Boscalid	0.05	0.02	0.007
Cyazofamid	0.02	0.001	0.0003
Cyprodinil	0.03	0.001	0.0003
Difenoconazole	0.02	0.001	0.0003
Dimetomorph	0.05	0.001	0.0003
Fenhexamid	0.05	0.001	0.0003
Fludioxonil	0.05	0.001	0.0003
Fluopicolide	0.03	0.005	0.002
Fluopyram	0.02	0.001	0.0003
Fluxapyroxad	0.02	0.001	0.0003
Folpel	0.25	0.001	0.0003
Iprodione	0.05	0.001	0.0003
Iprovalicarb	0.05	0.005	0.002
Metalaxyl-M	0.02	0.005	0.002
Metrafenone	0.02	0.001	0.0003
Penconazole	0.05	0.001	0.0003
Phosphorous acid	5	0.001	0.0003
Pyrimethanil	0.05	0.001	0.0003
Spiroxamine	0.02	0.1	0.003
Tebuconazole	0.03	0.001	0.0003
Tetraconazole	0.02	0.001	0.0003
Thiophanate-methyl	0.02	0.001	0.0003
Zoxamide	0.02	0.001	0.0003

4. Analytical methods

4.1. Yeast viability and population measurement

Yeast viability and population were assessed by harvesting 5 μ L samples, which were then diluted 100-fold in phosphate-buffered saline (PBS). To identify dead cells, 5 μ L of propidium iodide (PI) (100 \times stock solution) was added to each diluted sample, and labelling was performed for 20 minutes. Viability and population measurement were subsequently analysed using a flow cytometer (Attune NxT, Thermo Fisher Scientific) with the following parameters: FSC = 140 mV, SSC = 240 mV, and threshold = 1,000. Gating for the yeast quantification was performed on the window SSC-H vs FSC-H.

4.2. Copper concentration quantification

Copper concentration was quantified by collecting 3 mL samples at various time points during fermentation. Each sample was centrifuged at 4,500 rpm for 5 minutes at 4 $^{\circ}$ C, and the supernatant was carefully recovered by inverting the tube. Copper levels in the supernatant were determined by UV/visible spectrophotometry using the CUI-COL Copper Assay Kit (LIBIOS, VINDRY-SUR-TURDINE, France). The kit's protocol was adapted for use with 96-well microplates

(all volumes were scaled down by a factor of 5), and samples were diluted twofold to ensure copper concentrations were within the kit's linearity limit (5 mg/L Cu). All measurements were performed in triplicate.

4.3. Pesticide quantification

To quantify pesticide concentrations, 50 mL of partially fermented must was collected in glass tubes. Each sample was centrifuged at 4,000 rpm for 7 minutes, and the supernatant was carefully transferred to a clean glass bottle. The collected samples were stored at -20 $^{\circ}$ C prior to pesticide content analysis. The analysis employed gas chromatography coupled with mass spectrometry (GC-MS) using the QuEChERS (Quick, Easy, Cheap, Efficient, Rugged and Safe) extraction and clean-up technique. Certain pesticides were analysed using liquid chromatography-mass spectrometry (LC-MS). All analyses of pesticide concentrations were conducted by Dubernet laboratories (Laboratoires Dubernet, Narbonne, France).

4.4. Quantification of central carbon metabolites

For each sample collected for pesticide quantification, a 200 μ L aliquot of the supernatant (obtained after centrifugation) was recovered and diluted in a 2.5 mmol/L

sulfuric acid (H₂SO₄) solution. The precise dilution factor was determined by gravimetric measurement. The diluted samples were then analysed by High-Performance Liquid Chromatography (HPLC) using a Phenomenex Rezex ROA column. This column is packed with a styrene-divinylbenzene (SDVB) resin carrying H⁺ functional groups, and the primary separation mechanism is ion exclusion. The low pH required for HPLC separation was achieved by diluting the samples in the 2.5 mmol/L H₂SO₄ mobile phase. Organic acids (α -ketoglutarate, citrate, pyruvate, malate, succinate, and acetate) were quantified using an ultraviolet (UV) detector, while sugars and alcohols (glucose, fructose, glycerol, and ethanol) were quantified using a refractive index detector (RID).

5. Statistics analysis

All fermentations were conducted in biological triplicates ($n = 3$). For fermentations performed using the PhenoFerm robotic system (specifically those involving pesticides), kinetic parameters such as maximum fermentation rate (R_{max}) and lag time were determined using a custom R package, *alfisStatUtilR* v1.0.0, based on the regression model described by Duc *et al.* (2020). Comparisons between fermentations with and without xenobiotics were primarily performed using pairwise analyses for each strain. Some fermentation parameters (maximum quantity of CO₂ released, maximum rate R_{max} of CO₂ release, and the latency time) were statistically assessed. For fermentations in the presence of copper, the analysed parameters included cumulative CO₂ release, cell viability at day 7 and day 12, and cell concentration at day 7. For fermentations in the presence of pesticides, the examined parameters were R_{max}, lag time, and cumulative CO₂ production at 70 hours. For all parameters, the homogeneity of variances and the normality of residuals were evaluated using Levene's test and the Shapiro–Wilk test, respectively. When these assumptions were satisfied, an analysis of variance (ANOVA) followed by a Tukey post-hoc test was applied. In cases where the assumptions were not met, a non-parametric Kruskal–Wallis test was used to compare the control condition with the xenobiotic condition for each strain. All statistical analyses were performed using R software, with a significance level set at $p < 0.05$.

RESULTS AND DISCUSSION

This study aimed to characterise the ability of 13 *Saccharomyces cerevisiae* and non-*Saccharomyces* yeast strains from the species *M. pulcherrima*, *M. fructicola*, *M. chrysoperlae*, *M. zizyphicola*, *Starmerella bacillaris*, *Torulasporea delbrueckii*, *Lachancea thermotolerans*, and *Pichia* sp. to eliminate xenobiotics and to explore their potential application in the pre-fermentative treatment of grape musts. We compared their performance during fermentation in the presence or absence of copper or selected pesticides. Our experimental design involved monitoring fermentation dynamics, yeast growth, viability, and xenobiotic levels throughout the fermentation process. The initial phase of our analysis concentrated on the first 72 hours

of fermentation to determine the impact of xenobiotics on early growth and fermentation establishment, and to assess potential early detoxification. For copper treatments, we also examined a later stage after ethanol addition to evaluate the influence of ethanol-induced stress on yeast populations and copper retention. In pesticide treatments, we analysed central carbon metabolites (glycerol, acetate) to investigate and understand the potential impact of xenobiotics on sugar metabolism in yeast.

1. Impact of metals on the fermentation by different wine yeasts

Fermentations of 13 strains (three *S. cerevisiae*, seven non-*Saccharomyces* species, including four strains of the species NS1) were conducted in a synthetic medium (SM) with or without 7.5 mg/L copper. This value was selected based on the copper content quantified in grape musts. Under conventional agricultural practices, the copper concentration in must typically does not exceed 1 mg/L (Calin *et al.*, 2012; Sun *et al.*, 2018). However, in organic agriculture, due to the prohibition of fungicide and pesticide use, copper-based treatments are extensively employed, and the concentrations detected in musts can reach levels of 15 mg/L (Dournes *et al.*, 2023; García-Esparza *et al.*, 2006). Due to the limited fermentative capacity of NS species and to prevent excessively long fermentations, the ethanol concentration was adjusted to 12 % after seven days of fermentation, which corresponded to the typical ethanol concentration at the completion of fermentation.

The comparison of the fermentative activity of the 13 strains over seven days revealed that, in the absence of metals, three distinct fermentation profiles were observed (Figure 1). The *Saccharomyces* yeasts clustered within the first group, due to their highest fermentative capacities, reflected by a CO₂ release higher than 90 g/L over seven days of fermentation. Three non-*Saccharomyces* species (NS2, NS6, and NS7, second group) exhibited moderate to high fermentation performances, producing between 53 to 83 g/L of CO₂. Finally, four non-*Saccharomyces* species (NS1, NS3, NS4, NS5) presented low fermentation capacities and released less than 36 g/L of CO₂. Interestingly, for most of the strains, the population achieved a substantial level, ranging from 4.2×10^7 to 1.7×10^8 viable cells/mL, with viability remaining higher than 95 % after seven days of fermentation (Figure 2). No clear relationship was observed between the CO₂ production of NS yeasts and the viable cell population (Figure 1, Figure 2). In contrast, the strain NS5.a displayed markedly impaired growth, not exceeding 5.9×10^6 cells/mL. This reduced growth is potentially attributable to poor medium adaptation, as evidenced by lower viability from 24 hours fermentation (Figure 2). NS7.a, while exhibiting good growth capabilities (final population 1.0×10^8 cells/mL), exhibited a gradual decline in viability early in the fermentation process. The important loss of viability (43 % of viable cells) can explain the stop of the fermentative activity of this strain after seven days of fermentation.

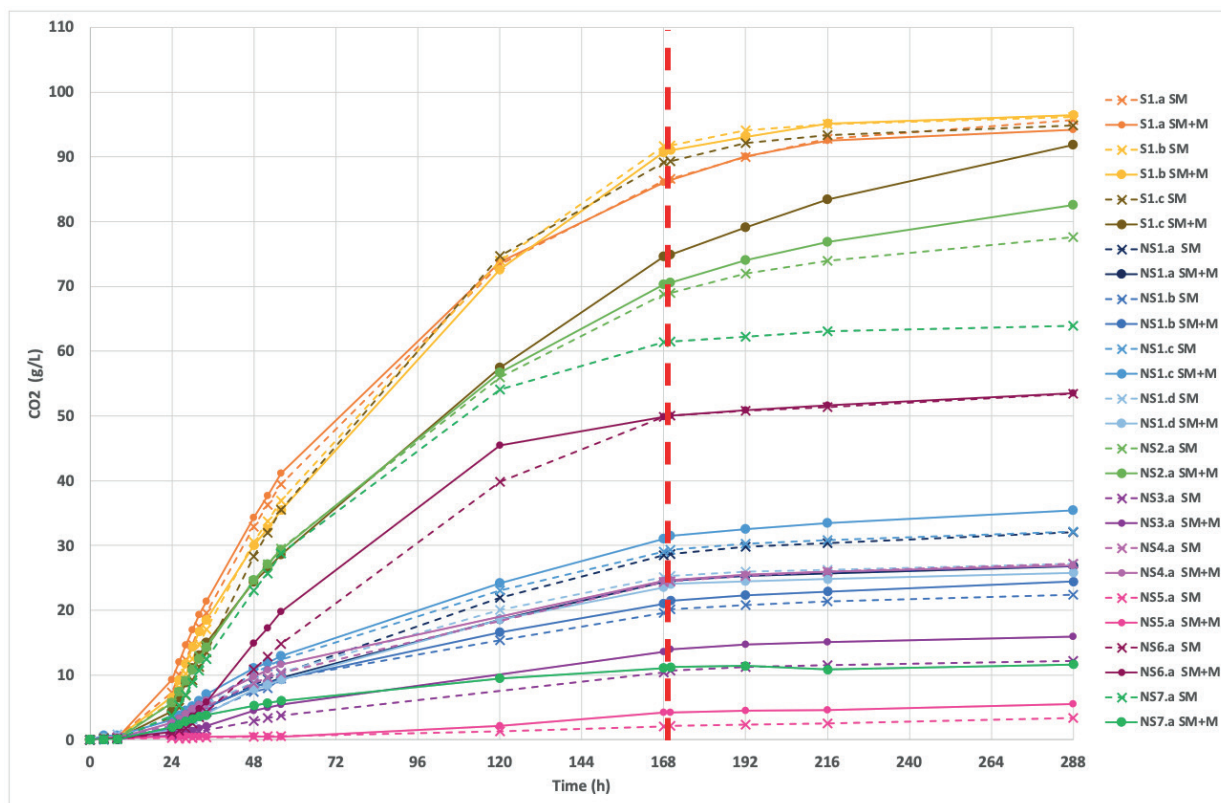


FIGURE 1. Fermentation kinetics of different yeast strains in the presence or absence of metals. The crosses represent the conditions of synthetic must without metals (SM), and the circles the conditions with copper (SM + M). Yeasts are grouped into colour groups by category: *Saccharomyces* yeasts are yellow/orange/brown; non-*Saccharomyces* yeasts of the same species are shown in blue. All other non-*Saccharomyces* species are shown in green/pink/purple. The red dots represent the moment of the addition of ethanol to reach 12 % alcohol in the fermenters.

Adding ethanol at the end of fermentation resulted in a rapid decrease in viability of all the non-*Saccharomyces* strains (1 hour), while *S. cerevisiae* strains demonstrated a marked resilience, maintaining high viability levels for at least two days after ethanol addition. Ethanol production has been reported as generating major stress for yeasts during fermentation, affecting their growth, viability, and metabolic activity (Leão & van Uden, 1982; Yang *et al.*, 2012; You *et al.*, 2003). The mechanisms underlying these effects notably include the impairment of the membrane transporter activity and the disruption of membrane integrity (Dinh *et al.*, 2008). A reduced capacity, recently documented in *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* relative to *S. cerevisiae*, to modulate membrane fluidity under high ethanol stress and preserve surface morphology, could contribute, in part, to the lower ethanol tolerance observed in non-*Saccharomyces* yeasts (Aiello *et al.*, 2024).

Overall, the growth and fermentation profiles observed during fermentation with copper were similar to those seen in the absence of this metal, showing that copper did not affect yeast growth, viability, or fermentation performance (Figures 1 and 2). Only three strains responded specifically to the presence of copper. The strain S1.c demonstrated a significant reduction in viability, with only 30 % viable cells after two days of fermentation. The population of viable cells further stabilised at 5.5×10^7 viable cells/mL compared

to 1.1×10^8 viable cells/mL in the absence of copper. This copper-induced decrease in cell viability directly impacted fermentation efficiency, as evidenced by a decrease in CO₂ production after seven days of fermentation from 90 g/L (without copper) to 75 g/L. However, this strain was still able to complete 92 g/L sugar fermentation in the presence of copper, indicating that the presence of this metal delayed but did not impair S1.c fermentation. Non-*Saccharomyces* strains NS5.a and NS7.a also experienced a decrease in viability in the presence of copper, combined with an altered growth capacity. Consequently, the viable population of NS7.a during fermentation in the presence of copper (5.5×10^6 cells/mL) accounted only for 15 % of that in the absence of copper (4.1×10^7 cells/mL). The fermentative activity was thus considerably affected, which resulted in a large decrease in the amount of CO₂ produced after seven days of fermentation from 63 g/L without copper to 11.6 g/L. This finding revealed the high sensitivity of the NS7.a strain to copper-induced stress.

The toxicity of copper on yeast growth and fermentative performance has been predominantly investigated in *S. cerevisiae*. Copper becomes toxic to most strains at concentrations exceeding 6 mg/L; however, certain strains demonstrate resistance to levels as high as 100 mg/L, while a few exceptions show sensitivity at concentrations below 3 mg/L (Capece *et al.*, 2017; Sica *et al.*, 2024).

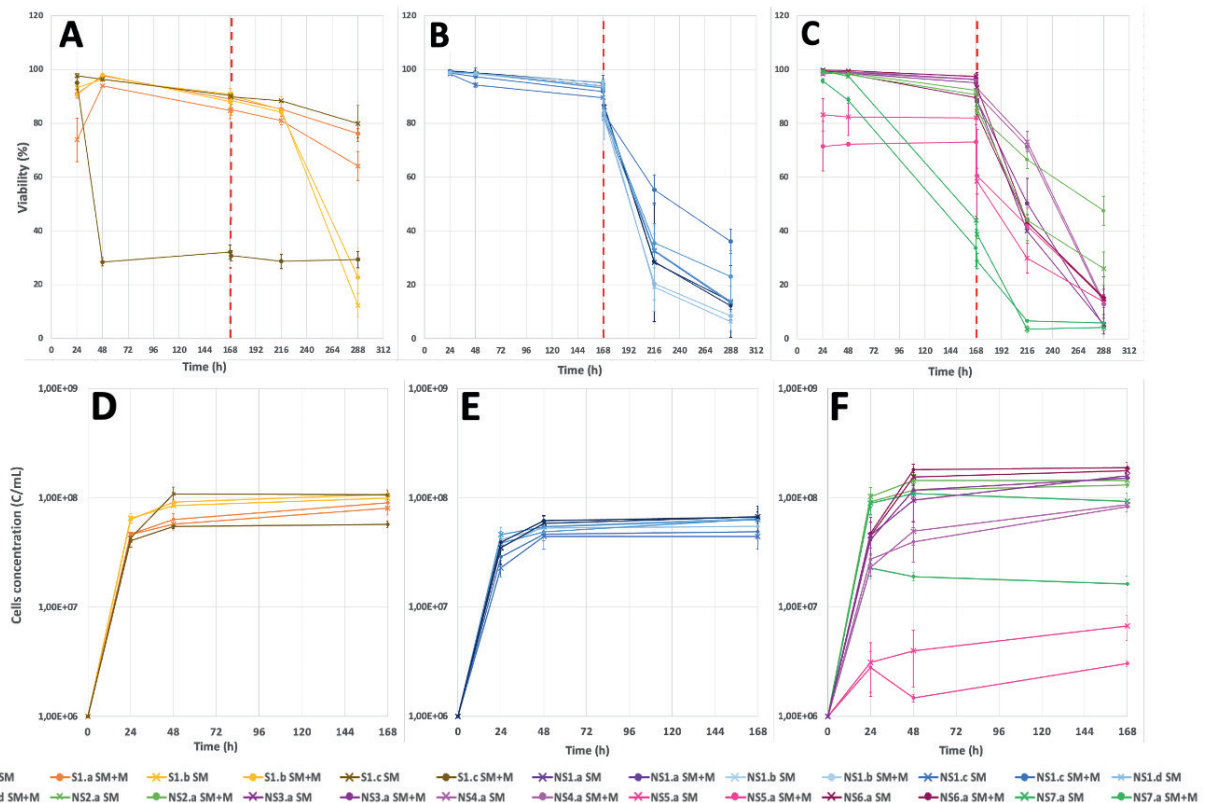


FIGURE 2. Viability (%) of different yeast strains as a function of fermentation time (h) in the presence or absence of metals (A–C). Number of cells (C/mL) of different yeast strains as a function of fermentation time (h) in the presence or absence of metals (D–F). The crosses represent synthetic must conditions without metals (SM), and the circles represent conditions with copper and iron (SM + M). The yeasts are grouped in three different graphs by category: *Saccharomyces* yeasts are represented in graphs A and D; non-*Saccharomyces* yeasts of the same species are represented in graphs B and E; all other species of non-*Saccharomyces* yeasts are represented in graphs C and F. The red dots represent the time of ethanol addition to reach 12 % alcohol in the fermenters.

These relatively high established toxicity thresholds explain the lack of a significant effect of the presence of 7.5 mg/L on the growth and fermentative capacity of most strains in this study, including both *S. cerevisiae* and non-*Saccharomyces* yeasts. In contrast to this general trend, the NS7.a strain stood out as copper presence severely impaired both its growth and fermentative performance. This distinct response could be due to specific characteristics of this yeast membrane composition or its copper metabolism. Notably, research in *S. cerevisiae* has revealed that this metal can compromise plasma membrane integrity, with cells rich in unsaturated fatty acids showing heightened vulnerability (Avery *et al.*, 1996). Furthermore, copper exposure induces oxidative stress, DNA damage, and metabolic perturbations in yeast cells (Farrés *et al.*, 2016). Cellular responses to copper toxicity encompass multiple pathways, including vacuolar detoxification and tryptophan biosynthesis (Jo *et al.*, 2008).

2. Copper detoxification capacity of different yeast strains

The copper removal capacity of 11 selected yeast strains was assessed by monitoring copper concentration over the first 48 hours of fermentation in the presence of 7.5 mg/L copper (Figure 3). For a subset of these yeasts, after seven days of fermentation, the ethanol concentration was adjusted to

12 % to simulate the ethanol level usually found at the end of fermentation and evaluate its toxicity. Copper concentration was monitored for a further five days to assess potential release from the cells (Figure 4). Due to cultivability problems with strains NS3.a and NS4.a, these strains were excluded from the following results.

A significant reduction in copper content was observed between 16 and 48 hours of fermentation with 9 of the 11 selected strains, leading to copper removal efficiencies of 93–98 % (Figure 3). However, strain NS5.a failed to sequester copper, a finding likely correlated with the absence of growth and the immediate loss of viability observed for this strain (Figure 2). Strain NS7.a also exhibited low copper-trapping efficiency (22 % removal, Figure 3), which aligns with its limited growth in the presence of copper (a reduction exceeding five-fold compared to copper-free fermentation after 48 hours) and substantial loss of viability. Nevertheless, copper detoxification by NS7.a continued throughout the fermentation period, with 67 % removal achieved after seven days.

The time to achieve 95 % copper elimination ranged from 26 (strain S1.a) to 48 hours (strain S1.b, S1.c, NS1.d, NS6). This substantial strain-dependent variability in copper elimination dynamics was primarily due to marked

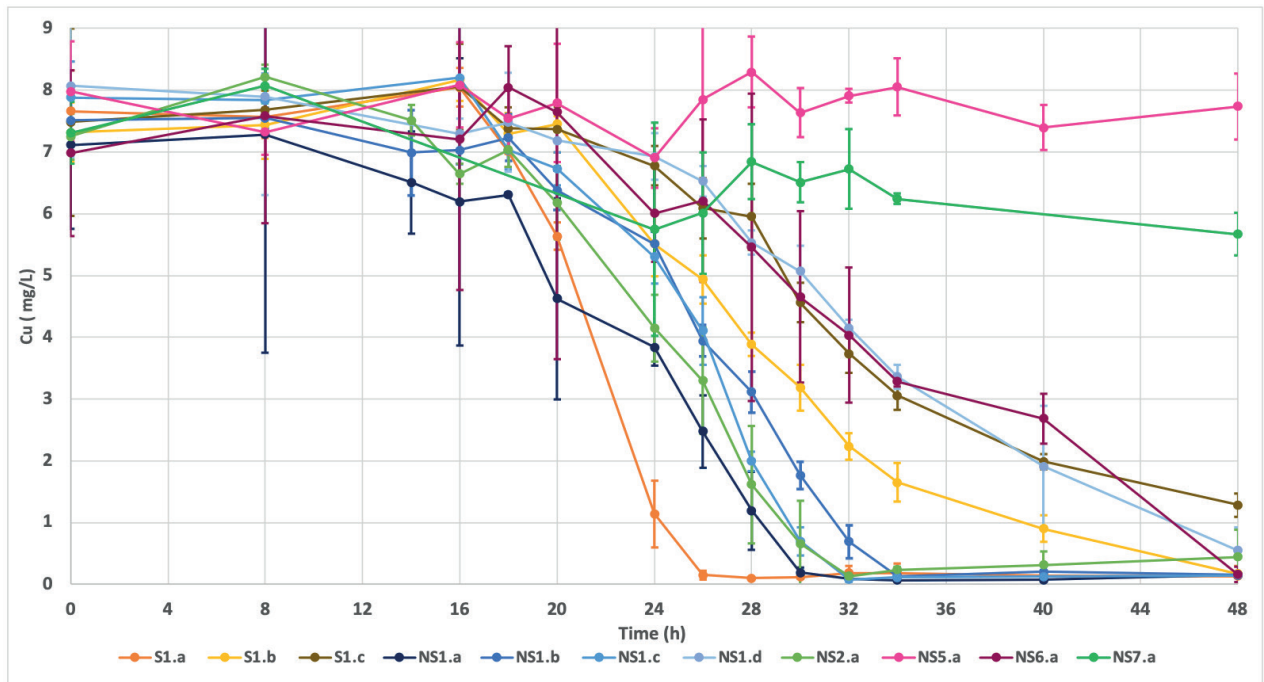


FIGURE 3. Copper concentration (mg/L) during the first 48 hours of fermentation (h) for the different strains. Yeasts are grouped into colour groups by category: *Saccharomyces* yeasts are yellow/orange/brown; non-*Saccharomyces* yeasts of the same species are shown in blue. All other non-*Saccharomyces* species are shown in green/pink/purple.

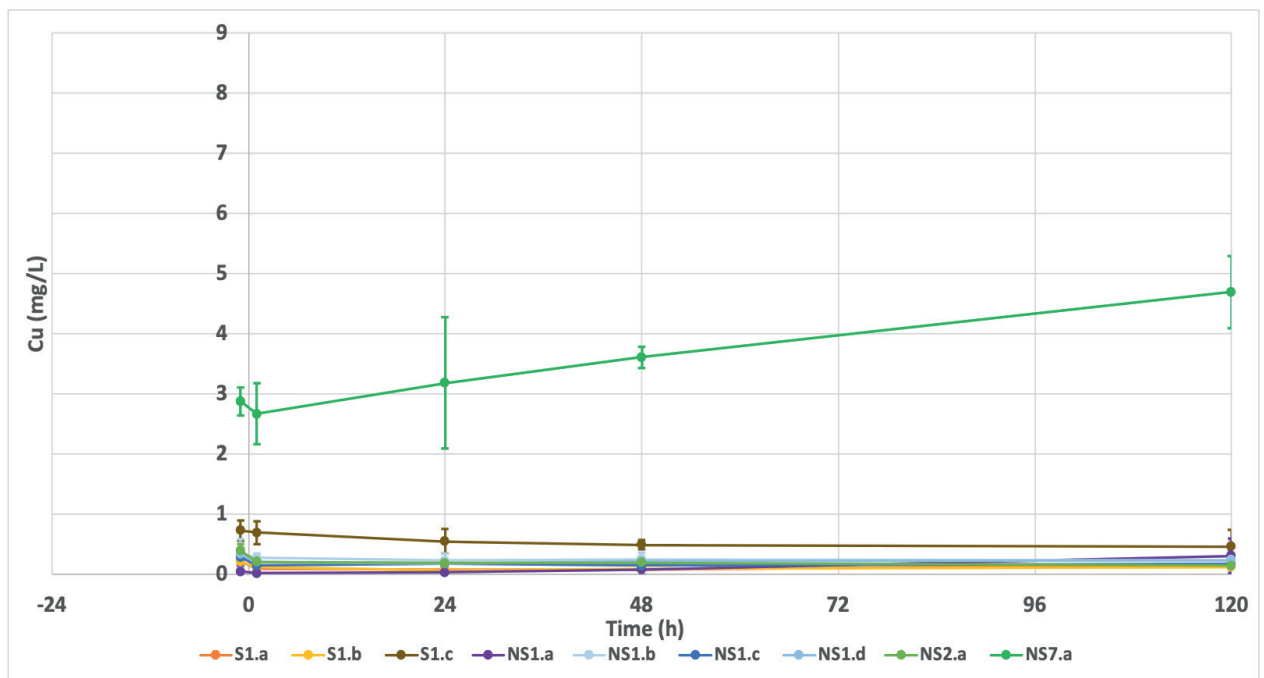


FIGURE 4. Copper concentration (mg/L) as a function of fermentation time (h) after ethanol addition. The negative value (-1 hour) represents the analysis just before ethanol addition (at 168 hours of fermentation). The value 0 corresponds to the time when the ethanol was added to obtain a concentration corresponding to 12 % (v/v) alcohol. Yeasts are grouped into colour groups by category: *Saccharomyces* yeasts are yellow/orange/brown; non-*Saccharomyces* yeasts of the same species are shown in blue. All other non-*Saccharomyces* species are shown in green.

differences in detoxification rates (Table 2). Strain S1.a exhibited a detoxification rate of 0.85 mg/L/h, while strains requiring more than 44 hours to detoxify copper have an average detoxification rate of 0.33 ± 0.09 mg/L/h. Furthermore, although the strains NS1.a, NS2, NS1.c, and

NS1.b detoxified copper at comparable rates, approximately 0.5 mg/L/h, they differed substantially in the time needed to achieve 95 % copper removal between 30 and 34 hours. This could be attributed to varying lag phases before detoxication commenced (Figure 3).

TABLE 2. Rate of detoxification (mg/L/h) by the different strains during their exponential phase of detoxification.

	S1.a	S1.b	S1.c	NS1.a	NS1.b	NS1.c	NS1.d	NS2.a	NS5.a	NS6.a	NS7.a
Detoxification rate (mg/L/h)	0.857	0.427	0.372	0.510	0.444	0.554	0.272	0.493	0.002	0.244	0.060
Standard deviation	0.022	0.025	0.009	0.004	0.020	0.018	0.006	0.017	0.015	0.028	0.002

Detoxification capacity in the studied yeasts was found to be highly strain-dependent, rather than species-specific. Notably, species NS1 and S1 encompassed strains exhibiting both the highest and lowest detoxification efficiencies. Moreover, the copper removal efficiency of yeasts did not correlate with their growth capacity. For instance, S1.a demonstrated superior copper detoxification capabilities compared to S1.b, despite exhibiting a lower cell population (6.3×10^7 cells/mL vs 9.1×10^7 cells/mL, respectively). Finally, it is worth noting that *S. cerevisiae* S1.c exhibited a distinct detoxification profile, achieving only 82 % copper removal within 48 hours. This limited efficiency likely resulted from a rapid decline in strain viability observed under copper stress at this time point (Figure 2).

For most strains, during the fermentation period from two to seven days, the copper content of the medium remained consistently below 0.4 mg/L, indicating a general absence of copper release (Figure 4). However, strain NS7 exhibited a notable exception to this trend, by continuing to remove copper, with levels declining from 5.7 to 2.9 mg/L. These data revealed that this strain was able to detoxify copper, but its limited growth, coupled with a substantial decrease in viability (Figure 2), impaired its global efficiency. It is well established that ethanol can compromise cell membrane integrity. Consequently, the elevated ethanol concentrations characteristic of the later stages of alcoholic fermentation (AF) may influence copper retention. Given that non-*Saccharomyces* yeasts typically produce lower ethanol levels (Mehломakulu *et al.*, 2021), the medium was adjusted to a final ethanol concentration of 12 % to specifically evaluate the potential for copper release under these conditions. For most of the strains, copper levels were unchanged for five days after the addition of ethanol, despite leading to a loss of cell viability that varied in severity depending on the strain (Figure 2). Copper concentration remained below 0.55 mg/L, indicating that copper removal was stable over time. The loss of yeast viability triggered by adding ethanol did not result in a re-increase in metal levels (“salting-out” phenomenon). Finally, NS7.a was the only strain for which copper levels increased (from 35 % to 65 % of the initial concentration) after ethanol addition, revealing a copper release by this species.

To summarise, we demonstrated that copper detoxification is a capacity exhibited by most *S. cerevisiae* and non-*Saccharomyces* strains, with an efficiency that depends more on the strain itself than on the species. This mechanism requires metabolically active cells and/or cells with high viability to achieve a stable and irreversible detoxification, even if yeast viability decreases after detoxification.

Copper exhibits a dual nature in microbial physiology. While an essential element for all life forms, serving as an enzymatic cofactor and structural protein stabiliser, it becomes cytotoxic in excess, primarily through the generation of reactive oxygen species (ROS), the alteration of cell membrane integrity, and the disruption of protein conformations (Avery *et al.*, 1996; Farrés *et al.*, 2016). Consequently, the regulation of intracellular copper concentration is a critical cellular process, extensively studied in *S. cerevisiae* (Shi *et al.*, 2021). Basically, to prevent toxicity, the cell sequesters excess copper ions by binding them to glutathione (GSH) and metallothioneins, and by transporting these complexes into vacuoles (Butt *et al.*, 1984; Shi *et al.*, 2021). Notably, the Cup1/Crs5 pathway facilitates the synthesis of metallothioneins—a family of low molecular weight, cysteine-rich proteins capable of binding metal ions like copper (Fogel & Welch, 1982; Winge *et al.*, 1985). Superoxide dismutase 1 (Sod1) also plays a key role in cytoplasmic copper complexation, mediated by the copper chaperone Ccs1 (Lamb *et al.*, 2000; Li *et al.*, 2010; Rae *et al.*, 1999; Slekár *et al.*, 1996). Therefore, these diverse detoxification mechanisms likely play a significant role in the observed removal and sequestration of copper from the surrounding environments. Moreover, we observed a brown-copper coloration of cells in the presence of copper (data not shown), potentially suggesting an additional detoxification mechanism involving the retention of this metal by cell walls or membranes. Further analysis is required to confirm these potential interactions between copper and cell walls or membranes. Notably, the only copper-sensitive strain, NS7.a, which showed altered growth, viability, and fermentation capacity when exposed to this metal, also presented a strikingly limited ability for copper detoxification, an effect that was reversed by ethanol supplementation. This observation could be attributed to a defect or absence of metallothionein synthesis in this yeast. As a result, copper detoxification might primarily occur via adsorption onto the cell surface or absorption without subsequent complexation mechanisms.

3. Screening the influence of pesticides on the fermentations of different yeast strains

Fermentations by 13 different yeast strains (*Saccharomyces* and non-*Saccharomyces*) were carried out in synthetic must (SM) in the presence (+P) or absence of pesticides to investigate the influence of pesticides on their growth and fermentation capacities. The pesticides were selected based on their frequency of use, their frequency of detection in the musts/wines, the diversity of their chemical structure, and their inability to be eliminated by other detoxification

methods. Their concentrations, ranging from 0.1 to 0.25 mg/L, were in general below their MRLs (between 0.25 % and 2.5 % of the MRLs), and the total concentration of pesticides is less than 6 mg/L. Phosphorous acid, employed at 5 mg/L to correspond with typical concentrations found in grape must, and iprodione, utilised at 0.05 mg/L to satisfy analytical requirements (to have 10 times the limit of quantification, corresponding to 0.005 mg/L), constitute the sole exceptions. The impact of pesticides on fermentation was evaluated for 70 hours, a duration sufficient for the complete growth of all yeast strains examined (Figure 5), by comparing fermentation kinetics (release of CO₂, fermentation rate), cell population, viability, and production of central carbon metabolites (glycerol, acetate).

Pesticides likely negatively affect fermentation kinetic parameters through two main pathways: they can directly slow down yeast cell activity (Jawich *et al.*, 2005) or they can impede yeast development, thus slowing fermentation by limiting cell number or viability (Cabras & Angioni, 2000; Calhella *et al.*, 2006). We then investigated the impact of pesticides on yeast population and viability after 72 hours of fermentation. There were no significant differences in viability and cell population between the conditions with and without pesticides (Table 3) for most strains. In particular, the presence of pesticides had no impact on yeast viability (higher than 90 %) apart from S1.c and NS7.a strains. These observations suggested a direct effect of these xenobiotics

on the yeast fermentative activity. However, the pesticide-induced slight decrease in S1.c viability (from 94.6 % to 89.9 %) and population (from 10.3 × 10⁷ cells/mL to 8.9 × 10⁷ cells/mL) likely contributed to the negative impact on its fermentation performance. Strain NS7.a exhibited the lowest viability under control conditions, suggesting a high sensitivity to ethanol, as is often observed in non-*Saccharomyces* yeast (Borren & Tian, 2021). Surprisingly, this strain displayed higher viability (+19.2 %) and cell population (+6.6 × 10⁷ cells/mL) after 70 hours of fermentation in the presence of pesticides compared to the pesticide-free control. This could be explained by the reduction in fermentative activity triggered by the pesticides (Figure 5). Consequently, compared to the control, ethanol production at 70 hours in the presence of pesticides was lower, thus preventing cell mortality. The NS5.a strain showed low viability in the absence of pesticides (83.9 %) and an even greater reduction in viability in the presence of pesticides (72.7 % viability). This is probably due to poor adaptation to the environment. These decreases in viability may explain the inability of the strain to undergo alcoholic fermentation (> 0.6 g/L of CO₂ released after 70 hours, Figure 5).

Finally, supplementation with pesticides resulted in an increased conversion of sugars to glycerol in the pesticide-sensitive yeasts NS1 and NS7.a (ranging from 10 to 70 %), for which fermentative activity was affected by these compounds.

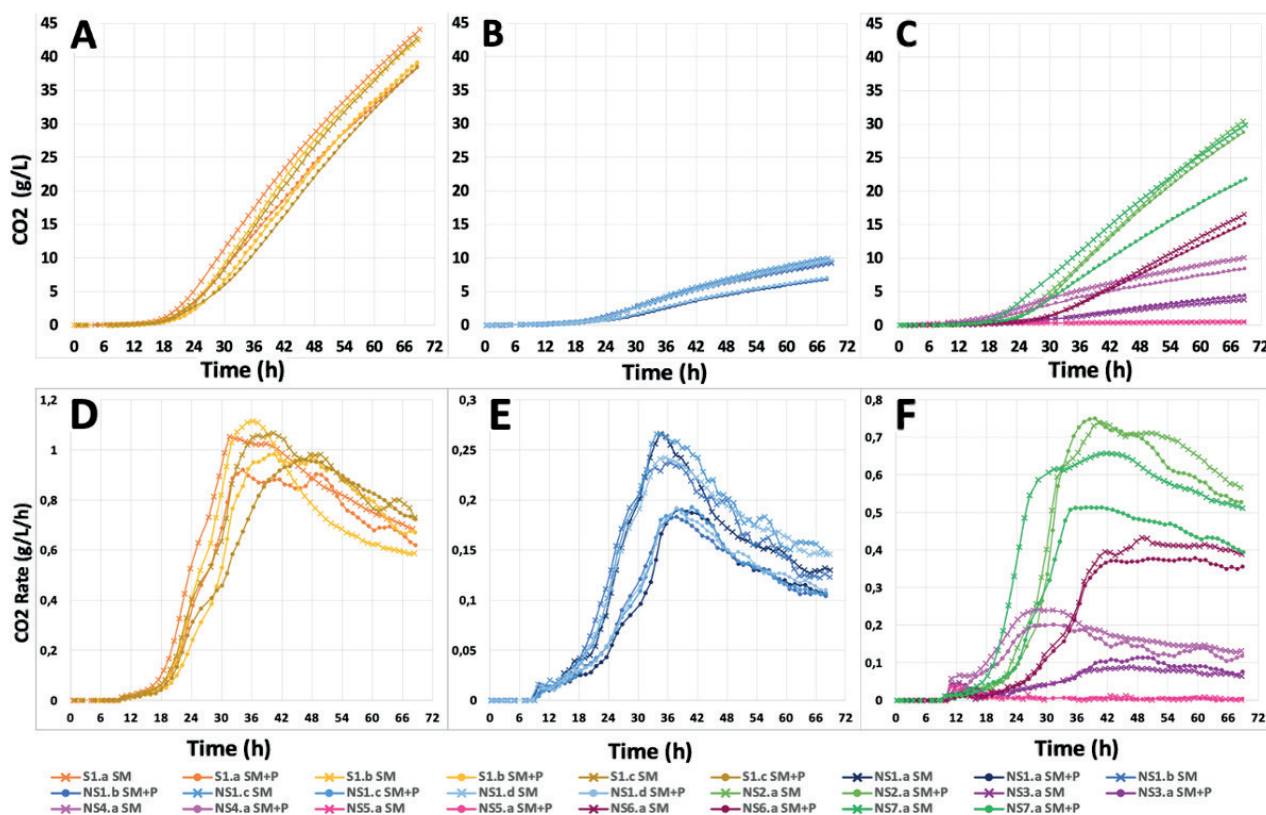


FIGURE 5. Kinetic parameters during fermentation of synthetic must (SM) in the presence (+P) or absence (-) of pesticides, represented in two forms: CO₂ release (g/L) over time (hours) (A–C) and CO₂ release rate (g/L/h) over time (hours) (D–F). The yeasts are grouped in three different graphs by category: *Saccharomyces* yeasts (A, D); non-*Saccharomyces* yeasts of the same species (B, E); all other species non-*Saccharomyces* yeasts (C, F).

TABLE 3. Parameters observed during the fermentation of synthetic must (SM) in the presence (+P) or absence of pesticides: Kinetic parameters such as the maximum rate of CO₂ release (Rmax in g/L/h), the lag time before the start of fermentation (Lag time in h) and population parameters after 70 hours of fermentation, such as the number of cells (Population in cells/mL) or the viability of populations (Viability in %) are shown. The p-values are shown in Table S7.

Strain	Condition	Rmax (g/L/h)		Lag time (h)		CO ₂ max		Population (cells/mL)	Viability (%)
		Means ± SD	Significance	Means ± SD	Significance	Means ± SD	Significance	Means ± SD	Means ± SD
S1.a	SM	1.01 ± 0.05	*	22.0 ± 4.2		40.96 ± 4.35		6.4E+07 ± 1.8E+06	97.6 ± 1.5
	SM + P	0.93 ± 0.03		20.7 ± 0.6		39.11 ± 0.69		6.2E+07 ± 3.0E+06	97.5 ± 0.5
S1.b	SM	1.09 ± 0.01	*	21.1 ± 0.6	*	41.64 ± 2.19		1.1E+08 ± 3.5E+06	97.0 ± 0.7
	SM + P	0.99 ± 0.02		22.8 ± 0.5		39.69 ± 0.57		9.6E+07 ± 5.1E+06	96.8 ± 0.5
S1.c	SM	1.07 ± 0.01	*	21.9 ± 0.4	*	43.51 ± 0.72	*	1.0E+08 ± 6.3E+06	94.6 ± 0.8
	SM + P	0.96 ± 0.01		24.4 ± 0.4		39.14 ± 0.54		8.9E+07 ± 5.3E+06	90.0 ± 0.8
NS1.a	SM	0.27 ± 0.01	*	19.8 ± 0.1	*	9.61 ± 0.28	*	4.1E+07 ± 6.7E+06	98.7 ± 0.3
	SM + P	0.20 ± 0.01		21.7 ± 1.0		7.37 ± 0.51		5.8E+07 ± 8.3E+06	98.8 ± 0.1
NS1.b	SM	0.24 ± 0.01	*	17.9 ± 0.4	*	9.49 ± 0.23	*	2.7E+07 ± 1.4E+06	93.2 ± 0.8
	SM + P	0.19 ± 0.01		20.1 ± 0.6		7.41 ± 0.49		4.5E+07 ± 2.8E+06	96.5 ± 0.5
NS1.c	SM	0.27 ± 0.01	*	19.4 ± 0.0	*	10.35 ± 0.52	*	4.0E+07 ± 3.6E+06	96.8 ± 1.0
	SM + P	0.20 ± 0.02		21.0 ± 1.1		7.57 ± 1.06		5.0E+07 ± 6.4E+06	98.5 ± 0.2
NS1.d	SM	0.25 ± 0.01	*	19.0 ± 0.4	*	9.99 ± 0.29	*	3.3E+07 ± 1.7E+06	97.4 ± 0.3
	SM + P	0.19 ± 0.01		21.3 ± 0.3		7.21 ± 0.19		5.7E+07 ± 8.7E+06	98.8 ± 0.1
NS2.a	SM	0.74 ± 0.01		22.7 ± 0.7	*	31.39 ± 0.85	*	1.4E+08 ± 4.6E+06	99.0 ± 0.4
	SM + P	0.70 ± 0.04		24.1 ± 0.3		27.85 ± 0.84		1.2E+08 ± 4.9E+06	98.2 ± 0.2
NS3.a	SM	0.09 ± 0.00	*	22.5 ± 1.5		3.73 ± 0.17	*	1.4E+08 ± 2.5E+07	98.7 ± 0.1
	SM + P	0.11 ± 0.00		24.3 ± 0.7		4.33 ± 0.11		1.3E+08 ± 5.6E+06	97.7 ± 0.2
NS4.a	SM	0.26 ± 0.02		15.0 ± 1.8		10.53 ± 0.39	*	4.3E+07 ± 7.9E+06	98.6 ± 0.2
	SM + P	0.22 ± 0.02		15.9 ± 1.3		8.98 ± 0.49		4.9E+07 ± 5.9E+06	97.8 ± 0.4
NS5.a	SM	0.03 ± 0.01		21.8 ± 22.0		0.40 ± 0.15		3.6E+06 ± 6.7E+05	83.9 ± 2.7
	SM + P	0.03 ± 0.01		1.9 ± 0.8		0.42 ± 0.03		2.1E+06 ± 4.1E+05	72.7 ± 7.2
NS6.a	SM	0.41 ± 0.01		27.6 ± 0.7		16.49 ± 0.09		1.3E+08 ± 1.3E+07	98.1 ± 0.2
	SM + P	0.39 ± 0.02		27.2 ± 0.8		15.94 ± 0.69		7.7E+07 ± 2.3E+07	98.0 ± 0.1
NS7.a	SM	0.65 ± 0.01	*	18.7 ± 0.3	*	29.89 ± 0.12	*	2.9E+07 ± 6.9E+06	78.7 ± 1.3
	SM + P	0.53 ± 0.02		21.6 ± 1.2		22.69 ± 1.12		9.5E+07 ± 7.9E+06	97.9 ± 0.5

* = p-value < 0.05

In contrast, glycerol production was diminished by 38 % and 25 % in the pesticide-insensitive yeasts NS3.a and NS6.a, respectively (Table S6). Furthermore, the yield of sugar conversion to acetate in the presence of pesticides was decreased in the NS3.a and NS6.a strains, but increased in NS7.a. Acetate plays a fundamental physiological role as the direct precursor to acetyl-CoA. This pivotal intermediate is essential for lipid biosynthesis, encompassing the production of membrane lipid components (Flikweert *et al.*, 1996; Pronk *et al.*, 1996). Consequently, our findings suggest a differential management of the acetate pool between pesticide-tolerant and -sensitive strains. Tolerant strains appeared to preferentially direct acetate towards acetyl-CoA and lipid biosynthesis when exposed to xenobiotics, possibly

to enhance the sequestration of these compounds and better maintain membrane integrity. Furthermore, considering the crucial role of glycerol in cellular physiology as an osmoregulatory compound and in the maintenance of redox homeostasis (Ansell *et al.*, 1997), these findings suggest that the yeasts sensitive to pesticides upregulate glycerol production to counteract compromised osmotic balance regulation resulting from membrane alteration. The NS7.a strain is the only yeast producing lactic acid (5 g/L in the absence of pesticides) (Table S6). Interestingly, the yield of sugar conversion to lactic acid in the presence of pesticides was increased by 20 %, suggesting a metabolic re-routing in this pesticide-sensitive strain.

4. Pesticide detoxification by wine yeasts

To evaluate the pesticide detoxification capacity of the 13 strains, the residual concentration of these 23 compounds was compared after 70 hours of growth in synthetic medium containing the pesticide mix (Table 1). A minimal, chemically defined medium was used to ensure that the observed detoxification capacities were not influenced by potential interactions between the pesticides and polyphenols or solid particles present in more complex natural grape juices. Furthermore, pesticide concentration remained stable during incubation for 10 days at 20 °C without yeasts, reflecting the absence of interaction between pesticides and SM compounds (Table S10).

When considering the totality of pesticides, the total residual concentration was consistently below the initial concentration (0.94 mg/L) for all tested strains (Figure 6), with considerable variability due to the large differences in concentrations for each pesticide (Figure 7). This indicates the capacity of these yeasts to at least partially eliminate these molecules from the synthetic grape juice. However, a substantial variability between strains in this ability was observed. Notably, the three *S. cerevisiae* strains displayed the lowest detoxification capabilities, with residual pesticide levels after 70 hours of fermentation accounting for 69 % to 76 % of the initial concentration. In contrast, the residual pesticide content after non-*Saccharomyces* fermentation was above 65 % of the initial concentration, with the strains NS3.a, NS4.a, and NS1.d demonstrating the highest performance (between 47 and 51 %). A high viability with limited variation between strains was observed after 70 hours of growth (90–99 %; Table 3). Consequently, these minor variations in cell viability are unlikely to account for

the differences in detoxification efficiency. Furthermore, the cell population did not correlate with detoxification capacity. Thus, strains NS4.a, NS1.d, and NS3.a exhibited similarly high levels of detoxification, despite their differing cell concentrations (6.1×10^6 , 5.1×10^7 , and 1.1×10^8 cells/mL, respectively). In addition, strains with comparable cell populations displayed varying detoxification levels. Therefore, inter-strain differences in residual pesticide levels are independent of both cell population and viability. Likewise, no clear correlation was observed between yeast fermentation activity and pesticide detoxification (Figure 5, Figure 7). For example, *S. cerevisiae* strains exhibited the highest CO₂ production after 70 hours but demonstrated the lowest detoxification rates (23–32 % reduction). Conversely, the NS5.a strain produced a low amount of CO₂ (0.46 g/L in 70 hours) yet achieved a moderate detoxification level (35 %).

The comparison of residual levels of each compound after 72 hours of culture revealed that the detoxification efficiency of yeasts also depended on the nature of the pesticide. In general, 17 of the 23 molecules were eliminated by more than 30 % after 70 hours of fermentation by all strains (Figure 7). However, it should be noted that some compounds were only weakly (metalaxyl, methylthiophanate, cyprodinil, pyrimethanil) or not at all (phosphorous acid and folpel) removed from the synthetic must, regardless of the yeast used (Figure 7). Conversely, some pesticides, such as cyazofamid and zoxamide, were strongly detoxified, with residual levels at 72 hours between 0 and 17 % of the initial level. The detoxification profile of other pesticides showed greater variability depending on the strain: residual levels of difenoconazole varied between 32 % (NS4.a) and 100 % (S1.b) of the initial concentration.

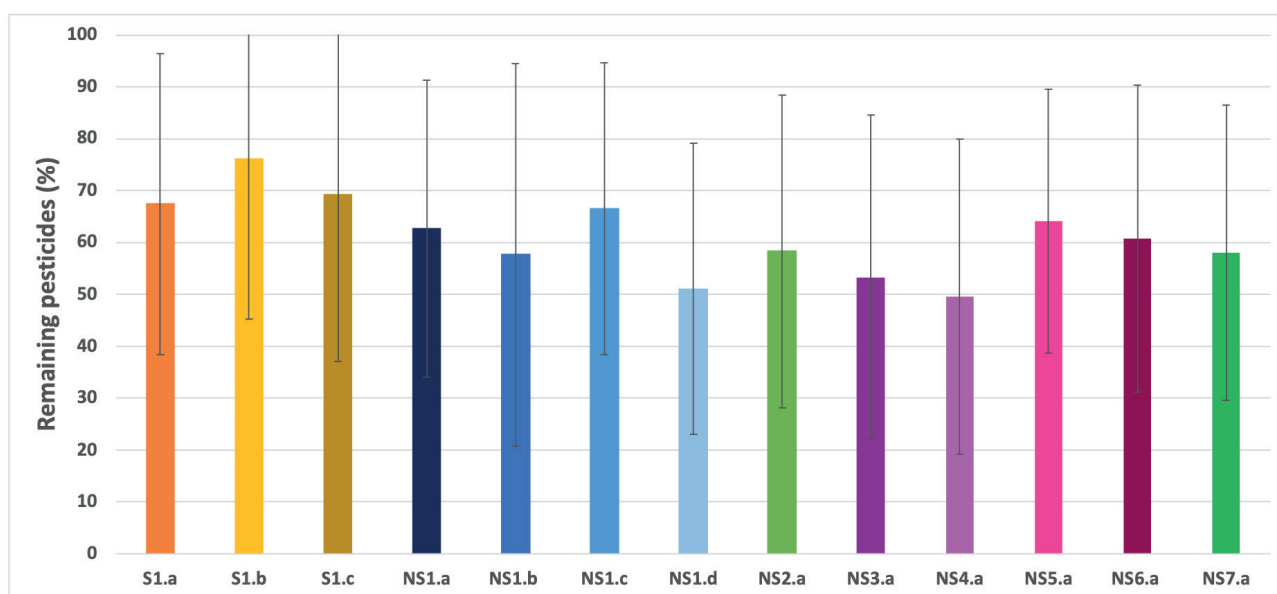


FIGURE 6. Pesticide levels (%) remaining after 70 hours of fermentation with the different strains. The percentage of pesticides remaining is calculated in relation to the quantity of pesticides detected in the control condition (must with pesticides without inoculation). Yeasts are grouped into colour groups by category: *Saccharomyces* yeasts are yellow/orange/brown; non-*Saccharomyces* yeasts of the same species are shown in blue. All other non-*Saccharomyces* species are shown in green/pink/purple.

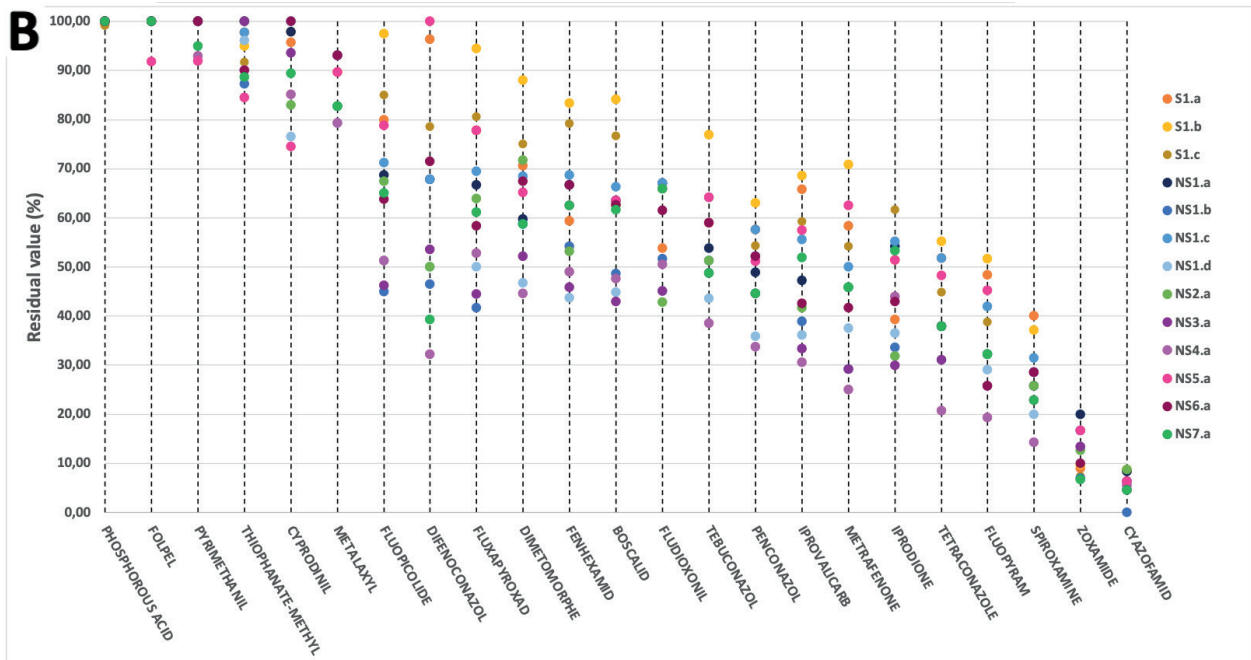
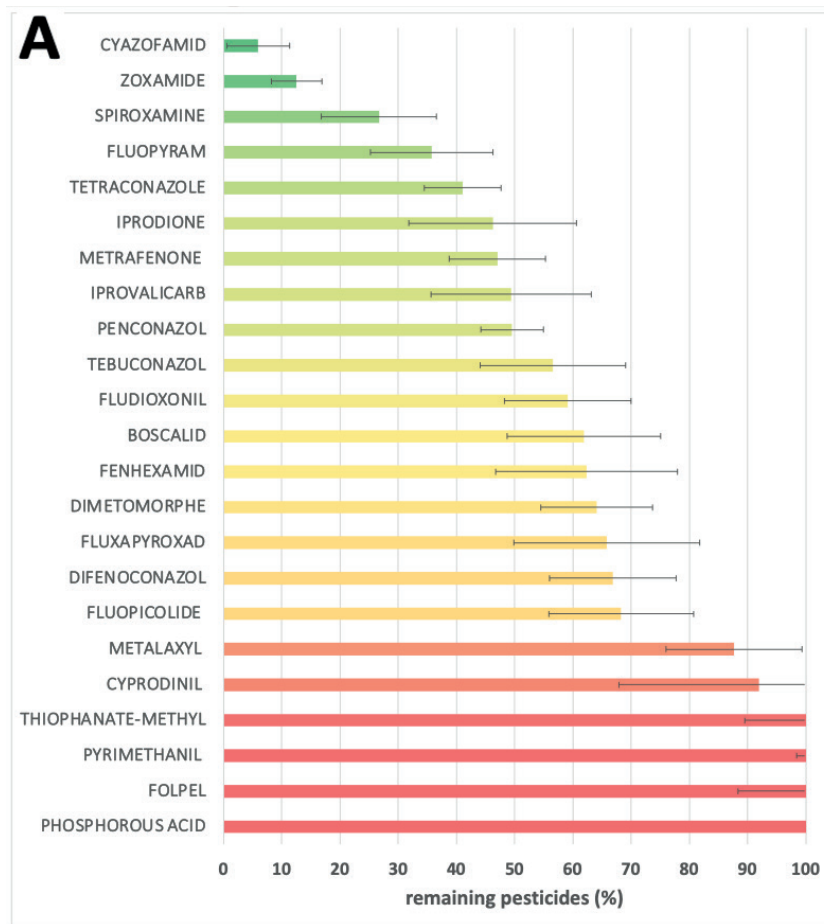


FIGURE 7. (A) Average levels of each pesticide (mg/L) remaining after 72 hours of fermentation for all strains. The percentage of residual pesticides is calculated by comparing the detected quantity to the amount in the control (without yeast). The fact that some levels are higher than 100 % is due to analytical uncertainty, as the concentrations (in mg/L) analysed are low. (B) Pesticide content (or residual value, expressed as %), after 72 hours of fermentation at 20 °C with the different strains tested. For each of the molecules (vertical names) present in the pesticide mix or degradation product. Yeasts are grouped into colour groups by category: *Saccharomyces* yeasts are yellow/orange/brown; non-*Saccharomyces* yeasts of the same species are shown in blue. All other non-*Saccharomyces* species are shown in green/pink/purple.

Furthermore, as a general rule, the classification of the strains based on their detoxification capacity was the same regardless of the pesticide (Figure 7B). However, some strains displayed some specific behaviour. Thus, NS4.a, the most efficient strain at detoxifying nine pesticides, exhibited a low capacity to remove iprodione. Conversely, the strain NS2.a, with moderate global detoxification capacity, showed the highest level of detoxification for fludioxonil (57.1 %).

Finally, intra-specific variability was found in the detoxification capacities of yeasts. In general, *S. cerevisiae* displayed a less efficient detoxification capacity compared to non-*Saccharomyces* yeasts. Notably, however, the strain S1.a occasionally outperformed some non-*Saccharomyces* yeasts, particularly in the removal of fenhexamid, fludioxonil, and iprodione. The NS1 species presented a clear dichotomy in detoxification efficiency. Strain NS1.d consistently demonstrated high activity, ranking among the three with the lowest remaining pesticide levels. Conversely, NS1.c consistently showed lower activity, frequently being among the three strains with the highest residual pesticide content.

Despite the growing global concern regarding pesticide contamination and the potential of bioremediation strategies, the detoxification of pesticides by yeasts remains a comparatively underexplored area within the scientific literature. Research efforts in this field focused on a restricted number of yeast species (*S. cerevisiae* and *M. pulcherrima*) and a limited spectrum of pesticide compounds (Bizaj *et al.*, 2011; Jawich *et al.*, 2005). By utilising 13 yeast strains from eight different species and a mixture of 23 xenobiotics, our approach significantly broadened the knowledge surrounding the promising potential of wine yeasts for pesticide elimination. A key finding was the remarkable capacity of yeast to detoxify a large number of pesticides even in a mixed solution, with 17 out of 23 compounds reduced by 35 % to 99–100 %. However, the efficiency of xenobiotic detoxification was markedly dependent on both the yeast strain employed and the nature of the pesticide.

Notably, non-*Saccharomyces* yeasts exhibit a greater capacity for pesticide reduction compared to *Saccharomyces cerevisiae*, despite the diversity of this phenotype among species and strains. Literature reported that pesticide detoxification by yeast involves a complex interplay of mechanisms, with a key role of interactions between these molecules and the yeast cell wall (Bizaj *et al.*, 2011) and its mannoprotein constituents (Caridi, 2007) in this process. Moreover, the ultimate fate of a pesticide is molecule-dependent, with observations ranging from simple cellular adsorption to absorption followed by intracellular storage or degradation (Caboni & Cabras, 2010; Jawich *et al.*, 2005). Consequently, the observed variations in pesticide detoxification efficiency across different yeast strains might be attributable to the inherent variability in their cell wall composition, particularly concerning the abundance and structure of mannoproteins (Caridi, 2007; Snyman *et al.*, 2023) or in their enzymatic machinery necessary for pesticide breakdown.

Despite examining the detoxification efficiency across a range of pesticides, no straightforward relationship emerged between the chemical attributes of these compounds and the effectiveness of their removal by yeasts (Table S8). First, neither the chemical family nor the molar mass of the pesticide is a reliable predictor of the efficiency of its yeast-mediated detoxification. Thus, the extent of detoxification varied considerably within the same chemical family, as exemplified by the amide compounds, zoxamide (87 % detoxification) and fluopicolide (32 % detoxification). Furthermore, molecular weight did not appear to be a primary determinant of detoxification efficiency (Table S9), as metrafenone and difenoconazole were removed at 53 and 33 % despite their similar molecular weights. Likewise, analysis of pesticide polarity and the presence of specific functional groups or substructures did not reveal a consistent pattern influencing their elimination (Tables S8 and S9). Intriguingly, a single trend emerged, pertaining to halogenated compounds (containing chlorine, fluorine, or bromine), showing a detoxification level above 30 %. Nevertheless, it is important to note, though, that the presence of a halogen moiety is not a prerequisite for effective detoxification, as iprovalicarb and spiroxamine, lacking halogens, exhibited comparable detoxification levels (50.6 % and 73.2 %, respectively). These findings, when considered together, suggest that pesticide detoxification is mediated by diverse mechanisms that are still to be elucidated. Furthermore, differences in detoxification between compounds may be due to the fact that all pesticides are tested simultaneously (in a mixture); different results may be possible if the pesticides are tested separately. Further studies are also needed to explore this hypothesis, and more generally to clarify the mechanisms involved in pesticide detoxification, including the identification of products of potential yeast-mediated degradation of pesticides, and transcriptomic analysis.

CONCLUSION

In summary, this study provides valuable insights into the differential effects of copper and pesticides on wine yeasts from the species *S. cerevisiae*, *M. pulcherrima*, *M. fructicola*, *M. chrysoperlae*, *M. zizyphicola*, *Starmerella bacillaris*, *Torulaspora delbrueckii*, *Lachancea thermotolerans*, *Pichia* sp., and their detoxification capabilities, highlighting the complexity of yeast responses to these common viticultural xenobiotics.

Copper at 7.5 mg/L generally did not impede the growth and fermentation of most yeast strains. Only specific negative impacts were observed on viability and fermentation efficiency in a few strains (S1.c, NS5.a, NS7.a). In contrast, the presence of a pesticide mixture at environmentally relevant concentrations led to a more widespread reduction in fermentation performance across the majority of tested yeasts, in particular of NS strains. This was primarily reflected in decreased CO₂ production and fermentation rates. However, yeast viability was poorly affected.

Most yeast strains, both *S. cerevisiae* and non-*Saccharomyces*, demonstrated a significant capacity to efficiently and irreversibly remove copper from the medium, achieving high levels within 48 hours. The rate of copper elimination was highly strain-dependent and linked to cell metabolic activity. Conversely, the detoxification of the pesticide mixture varied considerably by both yeast strain and the pesticide type. Some pesticides, such as folpel or phosphorous acid, are not eliminated by yeast, while others (zoxamide or cyazofamid) are eliminated by all yeasts. However, the mechanisms underlying detoxification remain unclear, without a clear correlation with pesticide chemical properties. Furthermore, NS yeasts generally exhibited a greater overall pesticide reduction capacity compared to *S. cerevisiae*.

However, the ability of yeast strains to detoxify pesticides did not correlate with their capacity to detoxify copper. For example, *S. cerevisiae* strains showed lower pesticide removal but efficient copper detoxification. Moreover, the most effective pesticide-detoxifying NS strain (NS1.d) was not necessarily the most tolerant or efficient at removing copper (NS1.a, NS1.c). This highlights distinct and potentially independent detoxification mechanisms for these two classes of xenobiotics, which remain to be elucidated.

These findings suggest that selecting specific yeast strains could enhance the bioremediation of copper- and pesticide-contaminated musts, potentially improving wine quality and reducing environmental impact. Further research could explore the physiological mechanisms and genetic basis of these detoxification modes to optimise yeast-based strategies for sustainable viticulture.

ACKNOWLEDGMENTS

We would like to thank the Dubernet laboratories (Laboratoires Dubernet, Narbonne, France) and, in particular, Vincent Bouazza (Dubernet laboratories) for their in-depth discussions and help.

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