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Characterisation of a sulphite-tolerant *Schizosaccharomyces pombe* strain: potential as spoilage in oversulphited must and as starter cultures in wine

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ABSTRACT

This study characterises a novel *Schizosaccharomyces pombe* (*Sc. pombe*) strain SP2 isolated from a winery where alcoholic fermentation has been prevented by sulphiting to obtain preserved grape must, in which spontaneous malo-alcoholic fermentation occurs despite high sulfur dioxide (SO_2) concentrations. This work demonstrated that the same yeast strain can have a dichotomous relevance in the same agri-food chain: a spoilage agent in preserved grape must and ii) as a candidate starter culture for wine production. In the study, levels of tolerance to SO_2 in the simulations of selective pressure exerted by this bacteriostatic agent are compatible with the phenomenon observed in the winery, demonstrating the spoilage potential of the strain in the preserved must industry. The selection of a highly resistant fission yeast strain highlights the importance of studying food niches to improve our understanding of evolutionary phenomena and phenotypic variability in model microorganisms. Fermentation trials in synthetic musts, white and red wines, contribute to assess the yeast behaviour under monoculture, co-inoculation, and sequential inoculation with *Saccharomyces cerevisiae*. *Sc. pombe* monocultures lagged in fermentation kinetics and produced more acetic acid, especially in red musts. Sequential and co-inoculation contributed to enhancing volatile profiles, especially esters and higher alcohols, contributing to superior aromatic complexity as illustrated by multivariate analysis. Sequential inoculation, in particular, enhances the production of key esters and alcohols, enabling efficient malic acid degradation with low acetic acid production. The results also underscore that the matrix type (synthetic must, red, or white wine) interacts strongly with the inoculation strategy in shaping the volatile composition.

1. Introduction

Yeasts represent the most important group of microorganisms to winemakers as they have a predominant role in oenology (Berbegal et al., 2017). They are commonly classified as *Saccharomyces* and non-*Saccharomyces*. Between them, non-*Saccharomyces* yeasts form a heterogeneous group representing several genera/species (Roudil et al., 2020), which can affect overall wine quality to varying extents (Tufariello et al., 2021) and have long been the subject of debate due to their protechnological features (Wang et al., 2023). In fact, non-*Saccharomyces* yeasts can contribute either positively or negatively to the sensory profile of wines, depending on several factors (Padilla et al., 2016).

Among these, *Schizosaccharomyces pombe*, traditionally considered a spoilage yeast, has been receiving increasing attention for protechnological applications (Á. Benito et al., 2018). The fission yeast *Sc. pombe*, together with *Saccharomyces cerevisiae*, not only shares the ecological niche associated with oenology but also shares the relevance as a crucial model organism for the study of eukaryotic and molecular biology (Wood et al., 2002). Studies on *Sc. pombe* have contributed to significant advances in cell biology, including the elucidation of biological mechanisms and gene functions, such as those associated with DNA replication, transcription, translation, and signal transduction (Jeffares et al., 2015). *Sc. pombe* received distinct interest for its particular metabolism,

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which gives it specific abilities, such as decreasing the acidity of wine through malo-alcoholic fermentation (Loira et al., 2015). The biological deacidification of wine is possible thanks to the capacity of this yeast species to consume the malic acid present in the grape must or wine with the corresponding stoichiometric production of ethanol and CO_2 (Loira et al., 2018). The use of *Sc. pombe* is becoming a valuable new tool to reduce malic acid in wines as an alternative to the malolactic fermentation carried out by lactic acid bacteria, especially in wines that are less permissive to the development of malolactic bacteria after alcoholic fermentation (Loira et al., 2018). Commercial strains are available nowadays, influencing the acidity and aromatic compound profiles of the resulting wines (Vicente et al., 2023).

On the other hand, *Sc. pombe* is also considered a spoilage yeast due to the production of specific *off*-flavours commonly associated with its metabolism (Á. Benito et al., 2018). *Sc. pombe* is frequently isolated from wines with organoleptic and chemical deficiencies through the presence of acetic acid, acetaldehyde, acetoin and ethyl acetate.

Remaining in the field of spoilage phenomena in oenology, little has been explored in the scientific literature on preserved grape musts. In the 'International Code of Oenological Practices' the 'International Organisation of Vine and Wine' defined a 'Preserved Grape Must' as a "Fresh grape must whose alcoholic fermentation has been prevented" (ethanol tolerated with a limit of 1 % vol) and include 'sulfiting' as one of the main strategies to obtain this kind of product (Delogu et al., 2022). In effect, adding SO_2 in high doses is one of the simplest ways to preserve a must at low energy costs and kill microorganisms. This category of product, called 'mute' must in Italy (Anfossi et al., 2012), finds various uses, such as deseasonalised oenological fermentations and the increase of sugar and other oenological properties in musts that need to be improved (when legally allowed) (Delogu et al., 2022). In this type of product, the development of microorganisms, in particular fermentative ones capable of producing ethanol, represents spoilage organisms that influence the management and quality of the product.

This study aims to characterise a new strain of *Sc. pombe* isolated as a spoilage microorganism from Apulian preserved grape must, which contains high amounts of SO_2 (approximately 3 g/L) and in which this strain has developed malo-alcoholic fermentation. Besides, to evaluate the potential use of this *Sc. pombe* strain in a single culture or in combination with *S. cerevisiae* during vinification in a red and a white must from the Puglia region, focusing mainly on the consumption of malic acid, the production of acetic acid and the formation of volatile compounds.

2. Material and methods

2.1. Yeasts strains

The *Schizosaccharomyces pombe* strain SP2 was isolated in the cellar from Apulian red grape must during the vintage 2017. The grape must, preserved by adding a high concentration of potassium bisulfite (about 3 g/L), had suffered the malo-alcoholic fermentation after. The *Sc. pombe* strain SP2 is available in the Industrial Microbiology Laboratory (DAFNE Department, University of Foggia) collection. The isolate was identified according to the length of the rDNA region spanning the 5.8S rRNA gene and flanking the internal transcribed spacers 1 and 2. The amplicon was subjected to sequence analysis and compared with the sequences in the GenBank database to determine species identity. The *Sc. pombe* strain (denoted as SP2) was grown in YPD at 28 °C, 72 h until the end of the exponential phase, and then the yeast culture and sterile glycerol at 30 % (v:v). The *Saccharomyces cerevisiae* DV10 (Lallemand, USA) was also used to promote alcoholic fermentation during experimental vinifications.

2.2. Evaluation of pH and SO_2 tolerance in synthetic must

Sc. pombe was pre-grown in YPD broth to the early stationary phase. An inoculum consisting of 1×10^6 CFU/mL was used to inoculate synthetic must (100 g/L glucose, 100 g/L fructose, 1 g/L yeast extract, 2 g/L $(NH_4)_2SO_4$, 0.3 g/L citric acid, 5 g/L L-malic acid, 5 g/L L-tartaric acid, 0.4 g/L $MgSO_4$, 5 g/L KH_2PO_4, 0.2 g/L NaCl, 0.05 g/L $MnSO_4$) (Rossouw et al., 2012), testing pH values of 3.0, 3.2, 3.4, 3.6 and 3.8, supplemented with total SO_2 at 400, 1000, 1300, 1700 and 2200 mg/L which corresponded approximately to the free SO_2 concentrations of 150, 350, 540, 680 and 880 mg/L respectively (Table S1). Synthetic must represents a representative model solution of must chemistry, formulated to facilitate the reproducibility of research in the field of wine microbiology. Fermentations were carried out at 25 °C, and their kinetics were monitored daily for 10 days, depicted as cumulative weight loss. Each fermentation experiment was carried out by performing three simultaneous and independent tests. Microbial growth was monitored by turbidimetry, measuring the optical density (OD) at 600 nm.

2.3. Growth in red grape must at high SO_2 concentration

Fermentations were undertaken using must from *Uva di Troia* grapes (21° Babo; 7.2 g/L total acidity; 2.57 g/L malic acid; pH 3.78). The must was centrifuged (1000 g, 3 min, 4 °C) and filtered twice through cheesecloth and a 0.22 μm membrane filter, and then combined with total SO_2 concentrations of 400, 1000, 1300, 1700, and 2200 mg/L. *Sc. pombe* SP2 and *S. cerevisae* DV10 were previously grown in YPD medium and inoculated in independent fermentation trials of 25 mL with an initial population of 10^4 CFU/mL. Fermentations were carried out at 25 °C, and their kinetics were monitored daily for 8 days by measuring the weight loss until the weight remained constant.

2.4. Alcoholic and malo-alcoholic fermentations in different musts by *S. cerevisiae* and *Sc. pombe* inoculations

The *Sc. pombe* SP2 and *S. cerevisiae* DV10 were tested by microfermentation assays conducted in different grape musts: red grape must (*Nero di Troia*) (22° Babo; 6.2 g/L malic acid; pH 3.6), white grape must (*Bombino Bianco*) (10° Babo; 0.9 g/L malic acid; pH 3.2) and, in synthetic must (pH 3.5) (Rossouw et al., 2012). Red and white grape musts were centrifuged (1000 g, 3 min, 4 °C) and filtered twice through cheesecloth and a 0.22 μm membrane filter and then were combined with 100 mg/L potassium metabisulphite. Grape must was placed in sterile Erlenmeyer 150-ml flasks and then inoculated with different microbial resources. In particular, four inoculation strategies were applied to inoculate in triplicate 100 mL for each different must: (*i*) inoculation of *Sc. pombe* (1×10^6 CFU/mL), (*ii*) inoculation of *S. cerevisiae* (1×10^6 CFU/mL), (*iii*) co-inoculation of *Sc. pombe* (1×10^6 CFU/mL) and *S. cerevisiae* (1×10^6 CFU/mL) and (*iv*) sequential inoculation of *Sc. pombe* (1×10^6 CFU/mL) and 48 h later on *S. cerevisiae* (1×10^6 CFU/mL) (Table 1). All fermentation trials were carried out at 25 °C, and their kinetics were monitored daily. Samples of must and wines were collected for further chemical and microbiological analysis.

2.5. Determination of microbial population

Plating cultures assessed the viable count of yeasts during the fermentation trials onto WL agar medium (Sigma-Aldrich, USA) at 28 °C for 72 h. This medium allowed discrimination between yeast species because of their specific colony morphology and colour (large white colonies for *S. cerevisiae,* green colonies for *Sc. pombe*).

2.6. Analytical determinations

L-malic acid and acetic acid were determined by enzymatic kits (Biogamma, Italy). The enzymatic method of the commercial assays was based on the enzymatic conversion of the analyte and the stoichiometric formation of NADH, whose absorbance is measured at 340 nm. Start pipetting 2 mL of the first kit reagent into each cuvette. Add 100 μL

Table 1
Microorganisms employed and inoculation approach in the different grape must fermentations.

Sample code	Must	Inoculated strain	Inoculation approach
W1	White	*S. cerevisiae* DV10	Monoculture
W2		*S. cerevisiae* DV10 + *Sc. pombe*	Co-inoculation
W3		*Sc. pombe* + *S. cerevisiae* DV10	Sequential inoculation
W4		*Sc. pombe*	Monoculture
R1	Red	*S. cerevisiae* DV10	Monoculture
R2		*S. cerevisiae* DV10 + *Sc. pombe*	Co-inoculation
R3		*Sc. pombe* + *S. cerevisiae* DV10	Sequential inoculation
R4		*Sc. pombe*	Monoculture
M1	Synthetic	*S. cerevisiae* DV10	Monoculture
M2		*S. cerevisiae* DV10 + *Sc. pombe*	Co-inoculation
M3		*Sc. pombe* + *S. cerevisiae* DV10	Sequential inoculation
M4		*Sc. pombe*	Monoculture

distilled water (blank) or 100 μL wine sample (test), mix, incubate for 3 min at 25 °C, and read absorbance (spectrophotometer) at 340 nm (A_1). Add 500 μL of the second kit reagent in each cuvette, mix, incubate for 15 min at 25 °C, and read absorbance in a spectrophotometer at 340 nm for each cuvette (A_2). Calculations have been performed using A_1 and A_2 values, according to the manufacturer's instructions. The principal constituents of wine and must during fermentation (ethanol, pH, tartaric acid, citric acid, volatile acidity, total acidity, glycerol, and SO_2) were analysed using Fourier Transform Infrared Spectroscopy (FTIR) with a WineScan Flex instrument (FOSS Analytical, Denmark). Prior to analysis, samples were centrifuged at 8000×*g* for 10 min and subsequently processed according to the manufacturer's guidelines.

2.6.1. Characterisation of organic volatile compounds

The procedure for both qualitative and quantitative determination of volatile compounds in wine samples employed headspace solid-phase microextraction coupled with gas chromatography–mass spectrometry (HS-SPME–GC–MS), and was adapted from the method described by Kong et al. (2019) with minor modifications. Eight milliliters of supernatant were transferred into a 20 mL headspace vial containing 40 μg/L of the internal standard (2-octanol) and 2 g of NaCl. The vial was hermetically sealed and incubated at 40 °C for 15 min on a heating block. Afterwards, the conditioned solid-phase fiber was inserted into the vial headspace at 40 °C for 30 min until equilibrium was achieved. The fiber was then thermally desorbed in the GC injector for 5 min at 230 °C. The DVB/CAR/PDMS (divinylbenzene/carboxen/polydimethylsiloxane) fiber was obtained from Supelco Corporation (Bellefonte, PA, USA).

Extraction of each sample was performed in triplicate. GC/MS was performed using an Agilent 6890 chromatograph equipped with a split/splitless injector (Agilent Technologies, Folsom, CA, USA), a J&W DB-Wax column (60 m length*0.25 i.d.* 0.25 film thickness; J&W Scientific, Folsom, CA), and 5973 Network series quadrupole mass spectrometric detector (Agilent Technologies, Folsom, CA, USA). The temperature program used was 40 °C for 3 min, raised at 4 °C min-1 to 220 °C, and held for 20 min at maximum temperature. Electron impact mass spectra were recorded with an ion source energy of 70 eV. A 1 μL aliquot of each concentrated extract was injected in splitless mode. Volatile compound identification was performed by comparing retention times and mass spectra obtained by analysing pure reference compounds under the same conditions. The identification was further confirmed by comparing mass spectra with those of the NIST database. Compounds for which pure reference standards were unavailable were tentatively identified based on mass spectra comparison. Semi-quantification of the volatile compounds by GC-MS was obtained using an internal standard method.

2.7. Statistical analysis

Principal component analysis based on Pearson correlation (n-1), i. e., applied to standardised data, was applied to volatile compounds concentrations identified in all samples. Both score plots and correlation circles, which report Pearson correlation coefficient values between scores along a specific PC and values of each individual variable, were considered, and the statistical significance of these correlations was assessed through the corresponding p-values. For each volatile compound, a two-way ANOVA with interaction was also performed in Matlab, considering wine type and treatment (inoculum strategy) as factors, including evaluation of p-values and the coefficient of determination (R^2), which represents the proportion of variance explained by the model. Statistical significance was considered at a threshold of p-value <0.05.

PCA and cluster heatmap were carried out using Matlab software.

3. Results

3.1. Evaluation of pH and SO_2 tolerance in synthetic and red wine grape must

Schizosaccharomyces pombe SP2 was isolated from a preserved grape must in which the alcoholic fermentation was prevented by a high concentration of SO_2. Under conditions that are weakly permissive for microbial growth, the company reported slight ethanol production and malic acid consumption. Indeed, following sampling under aseptic conditions and isolation in pure culture, the strain under study was isolated. Considering the hostile conditions for the development of microorganisms due to the high doses of SO_2, it was considered interesting to understand the spoilage potential of this strain, precisely because of its tolerance to sulphites. In order to study the resistance to SO_2 in terms of growth and fermentation capacity, the strain was inoculated into synthetic must with high concentrations of this antimicrobial. Considering that its antiseptic property depends on the pH of the media, a set of different pH values was also tested. The growth kinetics of the yeast populations are shown in Fig. 1. It was observed that a higher concentration of total SO2 affected growth, particularly with a lowering of pH. At pH levels of 3.8 and 3.6, this strain grew with high concentrations of total SO_2, from 400 to 2200 mg/L. At pH 3.4, a lag phase of 2 days was observed for 1700 and 2200 mg/L SO_2 concentrations, and at pH 3.2, the lag phase increased until 6 days after a total concentration of 2200 mg/L was added to the medium. The sharpest differences in growth were found when the yeast was inoculated at pH 3.0. Under these conditions, with the highest concentration of SO_2, *Sc. pombe* SP2 was not able to develop. Growth results were related to the fermentation capacity. As can be seen in Fig. 2 (with more details about statistically significant differences in Table S2), the progressive weight loss was slowed down when the SO_2 concentration was higher. This effect was observed more markedly when the synthetic must presented a pH of 3.2. Regarding the pH 3.0 level, the results showed that with 1700 and 2200 mg/L of total SO2 concentrations, the strain could not carry out the fermentation since there was no weight loss. These results show the towering resistance of this strain to high concentrations of SO_2, even at very low pH levels. With the aim of evaluating the ability of *Sc. pombe* to carry out the malo-alcoholic fermentation, L-malic acid was measured after 3 days of inoculation (Table 2) and at the end of the vinification (10 days). It was found that after 3 days, only pH 3.2 with 2200 mg/L of SO_2 and pH 3.0 with 1300, 1700, and 2200 mg/L of SO_2, more than 0.5 g/L of L-malic acid was left. After 10 days of incubation under the most extreme conditions (pH 3.0 and 2200 mg/L of SO_2), the strain was unable to grow, and 1.85 g/L of L-malic acid was observed. Under this last condition, less than 0.1 g/L of L-malic acid was detected in the other trials. The

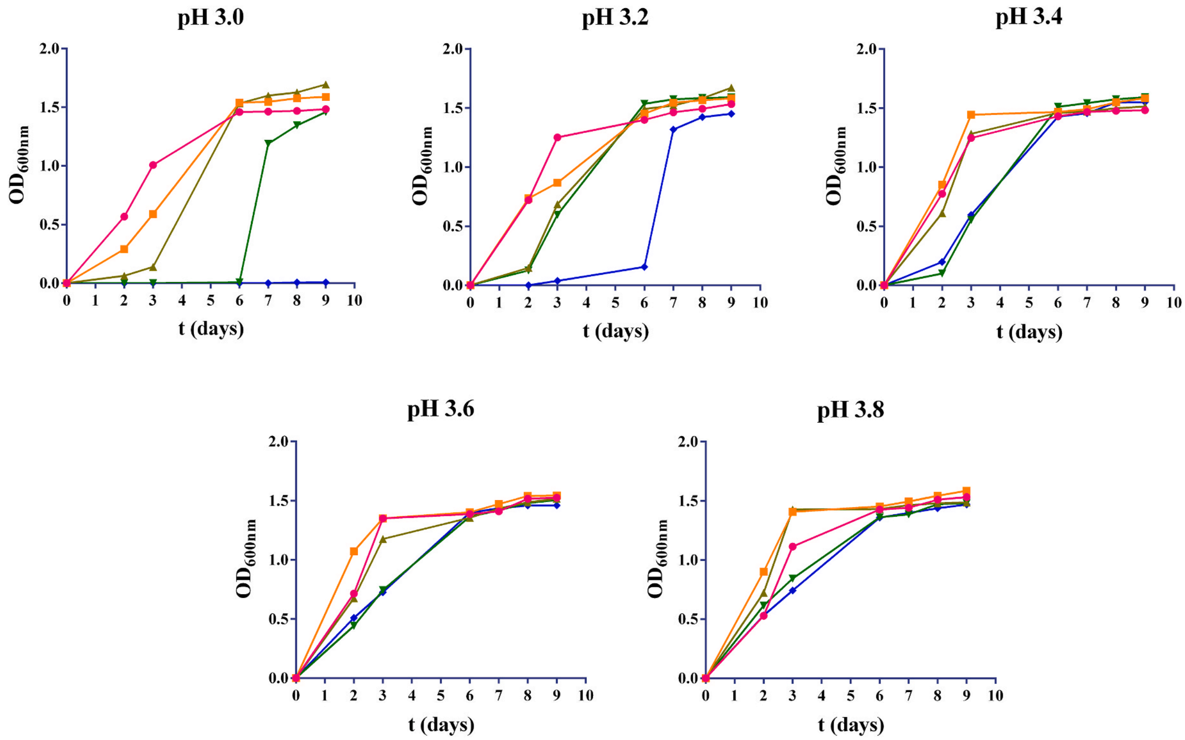

Fig. 1. Growth (OD_{600nm}) of *Sc. pombe* strain in synthetic must at pH 3.0, 3.2, 3.4, 3.6 and pH 3.8, supplemented with a total SO_2 concentration of 400 mg/L (●), 1000 mg/L (■), 1300 mg/L (▲), 1700 mg/L (▼), and 2200 mg/L (◆).

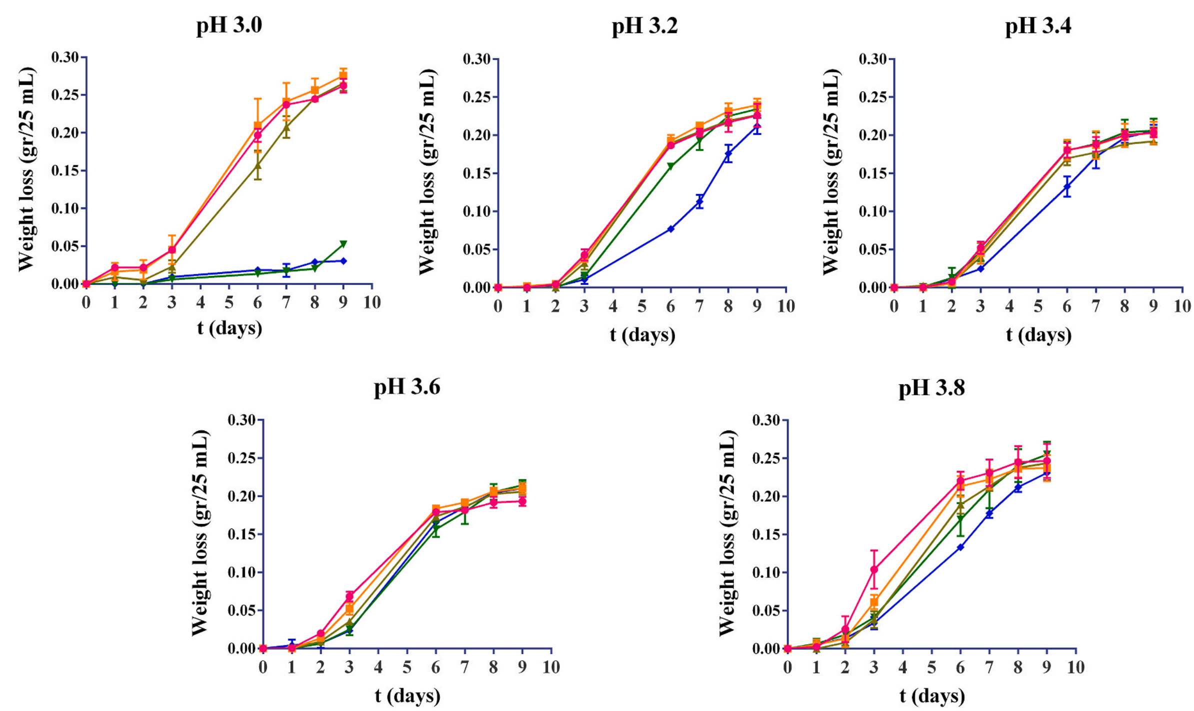

Fig. 2. Weight loss during vinification (g/25 mL) of *Sc. pombe* strain in synthetic must at pH 3.0, 3.2, 3.4, 3.6 and pH 3.8, supplemented with a total SO_2 concentration of 400 mg/L (●), 1000 mg/L (■), 1300 mg/L (▲), 1700 mg/L (▼), and 2200 mg/L (◆).

production of metabolites such as acetic acid followed the kinetics of growth (Table 3). It was almost undetectable in media with 2200 mg/L of total SO_2 and with pH levels of 3.0 or 3.2, where growth was not detected. The maximum acetic acid concentrations were reported in synthetic must at pH 3.8. Nevertheless, the acetic acid production never achieved levels over 1 g/L, a concentration associated with a negative impact on wine flavour. The *Sc. pombe* SP2 SO_2 resistance was evaluated in real vinification conditions. The yeast strain was inoculated in red grape must supplemented with total SO_2 at 400, 1000, 1300, 1700, and 2200 mg/L, and its behaviour was compared to *S. cerevisiae* DV10 (Fig. 3). *Sc. pombe* was able to grow in all cases, reaching a population greater than 10^8 CFU/mL in 8 days. In contrast, only *S. cerevisiae* was

Table 2
Concentration of L-malic acid after 3 days of *Sc. pombe* strain inoculation in synthetic must at pH 3.0, 3.2, 3.4, 3.6 and pH 3.8, supplemented with a total SO_2 concentration of 400 mg/L, 1000 mg/L, 1300 mg/L, 1700 mg/L, and 2200 mg/L.

pH	L-malic acid (g/L)				
	400 mg/L SO_2	1000 mg/L SO_2	1300 mg/L SO_2	1700 mg/L SO_2	2200 mg/L SO_2
3.0	0.366 ± 0.006	0.430 ± 0.051	1.901 ± 0.784	6.005 ± 1.244	6.145 ± 0.633
3.2	0.364 ± 0.010	0.355 ± 0.026	0.400 ± 0.054	0.377 ± 0.027	2.087 ± 0.122
3.4	0.327 ± 0.008	0.387 ± 0.029	0.355 ± 0.020	0.450 ± 0.098	0.408 ± 0.059
3.6	0.398 ± 0.075	0.404 ± 0.102	0.399 ± 0.031	0.512 ± 0.079	0.476 ± 0.096
3.8	0.405 ± 0.006	0.376 ± 0.023	0.343 ± 0.021	0.447 ± 0.026	0.375 ± 0.077

Table 3
Concentration of acetic acid after 10 days of *Sc. pombe* strain inoculation in synthetic must at pH 3.0, 3.2, 3.4, 3.6 and pH 3.8, supplemented with a total SO_2 concentration of 400 mg/L, 1000 mg/L, 1300 mg/L, 1700 mg/L, and 2200 mg/L.

	Acetic acid (g/L)				
	400 mg/L SO_2	1000 mg/L SO_2	1300 mg/L SO_2	1700 mg/L SO_2	2200 mg/L SO_2
pH 3.0	0.631 ± 0.043	0.642 ± 0.100	0.453 ± 0.097	0.268 ± 0.102	0.028 ± 0.039
pH 3.2	0.455 ± 0.146	0.440 ± 0.137	0.546 ± 0.014	0.308 ± 0.152	0.140 ± 0.138
pH 3.4	0.449 ± 0.056	0.395 ± 0.112	0.434 ± 0.048	0.359 ± 0.057	0.386 ± 0.069
pH 3.6	0.567 ± 0.077	0.633 ± 0.021	0.495 ± 0.143	0.416 ± 0.135	0.479 ± 0.047
pH 3.8	0.625 ± 0.117	0.792 ± 0.030	0.580 ± 0.055	0.596 ± 0.052	0.649 ± 0.003

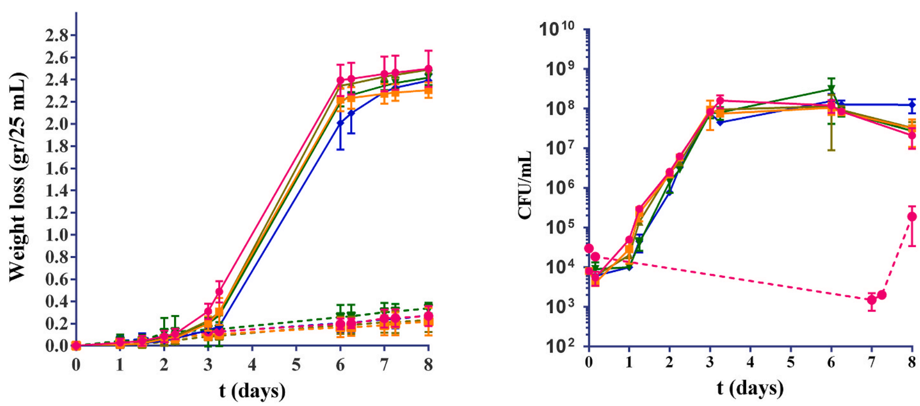

Fig. 3. (A) Weight loss (g/25 mL) and (B) growth (CFU/mL), of *Sc. pombe* (–) and *S. cerevisie* DV10 (—) in Primitivo red must supplemented with a total SO_2 concentration of 400 mg/L (●), 1000 mg/L (■), 1300 mg/L (▲), 1700 mg/L (▼), and 2200 mg/L (◆).

detected when the total SO_2 concentration was 400 mg/L, and after 8 days of incubation, no weight loss was observed in the samples in which *S. cerevisiae* had been inoculated. These results showed a significant difference in the resistance to SO_2 between the two tested *S. cerevisiae* and *Sc. pombe* strains, and they made the resistance capacity of *Sc. pombe* SP2 at towering concentrations of SO_2 relevant.

3.2. Alcoholic fermentation and malo-alcoholic fermentation in red, white, and synthetic musts

In light of the dual character of the species in oenology and considering that spoilage conditions have been detected in preserved grape must, we proceeded to evaluate the protechnological properties of the strain in wine production. Fig. 4 shows the weight loss over the

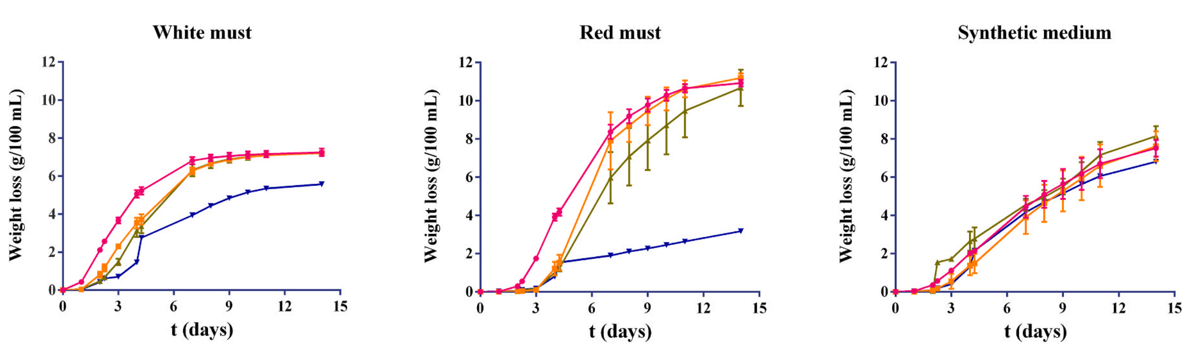

Fig. 4. Weight loss (g/100 mL) of the different fermentation trials in White (W), Red (R) and Synthetic (M) must following four inoculation approaches: (●) monoculture of *S. cerevisiae* DV10, (■) co-inoculation of *Sc. pombe* and *S. cerevisiae* DV10, (▲) sequential inoculation of *Sc. pombe* and *S. cerevisiae* DV10 and, (▼) monoculture of *Sc. pombe*.

fermentation period. The fermentation kinetics were different in the three musts as they presented very different initial conditions. White grape must exhibits a low pH and lower sugar concentrations compared with red grape must, which presented 22 °babo and a pH of 3.7. The lower concentration of initial sugars in the white must causes a lower final weight loss than in the red must. Considering all the evidence that includes *S. cerevisiae* DV10, the alcoholic fermentation ended approximately on the 7th day and the 14th day, when the yeast was inoculated in white and red must, respectively. In both grape musts, the fastest fermentations were carried out when *S. cerevisiae* was inoculated as a monoculture in W1 and R1 fermentation trials, followed by the combinations of *S. cerevisiae* DV10 and *Sc. pombe* SP2 (co-inoculated or in sequential inoculation). The fermentation kinetics of *Sc. pombe,* when inoculated in monoculture (W4 and R4), displayed a lag phase and never reached the loss of weight that trials with *S. cerevisiae* accomplished, and the differences between both yeasts were more pronounced in red must. On the other hand, the synthetic medium was less restrictive for *Sc. pombe* fermentation activity, and in the four trials, a very similar behaviour was observed. After 15 days of incubation, the final content of L-malic and acetic acid was analysed in all fermentation trials. The results of the different inoculation modes in white grape must show that when *Sc. pombe* was inoculated in monoculture (W4) or in combination with *S. cerevisiae* (W2 and W3), all malic acid was consumed, and it remained intact when *S. cerevisiae* was inoculated in monoculture form (W4). This was probably due to the high viability of *Sc. pombe*, when inoculated in white, must (Figure S1, Fig. 1). Although white grape must exhibits a low pH, sugars and L-malic acid concentrations were lower than red must, causing a rapid consumption of the L-malic acid in these fermentation trials. The content of L-malic acid was similar in the three fermentation trials where *Sc. pombe* was inoculated in red must. Although not all L-malic was consumed, there was three times less than in the fermentation trial inoculated with *S. cerevisiae* DV10. In the fermentation trials M3 and M4, no malic acid was detected, while 1.5 g/L and nearly 4 g/L were found in M2 and M1, respectively. In this case, differences in cell viability were found between M2 and M3 (Fig. S1, Fig. 1). When *Sc. pombe* and *S. cerevisiae* were sequentially inoculated, the lower concentrations of malic acid and acetic acid were detected compared to those obtained when the two species were co-inoculated. None of the fermentation trials detected acetic acid at a concentration higher than 1 g/L. In wines obtained from red and white musts, this compound's highest concentration was observed in the wine produced by the inoculation of *Sc. pombe* SP2 alone (R4 and M4). When the synthetic must was inoculated, the lowest concentration of acetic acid was found in M3.

The principal chemical parameters of the wines obtained by sequential inoculation were analysed by FT-IR one month after the end of the alcoholic fermentation (Table 4). Because the three musts were very different, the final parameters of the wines differed, as the musts presented distinct chemical characteristics, and the final products exhibited quantitative differences in the analysed parameters. Thus, R3 presented a higher degree of final ethanol than the wines obtained with white grape juice and synthetic grape juice. The final pH that was obtained was also higher, 4.47, compared to 3.44 and 3.61. In all the obtained fermented musts, volatile acidity, expressed as acetic acid, was quite low, ranging from 0.22 to 0.36 g/L. The differences in glycerol concentrations were also evident due to the variations in initial sugar concentrations. The primary purpose of the volatile compound analysis was to assess the contribution of each strain and the matrix type to the aromatic quality of the wine. A total of 30 different compounds were identified in the wine samples, as reported in Table 5a. The two-way analysis with interaction applied to volatiles is presented in Table 5b. Alcohols, esters, and acids were the main groups of volatiles identified. Higher concentrations of alcohols were predominantly observed in the experimental trials where both yeast strains (*S. cerevisiae* DV10 and *Sc. pombe* SP2) were present—either through co-inoculation (31.30 mg/L) or sequential inoculation (31.16 mg/L) (specifically, trials 3 and 4, respectively). In addition, alcohol production was greater in red and white wines compared to the must, highlighting the significant influence carried out by the fermentation matrix itself. Higher production of this compound was observed under co-inoculation conditions across all three matrices studied, with concentrations of 3.64 mg/L in red wines, 5.98 mg/L in white wines, and 5.93 mg/L in must, respectively. Esters, representing the second most abundant class of metabolites, were detected in higher concentrations under co-inoculation conditions in red wines (10.50 mg/L) and white wines (11.75 mg/L). Lower amounts were found in the must, with values ranging from 0.73 mg/L (M1) to 2.53 mg/L (M2). The acid content was especially high in white wines, ranging from 3.33 mg/L (trial W4) to 8.17 mg/L (W3), and the comprehensive evaluation of the impact of the factors (matrix and fermentation strategy) and their interaction on volatile compounds was obtained by applying multivariate analysis.

The two-way ANOVA results (Table 5b) show that most volatile compounds exhibit statistically significant differences between inoculation strategies, with 27 out of 30 compounds reaching significance at $p \leq 0.05$ or lower. In detail, strong effects were observed for key aroma-active compounds, including ethyl butanoate, isoamyl acetate, ethyl hexanoate, ethyl octanoate, diethyl succinate, phenylacetate, phenyl ethanol, 4-vinylguaiacol, furaneol, 2-methyl hexanoic acid, hexanoic acid, octanoic acid, terpinol-4-ol, α-terpineol, and 1-hexanol, all showing $p \leq 0.001$. The analysis also reveals significant matrix effects for most compounds and, importantly, significant matrix × inoculation strategy interactions for 26 compounds, indicating that the effectiveness of co-inoculation versus sequential inoculation varies depending on the wine matrix.

Fig. 5 reports the 3D principal components analysis (PCA). The PCA analysis was performed to explore the relationships between volatile compounds and the different fermentation strategies (1–4) applied across three matrices: must (M), white wine (W), and red wine (R). Fig. 5 shows the results of the PCA applied to the standardised concentrations. The 3D plot in Fig. 5a shows the scores of the different samples for the first three principal components. The three principal components (PC1, PC2, and PC3) explain 69.3 % of the total variance (35.2 % for PC1, 20.2 % for PC2, 13.9 % for PC3). On the other hand, the 3D correlation plot in Fig. 5b shows, for each molecule, the correlation values between the concentration and the three latent variables PC1, PC2, and PC3. The correlation plot represents the variables (volatile compounds) as vectors. The length and direction of each vector indicate the correlation of that compound with the first three principal components. The unit correlation circle for the first two principal components is shown as a visual reference. In particular, Table S3 provides quantitative statistical significance measures, reporting the correlations between the concentrations of each individual volatile compound and the scores of the first three principal components (PCs), along with indications of statistical significance. The table also provides the score values of each sample for

Table 4
Concentration of major chemical compounds in wines obtained with the sequential inoculation of *Sc. pombe* and *S. cerevisiae* in white, red and synthetic must.

Fermentation trial	Ethanol	pH	TA	VA	Tartaric	Citric	Glycerol
W3	9.64 ± 0.55	3.44±	3.38 ± 0.54	0.22 ± 0.21	2.75 ± 0.55	0.15 ± 0.05	6.23 ± 0.84
R3	14.41 ± 0.96	4.47±	4.14 ± 0.69	0.26 ± 0.42	2.53 ± 0.54	0.18 ± 0.04	8.69 ± 0.73
M3	9.27 ± 0.85	3.61±	4.23 ± 0.55	0.42 ± 0.95	2.85 ± 0.81	0.21 ± 0.07	7.21 ± 0.61

TA, total acidity. VA, volatile acidity. Values are expressed in g/L. The ethanol concentration is expressed in g/100 mL. The standard deviation values (±) are indicated.

Table 5a
Concentration of selected volatile compounds (mg/L±sd) determined by GC-MS in wines obtained by the different fermentation trials.

Volatiles (mg/L)	Red Wines								White Wines								Sintetic Must							
	R1	±*SD*	**R2**	±*SD*	**R3**	±*SD*	**R4**	±*SD*	**W1**	±*SD*	**W2**	±*SD*	**W3**	±*SD*	**W4**	±*SD*	**M1**	±*SD*	**M2**	±*SD*	**M3**	±*SD*	**M4**	±*SD*
Esters																								
Ethyl butanoate	0.21	±*0.04*	0.06	±*0.02*	0.43	±*0.12*	nd	nd	0.14	±*0.04*	0.18	±*0.05*	0.55	±*0.12*	0.24	±*0.07*	0.04	±*0.02*	0.17	±*0.04*	0.07	±0.03	0.06	±*0.02*
Isoamyl acetate	0.45	±*0.10*	0.16	±*0.04*	3.29	±*0.51*	1.21	±*0.07*	0.22	±*0.07*	0.12	±*0.04*	0.68	±*0.14*	0.1	±*0.02*	0.09	±*0.02*	0.12	±*0.05*	0.08	±0.02	0.11	±*0.03*
Ethyl hexanoate	0.34	±*0.08*	0.21	±*0.04*	2.07	±*0.14*	0.84	±*0.15*	0.25	±*0.05*	0.27	±*0.04*	2.38	±*0.37*	0.1	±*0.02*	nd		0.32	±*0.13*	0.31	±0.08	0.23	±*0.04*
Hexyl acetate	0.03	±*0.01*	0.12	±*0.03*	0.22	±*0.08*	nd		0.14	±*0.04*	0.17	±*0.03*	0.22	±*0.08*	0.08	±*0.02*	nd		nd		nd		nd	
Ethyl octanoate	0.51	±*0.10*	nd		2.61	±*0.34*	1.15	±*0.04*	0.91	±*0.14*	1.25	±*0.13*	1.78	±*0.24*	0.51	±*0.10*	0.51	±*0.15*	0.21	±*0.06*	0.65	±0.17	0.4	±*0.07*
Ethyl decanoate	0.32	±*0.07*	0.13	±*0.04*	0.87	±*0.14*	0.08	±*0.02*	1.27	±*0.17*	1.51	±*0.50*	2.14	±*0.14*	0.81	±*0.18*	nd		0.65		nd		nd	
Diethyl succinate	0.31	±*0.05*	nd		0.12	±*0.03*	nd		0.12	±*0.03*	0.1	±*0.02*	0.21	±*0.10*	0.14	±*0.04*	nd		0.7		nd		nd	
Ethyl-9-decenoate	0.14	±*0.04*	0.21	±*0.05*	0.27	±*0.05*	0.31	±*0.07*	0.11	±*0.03*	nd		nd		0.05	±*0.02*	nd		nd		nd		nd	
Phenylacetate	0.45	±*0.12*	0.81	±*0.11*	0.61	±*0.14*	0.14	±*0.04*	0.24	±*0.04*	0.13	±*0.03*	3.8	±*0.32*	7.64	±*1.64*	0.09	±*0.02*	0.36	±*0.08*	0.05	±0.02	0.07	±*0.02*
Total	**2.76**		**1.7**		**10.5**		**3.73**		**3.39**		**3.73**		**11.75**		**9.66**		**0.73**		**2.53**		**1.16**		**0.87**	
Alcohols																								
2-Methyl-1-propanol	0.08	±*0.02*	0.14	±*0.04*	0.21	±*0.07*	0.16	±*0.05*	0.05	±*0.02*	nd		0.34	±*0.04*	0.08	±*0.02*	0.07	±*0.02*	0.36	±*0.14*	0.08	±0.02	nd	
3-Methyl-1-butanol	13.59	±*4.31*	12.12	±*3.17*	25.73	±*4.12*	11.91	±*3.22*	11.78	±*1.75*	11.82	±*1.82*	24.04	±*4.10*	13.89	±*3.92*	12.07	*v2.14*	15.15	±*1.81*	11.79	±3.14	14.47	±*3.71*
1-Hexanol	0.22	±*0.04*	0.19	±*0.04*	1.21	±*0.04*	0.29	±*0.05*	0.05	±*0.02*	0.05	±*0.02*	0.49	±*0.08*	0.48	±*0.11*	nd		nd		nd		nd	
3-Hexen-1-ol (z)	nd	*nd*	0.04	±*0.02*	nd		0.07	±*0.02*	nd		nd		nd		nd		nd		nd		nd		nd	
Benzylalcohol	0.15	±*0.05*	0.44	±*0.11*	0.51	±*0.12*	0.17	±*0.04*	0.21	±*0.02*	0.25	±*0.05*	0.31	±*0.08*	0.14	±*0.04*	nd		nd		nd		nd	
Phenylethanol	1.24	±*0.07*	1.4	±*0.25*	3.64	±*0.61*	2.94	±*0.42*	2.78	±*0.78*	4.17	±*0.22*	5.98	±*0.15*	2.16	±*0.15*	3.48	±*0.51*	4.22	±*0.31*	5.93	±1.04	5.4	±*0.61*
Total	**15.28**		**14.33**		**31.3**		**15.54**		**14.87**		**16.29**		**31.16**		**16.75**		**15.61**		**19.73**		**17.8**		**19.87**	
Volatile phenols																								
4-Vinylguaiacol	0.19	±*0.04*	0.16	±*0.03*	nd		0.19	±*0.05*	0.05	±*0.02*	0.04	±*0.02*	nd		0.4	±*0.11*	0.77	±*0.18*	2.41	±*0.25*	0.23	±0.05	2.24	±*0.14*
	0.19		**0.16**		**0**		**0.19**		**0.05**		**0.04**		**0**		**0.39**		**0.77**		**2.41**		**0.23**		**2.24**	
Aldehydes-Ketons																								
Furfural	nd		nd		nd		nd		0.16	±*0.04*	0.15	±*0.03*	0.2	±*0.05*	0.16	±*0.04*	nd		nd		nd		nd	
Furaneol	0.22	±*0.07*	2.77	±*0.61*	1.25	±*0.17*	0.51	±*0.11*	nd		nd		nd		nd		nd		nd		nd		nd	
Total	**0.22**		**2.77**		**1.25**		**0.51**		**0.16**		**0.15**		**0.2**		**0.16**									
Acids																								
2-Methylpropanoic acid	nd		0.05	±*0.02*	0.09	±*0.03*	0.12	±*0.02*	2.77	±*0.17*	2.61	±*0.54*	3.15	±*0.15*	1.51	±*0.52*	0.14	±*0.04*	nd		nd		0.07	±*0.03*
Butanoic acid	0.1	±*0.03*	nd		nd		0.1	±*0.03*	nd		nd		nd		nd		0.12	±*0.03*	0.1	±*0.02*	nd		0.14	±*0.04*
2-Methylhexanoic acid	0.06	±*0.02*	nd		nd		0.08	±*0.03*	nd		nd		nd		0.07	±*0.02*	nd		nd		nd		nd	
3-Methyl butanoic acid	nd		0.15	±*0.04*	0.33	±*0.10*	nd		nd		0.12	±*0.03*	0.26	±*0.07*	0.11	±*0.02*	0.07	±*0.02*	nd		nd		0.23	±*0.05*
Hexanoic acid	0.68	±*0.18*	2.51	±*0.15*	2.37	±*0.17*	1.81	±*0.34*	0.42	±*0.08*	1.46	±*0.16*	3.52	±*0.35*	0.71	±*0.17*	0.49	±*0.07*	nd		0.71	±0.12	0.89	±*0.18*
Octanoic acid	1.04	±*0.15*	0.13	±*0.04*	nd		0.36	±*0.08*	1.04	±*0.02*	0.68	±*0.17*	1.24	±*0.14*	0.85	±*0.15*	0.76	±*0.16*	5.24	±*0.17*	1.1	±0.05	1.6	±*0.17*
Decanoic acid	0.55	±*0.05*	0.3	±*0.08*	nd		0.18	±*0.07*	0.42	±*0.13*	0.21	±*0.07*	nd		0.08	±*0.02*	0.26	±*0.05*	1.24	±*0.14*	0.52	±0.15	0.92	±*0.18*
Total	**2.43**		**3.15**		**2.78**		**2.64**		**4.65**		**5.08**		**8.17**		**3.33**		**1.85**		**6.58**		**2.32**		**3.85**	
Terpenes																								
Linalool	nd		0.21	±*0.03*	0.2	±*0.02*	0.11	±*0.03*	0.1	*0.02*	0.17	±*0.04*	nd		0.21	±*0.04*	nd		nd		nd		nd	
ho-Trienol	nd		0.15	±*0.04*	nd		nd		0.1	*0.03*	nd		nd		nd		nd		nd		nd		nd	
terpineon-4-ol	nd		0.08	±*0.03*	nd		0.16	±*0.03*	nd		nd		nd		nd		nd		nd		nd		nd	
trans β terpineol	nd		nd		0.06	±*0.02*	nd		nd		nd		nd		nd		nd		nd		nd		nd	
α-terpineol	0.12	±*0.03*	nd		0.11	±*0.02*	0.14	±*0.04*	0.12	*0.03*	0.18	±*0.03*	0.9	±*0.11*	0.76	±*0.17*	nd		nd		nd		nd	
Total	**0.12**		**0.44**		**0.37**		**0.41**		**0.32**		**0.35**		**0.9**		**0.97**									

nd: not detection; SD: standard deviation.

Table 5b
Two-Way ANOVA analysis with interaction.

	Matrix	Inoculation Strategy	Matrix*Inoculation Strategy	R^2
Ethyl butanoate	**	***	**	0.874
Isoamyl acetate	****	****	****	0.968
Ethyl hexanoate	****	****	****	0.969
Hexyl acetate	***	*	ns	0.839
Ethyl octanoate	****	****	****	0.953
Ethyl decanoate	****	**	*	0.936
Diethyl succinate	ns	***	****	0.962
Ethyl-9-decenoate	****	ns	*	0.921
Phenylacetate	****	***	****	0.952
2-Methyl-1-propanol	ns	*	**	0.829
3-Methyl-1-butanol	ns	*	ns	0.671
1-Hexanol	****	****	****	0.981
3-Hexen-1-ol (z)	***	*	**	0.873
Benzylalcohol	****	**	ns	0.889
Phenylethanol	****	***	*	0.902
4-Vinylguaiacol	****	****	****	0.983
Furfural	****	ns	ns	0.920
Furaneol	****	***	***	0.948
2-Methylpropanoic acid	****	ns	*	0.966
Butanoic acid	***	**	*	0.890
2-Methylhexanoic acid	**	***	*	0.868
3-Methyl butanoic acid	ns	**	***	0.878
Hexanoic acid	****	****	****	0.966
Octanoic acid	****	****	****	0.991
Decanoic acid	****	**	***	0.932
Linalool	****	**	***	0.943
ho-Trienol	**	**	****	0.916
terpineon-4-ol	****	***	***	0.938
trans β terpineol	**	**	***	0.892
α-terpineol	****	***	***	0.957

ns: not significative $p > 0.05$; $^*p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$; R^2 indicates the proportion of total variance in the dependent variable that is accounted for by the two factors and their interaction in the two-way ANOVA.

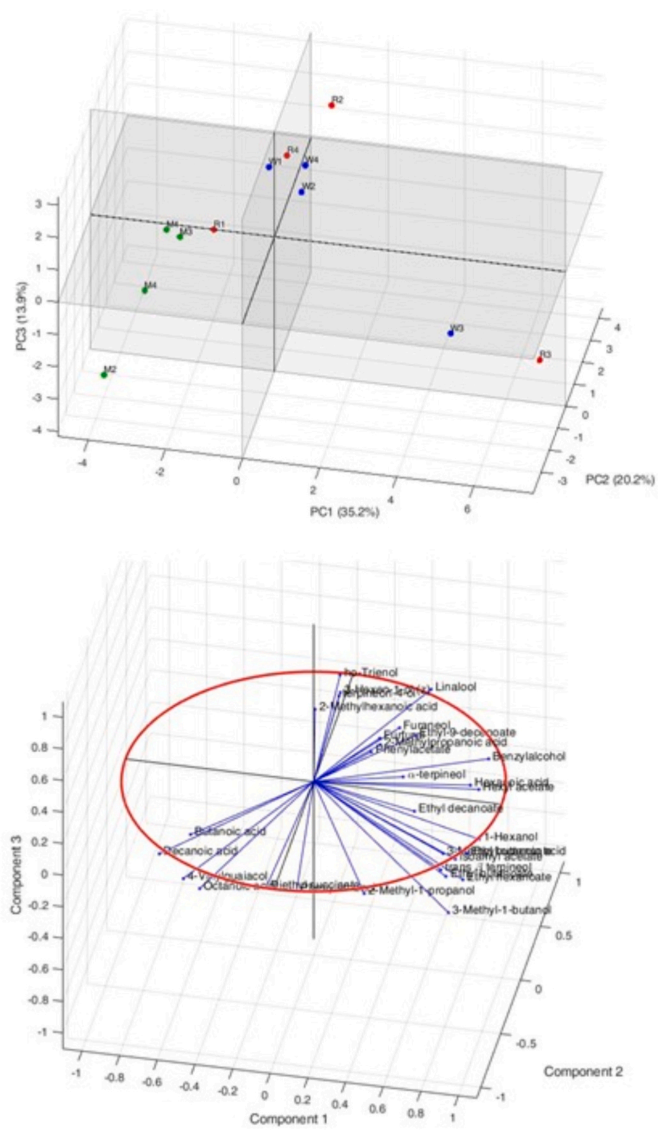

Fig. 5. Score plot and correlation circles calculated by Pearson correlation Principal Component Analysis (PCA) on the volatile compounds along the first three components in the different fermentation trials in White (W), Red (R) and Synthetic (M) must.

the first three PCs.

The compounds significantly and positively correlated (p-value≤ 0.001) with PC1 are: ethyl hexanoate, hexyl acetate, ethyl octanoate, 1-hexanol and benzylalcohol. These compounds showed correlations ranging between 0.8314 and 0.9072 and are the ones that contribute most to the differences obtained with sequential inoculation for red and white wines compared to the other treatments. R3 (red wine with strategy 3) and W3 (white wine with strategy 3) are clearly separated by higher than average values of PC1, indicating a strong association with the volatile compounds positioned in the same direction in the correlation plot—mainly esters and higher alcohols. White wines (W1–W4) tend to cluster in the lower-right quadrant (positive PC1, negative PC2), particularly W2, W3, and W4, aligning them with ester- and alcohol-rich profiles, consistent with observations from the correlation plot. On the other hand, butanoic acid (−0.6607), octanoic acid (−0.4841), decanoic acid (−0.7623), and vinylguaiacol (−0.6203) are located on the negative side of PC1, suggesting an inverse relationship with the fermentation strategies or matrices that load positively on PC1. As can be observed, must samples (M1–M4) cluster more closely to the origin or in the negative quadrant of PC1, especially M2, suggesting a lower overall impact from the compounds driving the PC1. Additionally, phenylethanol, diethyl succinate, and α-terpineol are less correlated with PC1 and PC2, implying a more balanced contribution. R2 and R4 (red wines with strategies 2 and 4) are located in the upper part of the plot (higher than average PC2), suggesting a different volatile profile, potentially influenced by compounds like linalool. Principal Component 3 (PC3), which accounts for 13.9 % of the total variance, adds an additional layer of interpretation to the dataset by revealing differences that PC1 and PC2 do not fully capture. When visualised alongside PC1, PC3 offers a more nuanced view of how the inoculation strategies influence the volatile profiles within each matrix. In particular, white wine samples tend to exhibit a clear positive projection along PC3, particularly for W1, W2, and W4. This suggests that these samples share certain volatile characteristics—likely involving compounds that correlate positively on this component, such as specific esters or higher alcohols contributing to fruity or floral notes. W3, while strongly separated along PC1, remains neutral along PC3, indicating that its unique aromatic profile—likely shaped by strategy 3—is primarily associated with compounds influencing PC1 rather than PC3. Red wine samples exhibit more diverse behaviour along this axis. R2, which lies in the upper region of the plot, appears to share some similarities with the white wines in terms of PC3-related compounds, possibly indicating a lighter or more floral aromatic character than the other reds. On the opposite end, R3 is located far along the positive side of PC1 but shows a strongly negative value on PC3, suggesting an intense production of esters or other volatiles that contribute to PC1, accompanied by a suppression or lower concentration of those defining PC3. R1 and R4 remain closer to the origin, suggesting a more balanced volatile profile across the first three components. In contrast, the must samples are predominantly located in the lower part of the PC3 axis. M2 and M4, in particular, show the most negative values, reinforcing the idea that musts, as unfermented or partially fermented matrices, generally lack the compounds that define PC3. M1 and M3, while also on the negative side, are positioned closer to the center, potentially indicating some minor development of volatiles depending on the inoculation strategy applied. Overall, PC3 enhances the interpretability of the PCA by separating samples not only by matrix but also by fermentation strategy. It is particularly useful in distinguishing white wines from musts and in highlighting the specific effects of certain inoculation approaches, such as strategy 3 (i.e. sequential inoculation), which seems to have a strong influence on the volatile composition across all matrices. These results were confirmed by the cluster-heatmap shown in Fig. S2. The cluster heatmap showed the standardised concentrations of volatile compounds identified using GC–MS. Samples are represented on the vertical axis and individual volatiles on the horizontal axis. The colour scale reflected relative abundance, with red shades indicating concentrations above the mean and blue shades indicating concentrations below it. Hierarchical clustering was applied to both samples and compounds, grouping together those with similar volatile profiles. This approach enabled patterns of similarity to be visualised, demonstrating the influence of treatments and wine matrices (white *versus* red) on volatile composition. Distinct clusters highlighted differences driven by treatment, while compound clustering reveals groups of volatiles that co-vary across samples. The treatment 3 (i.e., W3, R3, M3) exhibited a distinct volatile profile compared to all other treatments, both in white and red samples. It is characterised by a generally higher-than-average relative concentration of almost all detected volatiles, highlighting its pronounced impact on the overall volatile composition.

4. Discussion

Yeasts are fundamental biological agents responsible for converting grape must into wine, orchestrating complex biochemical processes that determine the chemical and sensory qualities of the final product (Berbegal et al., 2017). While *Saccharomyces cerevisiae* remains the dominant species employed in winemaking due to its robust fermentative power and predictable outcomes, non-*Saccharomyces* yeasts, such as *Schizosaccharomyces pombe* (Loira et al., 2018), have garnered increasing interest for their unique metabolic capacities (Roudil et al., 2020). However, these yeast species are often considered double-edged swords, offering both spoilage risks and technological potential (Á. Benito et al., 2018), including both spoilage and protechnological strains. In this study, the *Sc. pombe* strain SP2 was isolated from Apulian preserved grape must previously by high concentrations of sulfur dioxide (SO_2), in which the winery reported oenological parameters comparable to an initiated malo-alcoholic fermentation, a remarkable event considering the microbiostatic pressure exerted by SO_2 (Z. Li et al., 2024). There are microbial species that have a dual character, so that the same strain can be a spoilage agent or protechnological resource, depending on the supply chain, as is the case of *Brettanomyces bruxellensis* in wine and some beer styles, respectively (Avramova et al., 2018). Intriguingly, this study characterises, for the first time, the dichotomic potential of a yeast strain, Sc. pombe strain SP2, as both spoilage agents and potential starter culture candidates in different production phases relevant to the wine industry (i.e., preservation of grape must and alcoholic fermentation).

Sulfur dioxide remains the cornerstone of microbial control in winemaking, utilised for its antimicrobial, antioxidant, and enzyme-inhibiting properties. Yet, its effectiveness is closely tied to pH levels and microbial resistance. The study of microbial spoilage in various food and beverage sectors represents a field of considerable interest for understanding biological evolution phenomena in relation to specific ecological niches and for developing knowledge that can improve the sustainability of food systems (Snyder et al., 2024). The sector of preserved grape must is poorly studied in the literature, but concerns significant production volumes (Delogu et al., 2022). Numerous studies have confirmed that resistance to SO_2 varies significantly across yeast species of oenological significance and even among strains within the same species, both for protechnological species (e.g., *S. cerevisiae*) and for spoilage species (e.g., *B. bruxellensis*) (Capozzi et al., 2016). In line with this intraspecific variability, the isolated *Sc. pombe* strain SP2 exhibited an unexpected resistance threshold, tolerating more than 2 g/L of total SO_2 even at low pH, reaching intensities beyond levels that are harsh to most oenological yeasts, including *S. cerevisiae*. The tolerance levels to SO_2 in the simulations of selective pressure by these bacteriostatic agents are compatible with the spoilage phenomenon observed in the winery. The model microorganism for the study of SO_2 resistance mechanisms is *S. cerevisiae* (Divol et al., 2012), in which mechanisms related to transporters, acetaldehyde production, reduction processes of the compound, entry into a viable but non-culturable state, and chromosomal rearrangement have been found (García-Ríos et al., 2019). This work suggests a high interest in studying the variability of this phenotype in relation to intraspecific diversity in *Sc. pombe* and in investigating these mechanisms in a species that is interesting as a model in eukaryotic biology (Jeffares et al., 2015), describing a specific behavior at the strain level relevant in demonstrating how co-evolution of yeasts with humans can be a driver not only for the domestication (De Guidi et al., 2023; Marsit & Dequin, 2015) but also in selecting for undesired properties with industrial relevance. From this perspective, the work presents promising evidence for future investigations to elucidate the molecular basis of sulphite stress response, including genetic, transcriptomic, proteomic, and metabolic analyses, also including non-SO2-resistant strains.

Considering the assessment of protechnological characteristics, we observed that *Sc. pombe* could degrade L-malic acid under various conditions, including in red, white, and synthetic musts. Notably, its efficacy in low-pH white musts and synthetic media was significantly higher than in red musts, which presented higher sugar and acid content (but also a different polyphenol content). This suggests that while *Sc. pombe* is metabolically competent under stress conditions, its performance may be modulated by the chemical matrix of the must, including osmotic pressure and nutrient composition (Loira et al., 2015). In trials involving red must, fermentation by *Sc. pombe* in monoculture was slower and associated with increased acetic acid levels, echoing concerns in previous literature about its spoilage potential in wine (Roca-Domènech et al., 2018) and suggesting that, as with most non-*Saccharomyces*, the preferred use in winemaking is in combination with saccharomycetes (del Fresno et al., 2025). The findings also show limitations related to the use of synthetic must, suggesting the need for an integrated approach that includes model solutions to prioritise reproducibility and samples to evaluate behaviour under real conditions. The investigation of VOCs is also crucial, as they contribute significantly to the final sensory quality of fermented beverages. The most common ones identified in young wines belong to different chemical families, including esters, higher alcohols, fatty acids, and, in smaller quantities, phenolic compounds and terpenes. The co-inoculum strategy allowed a potential increase of fruity aromas in the wine by synthesising larger amounts of esters (ethyl butanoate, isoamyl acetate, hexyl acetate, ethyl hexanoate, ethyl octanoat,e and ethyl decanoate), especially in red and white wines (Rubio-Bretón et al., 2019). In addition, the co-inoculum enhances herbaceous aromas (1-hexanol) and total higher alcohols. Certain higher alcohols such as 2-methyl-1-propanol, 1-hexanol, benzyl alcohol, and 2-phenylethanol possess particular aromas that help improve the aromatic profile of the wine (Vilanova & Martínez, 2007). However, quantitatively, the main higher alcohols in wine are 2- and 3-methyl-1-butanol, both characterised by a strong alcohol aroma in a concentration above 300 mg/L (Sánchez-Palomo et al., 2012). In all our trials, this threshold value was not exceeded. As mentioned above, 2-phenylethanol is an interesting compound for red wine because it contributes to a floral aroma (rose petals) while adding a touch of honey (Mendes et al., 2012). Fatty acids can play a role in the aroma of wines, enhancing the complexity and aromatic balance since they are characterised by notes of fruit, cheese, and rancidity, even if they typically have high odour detection thresholds (Peng et al., 2015). In this study, the main fatty acids (hexanoic acid, octanoic acid, and decanoic acid) were quantified in all samples, but higher values were detected in W1-W2-W3. Regarding the terpenes, these compounds are associated with the flowery flavour of cider, and their relevance is linked to their low odour threshold (Yu et al., 2022). The terpene content was reported to vary with yeast strains and apple varieties, as supported by literature (Á. Benito et al., 2019). PCA reveals a clear differentiation of samples based on both fermentation strategy and matrix. Strategy 3 appears to significantly enhance the production of key esters and alcohols, especially in red and white wine, while must samples show a less pronounced profile, possibly due to a lower metabolic activity or compound retention. Several studies highlight how the inoculation strategy influences wine-associated volatiles (Vilela, 2020; Yang et al., 2024), but few have demonstrated that matrix type (synthetic must, red, white wine) interacts strongly with the inoculation strategy in shaping the volatile composition. Mixed fermentations using *Sc. pombe* in combination with *S. cerevisiae* represent a promising approach, with the results demonstrating that sequential inoculation, where *Sc. pombe* was introduced 48 h prior to *S. cerevisiae*, offered a balanced outcome, confirming the interest in this type of management in other fermented beverages (Grieco et al., 2024; A. Li et al., 2025). This protocol allowed for effective malic acid degradation while maintaining control over acetic acid levels. This type of management has been widely valued in the literature, as it allows maximising the diversification introduced by non-*Saccharomyces*, combining the potential of *S. cerevisiae* in terms of safety and closure of fermentations, avoiding abnormal fermentation stuck and deviations (Medina et al., 2012). This type of management has been widely valued in the literature, as it allows maximising the diversification introduced by non-*Saccharomyces*, combining the potential of *S. cerevisiae* in terms of safety and closure of fermentations, avoiding abnormal stops and deviations. In terms of future perspectives in protechnological application, several aspects of interest are potentially explorable, from the interaction with other microbial resources of interest to oenology (De Gioia et al., 2022) to the evaluation in mixed starter cultures with other non-*Saccharomyces* species such as *Lachancea thermotolerans* (Vicente et al., 2023). From the valorisation in sparkling wines (Capozzi et al., 2022) to a broader exploitation of traits of the oenological context (S. Benito et al., 2012). It is also interesting to apply *Sc. pombe* to positively modulate the volatile profile in fruity wine (i.e. mandarin wine) (Luo et al., 2023). Considering both research topics explored in this work, *Sc. pombe* might be a valuable ally to promote fermentation in musts that require elevated SO_2 additions for microbial control and/or might benefit from sulphitation to improve the dominance of this strain during fermentation. In general, considering the protechnological properties, the findings confirm that, under controlled inoculation strategies, Sc. pombe can serve as a biotechnological asset in winemaking, particularly for wines where malic acid reduction and aromatic enhancement are desired.

CRediT authorship contribution statement

Carmen Berbegal: Writing – original draft, Investigation, Data curation, Conceptualization. **Maria Tufariello:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Francesco Grieco:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Barbara la Gatta:** Writing – review & editing, Methodology, Formal analysis, Conceptualization. **Vittorio Capozzi:** Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualization. **Mariagiovanna Fragasso:** Writing – original draft, Supervision, Resources, Project administration, Methodology, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2025.107803.

Data availability

Data will be made available on request.

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