Biological Deacidification Strategies for White Wines

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Traditionally, the use of malolactic fermentation gives rise to microbiologically stable wines. However, malolactic fermentation is not free from possible collateral effects that can take place under specific scenarios. The present work tests the influence of different biological deacidification strategies on the volatile and non-volatile components of white must from Germany. The study compared mixed cultures of *Lachancea thermotolerans* and *Schizosaccharomyces pombe* and a pure culture of *Sc. pombe* to the classical biological deacidification process performed by lactic acid bacteria. Strains of *Oenococcus oeni* and *Lactiplantibacillus plantarum* were co- or sequentially inoculated with *S. cerevisiae* to carry out malolactic fermentation. Different fermentation treatments took place at a laboratory scale of 0.6 L in vessels of 0.75 L. The instrumental techniques Fourier-transform mid-infrared spectroscopy (FT-MIR), high performance liquid chromatography (HPLC) and gas chromatography–mass spectrometry (GC-MS) were used to evaluate different chemical parameters in the final wines. The results showed the ability of *Sc. pombe* to consume malic acid in combination with *L. thermotolerans* without using *S. cerevisiae* or lactic acid bacteria. Fermentations involving *Sc. pombe* consumed all the malic acid, although they reduced the concentrations of higher alcohols, fatty acids and acetic acid. Simultaneous alcoholic and malolactic fermentations reduced malic acid by about 80%, while classical malolactic fermentation reduced it by 100%. Fermentations involving *L. thermotolerans* produced the highest lactic acid, ester and glycerol concentrations.

INTRODUCTION

The classical winemaking process may include two main microbiological steps. The first one is alcoholic fermentation, which is carried out mostly by *S. cerevisiae*, but sometimes by other yeast species. The second one is malolactic fermentation (MLF), which usually is induced by lactic acid bacteria (LAB), such as *Oenococcus oeni* (Knoll *et al*., 2011, 2012; Sumby *et al*., 2014; Bartowsky *et al*., 2015; Du Plessis *et al*., 2017a) and *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*) (Du Toit *et al*., 2011; Iorizzo *et al*., 2016; Lucio *et al*., 2016, 2018; Brizuela *et al*., 2018; Du Plessis *et al*., 2019; Olguin *et al*., 2020). Traditionally, MLF is used to obtain microbiologically stable wines – both red wines and some white wines, and especially those that undergo barrel or bottle ageing (Bartowsky *et al*., 2015; Sumby *et al*., 2019). However, MLF may present some collateral effects in specific situations, such as off-flavour development and the production of potentially toxic products, i.e. biogenic amines (Smit & Du Toit, 2013) and ethyl carbamate (Benito *et al*., 2015a; Sumby *et al*., 2019).

Depending on the initial pH, the removal of L-malic acid can be detrimental to wine, especially wines coming from warm regions that may show high pH and low acidity (Volschenk *et al*., 2006; Dicks & Endo, 2009; Benito *et al*., 2016a). This scenario has opened the doors for the study of new biological deacidification strategies with specific *non-Saccharomyces* yeasts (Vilela, 2019; Benito, 2019). The *Schizosaccharomyces* genus shows the highest efficiency in wine deacidification. *Schizosaccharomyces* spp. can degrade malic acid (Jolly *et al*., 2014; Domizio *et al*., 2017, 2018; Minnaar *et al*., 2017a) via malo-ethanolic fermentation, leading to the production of ethanol and carbon dioxide (Benito, 2019).

Due to an increasingly sophisticated and fragmented market, consumers request different wine styles with healthier and more sustainable appeal. In addition, because of the change in wine composition due to the increase in the average global temperature, some strains, such as *Sc. pombe* and *Lachancea thermotolerans*, are receiving particular

attention (Jolly *et al*., 2014; Roudil *et al*., 2019; Benito, 2020).

The aim of this study was to compare the different available biological deacidification strategies and their influence on white wine production.

MATERIALS AND METHODS
Microorganisms
The following yeast and bacterial strains were used for the experimental fermentations of the white grape musts: *Saccharomyces cerevisiae* Lalvin QA23® (Lallemand, Montreal, Canada), *Schizosaccharomyces pombe* V2 (Benito *et al*., 2014a, 2016b; Scansani *et al*., 2020), *Lachancea thermotolerans* Concerto™ (Hansen, Hoersholm, Denmark), *Oenococcus oeni* (commercial strain) and *Lactiplantibacillus plantarum* (commercial strain). Due to a confidentiality agreement with the supplier, the names of the lactic acid bacteria may not be mentioned. The authors can be contacted if anyone needs further information.

Vinification process
The microvinifications were performed according to a previously described methodology (Dutraive *et al*., 2019), which was adapted to 0.75 L fermentation vessels. Must of the Riesling grape variety (Hochschule Geisenheim University, Germany) was used for all the fermentations. Constituent concentrations in the initial must were as follows: total sugar, 201.7 g/L; tartaric acid, 5.0 g/L; malic acid, 4.7 g/L; total acidity, 10.48 g/L; lactic and acetic acid, < 0.1 g/L; pH, 3.09; yeast-assimilable nitrogen content (YAN), 140 mg/L.

OptiMUM WHITE™ (0.4 g/L, Lallemand, Montreal, Canada) and FERMAID E™ (0.4 g/L, Lallemand) were added to the must to provide nutrition to the microorganisms. No sulphur dioxide was added, as the initial must was previously sterilised by fine filtration (0.22 μm), subsequently saturated with carbon dioxide gas and stored at a pressure of 600 kPa and a temperature of 0°C (Kanter *et al*., 2020). Then, 0.6 L of must were placed in 0.75 L sterilised glass fermentation vessels sealed with a fermentation airlock. The fermentation airlocks were filled with an aqueous solution of 20 g/L of potassium metabisulfite (Merck, Darmstadt, Germany), which allowed for the release of CO_2 while avoiding microbial contamination. Six treatments were performed in triplicate: (i) pure culture of *S. cerevisiae* (*SC*); (ii) *S. cerevisiae* followed by *O. oeni* at the end of alcoholic fermentation (*SC...OE*); (iii) *S. cerevisiae* followed by *O. oeni* after five hours (*SCxOE*); (iv) *S. cerevisiae* followed by *L. plantarum* after five hours (*SCxLB*); (v) *L. thermotolerans* followed by *Sc. pombe* after 96 hours (*LT...SP*); and (vi) pure culture of *Sc. pombe* (*SP*). All the strains were inoculated under aseptic conditions. The yeast and bacteria were inoculated at a population of 10^6 cfu/mL. The inoculums were prepared by rehydrating 100 mg of the corresponding commercial strain product in 10 mL of sterilised water under sterile laboratory conditions. The number of cells was evaluated by cell counting using a Thoma counting chamber, Blaubrand® (Brand, Wertheim, Germany), in a Leica DM 500 microscope (Wetzlar, Germany). The sequential MLF treatment, with *S. cerevisiae* followed by *O. oeni* after alcoholic fermentation (*SC...OE*), was inoculated with *O. oeni* at 10^7 cfu/mL. MLF

was performed in 0.5 L vessels at 18°C until malic acid was totally degraded. Only the sequential fermentation (*SC... OE*) performed MLF at 18°C after alcoholic fermentation by *S. cerevisiae*. The other alcoholic fermentations and simultaneous MLF (*SCxOE*; *SCxLP*) treatments were performed at 20°C. Once the weight loss remained constant for 48 hours, the wines were racked and stabilised for 15 days at 4°C, and the final product was bottled in 125 mL bottles. Potassium metabisulfite (Merck, Darmstadt, Germany) was added to achieve a concentration of 80 mg/L total sulphur dioxide. The bottles were sealed with aluminium screw caps and were placed in a climate chamber at 4°C until the chemical analyses were performed.

Analytical determination of non-volatile compounds
The determination of total acidity, residual sugar, density, ethanol, glycerol, extract, sugar-free extract and the pH of the must and wine was carried out with the standard operating procedure SOP-WG1-84 of the Department of Beverage Research of Hochschule Geisenheim University (HGU; Germany) based on Fourier-transform mid-infrared spectroscopy (FT-MIR) (Patz *et al*., 2004; Friedel *et al*., 2013; Kanter *et al*., 2020). The determination of L-malic acid, L-lactic acid and acetic acid was performed by HPLC (Kanter *et al*., 2020). For this purpose, we used the 1100 Series system of Agilent Technologies (Santa Clara, USA), equipped with an Allure Organic Acids™ column (Restek GmbH, Germany) (250 mm x 4.6 mm I.D. x 5 μm grain size x 60 Å pore size), preceded by a 4 mm x 3.0 mm inner diameter precolumn (Security Guard C18™, Phenomenex, Germany). Analytes were detected by a refractive index detector (RID) and a multiwavelength detector (MWD). The samples were analysed in scan mode. pH values were measured using a pH electrode (WTW pH meter pH 526) (Xylem Analytics Germany Sales GmbH & Co. KG, Weilheim, Germany).

Analytical determination of YAN
YAN levels in the must were measured using the NOPA technique (nitrogen by derivatisation with ortho-phthaldialdehyde (OPA)), following the directions of Dukes and Butzke (2013). NOPA determination was carried out using the Thermo Scientific™ Evolution 220 UV-visible Spectrophotometer (Fisher Scientific, New Hampshire, USA).

Analytical determination of volatile compounds
The determination of ester, higher alcohol and fatty acid concentrations was carried out at Hochschule Geisenheim University (Germany) by the Department of Microbiology and Biochemistry according to Rapp *et al*. (1994) with modifications (Kanter *et al*., 2020; Scansani *et al*., 2020). A gas chromatograph GC 5890 Series II (Hewlett-Packard, Palo Alto, USA) was used. For the sample preparation, 2 g of NaCl (Carl Roth, Karlsruhe, Germany) was weighed into a 15 mL sample vessel and 10 mL of wine was added. Then, 10 μL of the internal standard, 2,6-dimethyl-5-hepten-2-ol (DMH) (stock concentration 1 219 μg/L) (Carl Roth, Karlsruhe, Germany), was added for the quantification, 10 μL of the internal standard Cumol (Honeywell, Morris Plains, USA) (stock concentration 170 μg/L) was added for the

control, and 160 µL of 1,1,2-trichlorotrifluoroethane (Merck, Darmstadt, Germany) was added as extracting agent. The mixture was agitated for 20 min and centrifuged for 8 min (3 000 rpm; 1 700 g). The extract was removed with a glass pipette and transferred to a sample vial for analysis. The cold injection system, KAS 3 (Gerstel, Mülheim an der Ruhr, Germany), was used for sample injection. Two microlitres of the sample were injected in splitless mode (starting temperature 40°C; heating rate 3°C/min to 125 °C; holding time 4 min and 6°C/min to 200°C; holding time 14.2 min). The instrument was fitted with the Varian VF-5 MS column from Agilent (Santa Clara, USA) with dimensions of 60 m x 320 µm x 1 µm. Helium (Linde Gas, Bingen, Germany) was used as the carrier gas at a flow rate of 1 mL/min. The detection was performed by mass spectrometry (5972 MSD, Hewlett-Packard) in scan mode, covering a mass-to-charge ratio from 35 to 250. The voltage of electron impact was set at 70 mV.

Statistical analysis
For the statistical evaluation of the data, the means and standard deviations of the wine sample triplicates were calculated. One-way ANOVA and multiple range tests were performed using Statgraphics Centurion V17.2.05 software (Graphics Software Systems, Rockville, MD, USA). The significance level was set at $p < 0.05$. A multiple range test was used to compare and group the mean values of the variants according to the Fisher's least significant difference (LSD) method. These are identified by the letters a to d in the tables.

RESULTS AND DISCUSSION
Non-volatile compounds
Ethanol production
The final ethanol levels varied from 11.75% to 12.21% (v/v). The final density, together with the residual sugar values, showed that all the treatments reached dryness (Table 1). Alcoholic fermentations involving *Sc. pombe* fermented slower than the fermentations involving *S. cerevisiae* (Fig. 1). The *Sc. pombe* fermentation produced the highest final concentration of ethanol, while the other fermentations did not show significant statistical differences. This effect took place because the conversion of malic acid into ethanol is clearer for *Sc. pombe* when the initial content of malic acid is high (Minnaar *et al.*, 2017a). *LT...SP* produced the lowest concentration of ethanol (Table 1). Fermentations involving *L. thermotolerans* consistently contained lower ethanol concentrations, even in sequential inoculations with *S. cerevisiae* (Gobbi *et al.*, 2013). In the present study, *L. thermotolerans* proved to be a suitable tool to mitigate the higher ethanol production obtained from *Sc. pombe* fermentation. Yeast may use sugars as a source for the synthesis of molecules other than ethanol, including glycerol, pyruvic acid and lactic acid, or to increase yeast biomass (Benito *et al.*, 2015a; Benito, 2018; Hranilovic *et al.*, 2018).

Glycerol
Glycerol is one of the major yeast metabolites synthesised during fermentation and is the third most important metabolite after ethanol and CO_2. It may contribute to the smoothness, sweetness and complexity of wine (Jolly *et al.*, 2014). In this study, the final concentrations of glycerol varied from 5.9 to 7.3 g/L (Table 1). Treatments involving *S. cerevisiae* did not differ significantly from each other (Table 1), while sequential fermentation with *L. thermotolerans* and *S. pombe* (*LT...SP*) showed the highest final glycerol content. This agrees with previous studies that reported *L. thermotolerans* as a high glycerol producer (Comitini *et al.*, 2011; Gobbi *et al.*, 2013; Benito, 2018; Porter *et al.*, 2019b). Using *L. thermotolerans* in mixed fermentation with *S. cerevisiae* positively increases glycerol production (Gobbi *et al.*, 2013). The selected *S. cerevisiae* and *L. thermotolerans* strains might have had better-developed glycerol-pyruvic pathways than the selected *Sc. pombe* strain, as they generated lower final concentrations of ethanol and higher concentrations of glycerol (Table 1). Although some studies report *Sc. pombe* to synthesise higher amounts of glycerol than some *Saccharomyces* strains (Benito *et al.*, 2014b; Domizio *et al.*, 2017; Benito, 2019), the *Sc. pombe* treatments (*SP*) in this study produced the lowest final concentrations of glycerol. The high strain variability of this parameter (up to 20%) in *Sc. pombe* and *S. cerevisiae* (Benito *et al.*, 2014a, 2016b) explains this observation.

L-lactic acid
The final concentrations of lactic acid varied from 0 to 3.37 g/L, depending on the microorganisms involved in the fermentation (Table 1). The *LT...SP* treatment showed the highest concentration of lactic acid, the levels of which were even higher than the lactic acid levels reported in the treatments that underwent MLF by LAB. The ability of *L. thermotolerans* to generate lactic acid from substrates other than malic acid explains this result (Kapsopoulou *et al.*, 2005; Hranilovic *et al.*, 2017, 2018; Benito, 2018; Vilela, 2018; Porter *et al.*, 2019b; Fairbairn *et al.*, 2021; Hranilovic *et al.*, 2021). Previous studies that combined *L. thermotolerans* and *Sc. pombe* reported final levels of L-lactic acid varying from 2.77 to 3.41 g/L (Benito *et al.*, 2015a, 2016a, 2017, 2019; Escott *et al.*, 2018; Wang *et al.*, 2019; Benito, 2020). A wider range in the final level of lactic acid, varying from 0.22 to 5.13 g/L, depending on the inoculation strategy and timing, is reported in the literature regarding mixed cultures of *L. thermotolerans* and *S. cerevisiae* (Kapsopoulou *et al.*, 2007; Gobbi *et al.*, 2013; Benito *et al.*, 2015b). The inoculation time of *S. cerevisiae* affects the growth and survival of *L. thermotolerans*, and consequently the final amount of lactic acid in the wine (Kapsopoulou *et al.*, 2007). The later the inoculation with *S. cerevisiae*, the higher the final L-lactic acid concentration. The final L-lactic acid levels of the *SC...OE* and *SCxOE* treatments did not differ statistically (Table 1).
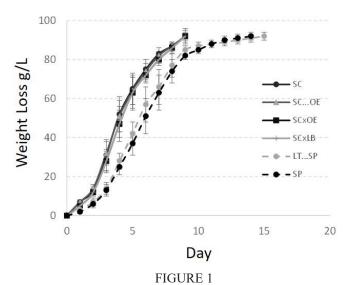
Malic acid and total acidity
The malic acid levels varied from 0 to 4.63 g/L, depending on the microorganisms involved in the fermentations (Table 1). *Sc. pombe* consumed all the malic acid in the *SP* and *LT...SP* treatments. This result shows the ability of some *Sc. pombe* strains to degrade up to 100% malic acid during alcoholic fermentation (Benito *et al.*, 2014b, 2015a, 2016a; Domizio *et al.*, 2017; Minnaar *et al.*, 2017a). The *SCxOE* and SCxLB

TABLE 1

Final analysis of fermentations of white must grapes: *S. cerevisiae* alone (SC), sequential fermentation with *S. pombe* and *L. thermotolerans* (LT…SP), co-inoculation of *S. cerevisiae* and *O. oeni* (SCxOE), co-inoculation of *S. cerevisiae* and *L. plantarum* (SCxLP), *Sc. pombe* alone (SP), and fermentations after a malolactic fermentation with *O. oeni* (SC…OE).

Parameter	SC	SC…OE	SCxOE	SCXLP	LT…SP	SP
L-lactic acid (g/L)	< 0.1 a	2.8 ± 0.17 b	2.87 ± 0.21 bc	2.57 ± 0.22 b	3.37 ± 0.11 c	< 0.1 a
L-malic acid (g/L)	4.63 ± 0.06 c	< 0.1 a	0.53 ± 0.32 b	0.8 ± 0.36 b	< 0.1 a	< 0.1 a
Acetic acid (g/L)	0.63 ± 0.06 b	0.80 ± 0.08 c	0.67 ± 0.06 b	0.60 ± 0.07 b	0.59 ± 0.05 b	0.23 ± 0.06 a
Total acidity (g/L)	9.23 ± 0.06 d	6.55 ± 0.15 bc	6.77 ± 0.22 c	7.07 ± 0.17 c	6.20 ± 0.16 b	3.07 ± 0.06 a
pH	3.1 ± 0 a	3.25 ± 0.07 b	3.27 ± 0.06 b	3.27 ± 0.06 b	3.3 ± 0 b	3.4 ± 0 c
Ethanol (% v/v)	11.89 ± 0.14 a	11.86 ± 0.21 a	11.80 ± 0.25 a	11.82 ± 0.20 a	11.75 ± 0.21 a	12.21 ± 0.15 b
Residual sugar (g/L)	1.57 ± 0.35 d	1.35 ± 0.35 bc	2.3 ± 0 d	2.0 ± 0.3 d	1.1 ± 0.1 b	0.13 ± 0.11 a
Glucose (g/L)	0.30 ± 0.09 b	0.35 ± 0.21 b	0.70 ± 0.12 d	0.62 ± 0.14 cd	0.50 ± 0.19 c	< 0.1 a
Fructose (g/L)	1.20 ± 0.30 cd	1.00 ± 0.14 c	1.6 ± 0.01 e	1.4 ± 0.30 de	0.57 ± 0.06 b	0.13 ± 0.11 a
Density	0.993	0.992	0.992	0.992	0.991	0.989
Glycerol (g/L)	6.70 ± 0.17 b	6.70 ± 0.22 b	6.57 ± 0.06 b	6.57 ± 0.06 b	7.30 ± 0.11 c	5.90 ± 0.20 a
Extract	22.27 ± 0.31 d	18.85 ± 1.34 b	20.27 ± 0.78 c	20.0 ± 0.36 c	18.93 ± 0.15 b	13.43 ± 0.15 a
Sugar-free extract (g/L)	20.77 ± 0.25 c	17.5 ± 0.99 b	17.63 ± 0.84b	18.03 ± 0.15b	17.83 ± 0.15 b	13.27 ± 0.15 a

Results are the mean ± SD of three replicates. Means in the same row with the same letter are not significantly different (p < 0.05)

FIGURE 1

Fermentation kinetics of the different treatments measured gravimetrically by the total weight loss during the development of the alcoholic fermentation: *S. cerevisiae* alone (*SC*), sequential fermentation with *S. pombe* and *L. thermotolerans* (*LT…SP*), co-inoculation of *S. cerevisiae* and *O. oeni* (*SCxOE*), co-inoculation of *S. cerevisiae* and *L. plantarum* (*SCxLP*), *Sc. pombe* alone (*SP*), and fermentations after malolactic fermentation with *O. oeni* (*SC…OE*).

treatments consumed 88% and 82% of the initial malic acid, respectively. Previous studies reported deacidifications of approximately 89% for similar fermentation strategies involving *L. plantarum*, and from 77% to 90% for similar strategies involving *O. oeni* from must with initial contents of malic acid of approximately 2 g/L (Minnaar *et al*., 2017b, 2019; Brizuela *et al*., 2018). It should also be considered that co-inoculations involving *L. plantarum* are thought to mainly consume malic acid in low acidic wines with a pH of over 3.5 (G-Alegría *et al*., 2004; Lucio *et al*., 2016, 2018). The original level of malic acid in the present study was 4.7 g/L and the initial pH was 3.09. Other authors have reported important strain variability and pH influence in malic acid consumption that varies from 50% to 100% for *L. plantarum* in synthetic wine (Iorizzo *et al*., 2016).

Malic acid consumption influenced the final levels of total acidity in the resulting wines. The final total acidity levels varied from 3.07 g/L to 9.23 g/L (Table 1). Compared to the pure culture of *S. cerevisiae* (*SC*), the pure culture of *Sc. pombe* (*SP*) reported a threefold decrease in the total acidity, while the malolactic fermentations performed by *O. oeni* and *L. plantarum* showed a one-fold decrease. The fact that *Sc. pombe* metabolised all malic acid to ethanol and carbon dioxide by producing no lactic acid explains these enormous differences (Minnaar *et al*., 2017a; Benito, 2019).

pH

The reduction in malic acid affected the pH of the *Sc. pombe* pure fermentation (*SP*); the pH increased by 0.3 units compared to that of the *S. cerevisiae* pure alcoholic fermentation (*SC*). Previous studies reported the potential of *L. thermotolerans* as a biological acidifier agent (Kapsopoulou *et al*., 2005, 2007; Balikci *et al*., 2016; Porter *et al*., 2019a). Although fermentations involving *L. thermotolerans* produced more lactic acid than the treatments involving LAB (Table 1), no significant differences were observed for pH because

Sc. pombe consumed the malic acid during the alcoholic fermentation.

Acetic acid

The acetic acid levels varied from 0.23 to 0.8 g/L (Table 1). The levels detected with the sequential inoculation of *L. thermotolerans* and *Sc. pombe* (*LT…SP*) did not differ significantly from the treatments fermented by *S. cerevisiae*. Although *Sc. pombe* is historically known as a high producer of acetic acid, reaching levels of 1 g/L (Benito *et al*., 2014b; Minnaar *et al*., 2017a; Miljić *et al*., 2017), recent studies have shown satisfactory results with regard to acetic acid levels. Some studies reported final acetic concentrations varying from 0.07 g/L to 0.2 g/L (Benito *et al*., 2016b; Du Plessis *et al*., 2017b; Scansani *et al*., 2020). The low acetic acid production by *Sc. pombe* is related to strain selection processes (Benito *et al*., 2016b), combined fermentations with *S. cerevisiae* (Benito *et al*., 2014b; Benito, 2019) or fed-batch fermentation technology (Roca-Domènech *et al*., 2018). In this study, the selected *Sc. pombe* strain on its own (*SP*) produced the lowest concentration of acetic acid, of 0.23 g/L (Table 1). The *SC…OE* treatment showed the highest acetic acid concentration.

Volatile compounds
Higher alcohols

In accordance with previous works (Benito *et al*., 2016a; Scansani *et al*., 2020), the *Sc. pombe* pure culture (*SP*) produced lower concentrations of most higher alcohols compared to the treatments involving *S. cerevisiae* or involving the sequential inoculation of *L. thermotolerans* and *Sc. pombe* (*LT…SP*). The *SP* fermentation produced approximately 30%, 50% and 50% less i-butanol, 3-methyl butanol and 2-phenyl ethanol, respectively than the fermentations involving *S. cerevisiae* (Table 2). Low levels of higher alcohols may increase the aromatic complexity.

TABLE 2

Final concentration of volatile compounds in fermentations from white grape musts: *S. cerevisiae* alone (*SC*), sequential fermentation with *Sc. pombe* and *L. thermotolerans* (*LT...SP*), co-inoculation of *S. cerevisiae* and *O. oeni* (*SCxOE*), co-inoculation of *S. cerevisiae* and *L. plantarum* (*SCxLP*), *S. pombe* alone (*SP*), and fermentations after malolactic fermentation with *O. oeni* (*SC...OE*).

Volatile compounds	SC	SC...OE	SCxOE	SCxLP	LT...SP	SP
Esters						
Ethyl lactate (mg/L)	11.16 ± 1.26 a	159 ± 45.96 bc	196.83 ± 36.48 bc	156.5 ± 22.02 b	202.83 ± 7.11 c	5.83 ± 0.29 a
Ethyl butanoate (µg/L)	507.83 ± 117.43 a	472 ± 15.56 a	451 ± 119.41 a	410.83 ± 148.37 a	878 ± 35.95 b	1009.83 ± 77.80 b
Ethyl isobutanoate (µg/L)	507.77 ± 117.55 b	472 ± 15.56 b	452.67 ± 122.28 b	410.83 ± 148.37 b	34.67 ± 3.69 a	n.q.*
Ethyl octanoate (µg/L)	1343 ± 432.51 ab	1602.25 ± 239.36 b	1315.67 ± 634.23 ab	1150.17 ± 634.23 ab	906.17 ± 111.4 a	1079.67 ± 43.61 ab
Ethyl propanoate (µg/L)	193.17 ± 34.29 b	253.25 ± 3.89 c	152.82 ± 21.26 b	157.5 ± 33.55 b	238.33 ± 14.93 c	83.93 ± 19.14 a
Ethyl benzene acetate (µg/L)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ethyl decanoate (µg/L)	352.83 ± 154.36 a	505.5 ± 161.22 ab	567.5 ± 120.21 b	451.33 ± 19.76 ab	387.5 ± 33.78 ab	327.16 ± 10.20 a
Ethyl hexanoate (µg/L)	960.5 ± 434.69 a	966.25 ± 61.87 a	660.17 ± 421.31 a	656.83 ± 398.69 a	1215.5 ± 30.33 a	1220.83 ± 178.08 a
Diethyl succinate (µg/L)	n.q.*	378.75 ± 21.57	n.q.*	n.q.*	n.q.*	n.q.*
3-Methylbutyl acetate (µg/L)	3621.83 ± 1216.64 a	2168 ± 356.74 a	3614.5 ± 1500.31 a	3126.83 ± 1500.31 a	10150.5 ± 1356.18 c	6532.33 ± 439.77 b
2-Methylbutyl acetate (µg/L)	164.5 ± 58.32 a	93.75 ± 12.37 a	152.67 ± 77.68 a	132.33 ± 59.98 a	383.83 ± 29.3 b	363.17 ± 15.45 b
Phenethyl acetate (µg/L)	281 ± 14,57 b	185.5 ± 25.46 a	318.83 ± 49.95 b	291 ± 16.07 b	503 ± 47.77 c	283.67 ± 19.53 b
Higher alcohols						
i-Butanol (mg/L)	28.0 ± 2.29 b	24.5 ± 0.71 b	26.3 ± 1.04 b	25.0 ± 3.77 b	28.33 ± 1.61 b	18.67 ± 0.29 a
2-Methyl butanol (mg/L)	25.33 ± 3.01 ab	23 ± 0 a	25.33 ± 1.53 ab	22.5 ± 3.12 a	28.33 ± 1.53 b	23.17 ± 1.53 a
3-Methyl butanol (mg/L)	212.5 ± 21.11 c	180.75 ± 3.89 b	219.5 ± 14.81 c	198.83 ± 19.26 bc	221.67 ± 12.98 c	100.17 ± 5.20 a
Hexanol (µg/L)	2033.17 ± 79.62 b	2024.75 ± 133.29 b	1944 ± 2.18 ab	1825.17 ± 129.29 a	2052 ± 44.6 b	1997.5 ± 88.24 b
2-Phenyl ethanol (mg/L)	25.5 ± 2.78 c	23 ± 0 c	25 ± 1.32 c	23.67 ± 1.04 c	18.67 ± 0.28 b	10.83 ± 0.29 a
Fatty acids						
Isovaleric acid (µg/L)	2624.67 ± 154.33 cd	2469.75 ± 14.50 c	2714.33 ± 96.78 d	2530.67 ± 37.19 c	2124 ± 17.73 b	1764.67 ± 13.20 a
Hexanoic acid (caproic acid) (mg/L)	13.67 ± 0.58 bc	14 ± 0 bc	14.5 ± 0.87 c	13.5 ± 0.5 b	10.83 ± 0.29 a	11.5 ± 0.5 a

TABLE 2 (CONTINUED)

Volatile compounds	SC	SC...OE	SCxOE	SCxLP	LT...SP	SP
Octanoic acid (caprylic acid) (mg/L)	11.83 ± 0.76b	12 ± 0 b	12.17 ± 1.26 b	11 ± 0.5 b	8.83 ± 0.29 a	7.67 ± 0.76 a
Decanoic acid (µg/L)	4 ± 0 b	4 ± 0 b	3.67 ± 0.58 b	3.67 ± 0.29 b	3.33 ± 0.58 b	2.33 ± 0.58 a
Terpenes						
Linalool oxide-1 (µg/L)	11.83 ± 0.29 ab	12 ± 0.71 ab	12.67 ± 0.76 b	11.5 ± 0.5 a	12.67 ± 0.76 b	11.5 ± 0.5 a
Linalool oxide-2 (µg/L)	6.0 ± 0.5 a	6.0 ± 0 a	6.5 ± 0.5 a	6.0 ± 0.71 a	6.5 ± 0.5 a	6 ± 0 a
Linalool (µg/L)	4.33 ± 1.44 a	3.5 ± 0.71 a	4.5 ± 1.32 ab	4 ± 0 a	5.17 ± 0.29 ab	6 ± 0 ab
α-Terpinol (µg/L)	7.17 ± 1.04 a	6 ± 0 a	7.5 ± 1.32 a	6.33 ± 0.58 a	7 ± 0.5 a	6.5 ± 0 a

Results are the mean ± SD of three replicates. Means in the same row with the same letter are not significantly different ($p < 0.05$)

n.q. = not quantifiable

However, high concentrations mask the wine bouquet. The production of wine with a low concentration of higher alcohols allows the specific varietal aroma of grapes to be fully expressed (Ruiz *et al*., 2019).

As noted in other studies, mixed fermentations with *L. thermotolerans* and *Sc. pombe* (*LT...SP*) increase the concentration of all higher alcohols compared to the case with *Sc. pombe* pure culture (*SP*) (Benito *et al*., 2016a). The ability of *L. thermotolerans* to increase the concentration of higher alcohols was observed in a previous study using mixed fermentation by *L. thermotolerans* and *S. cerevisiae* (Comitini *et al*., 2011). Subsequent studies using the same mixed culture showed increments in higher alcohol concentrations independent of the inoculation mode of co-inoculation or sequential fermentation employed (Gobbi *et al*., 2013). Other authors have reported an increase in the levels of higher alcohol only in co-inoculation cultures (Balikci *et al*., 2016).

Esters

Esters can affect wine quality positively, imparting a fruity character to wine bouquets, especially wine from varieties with a neutral flavour (Ruiz *et al*., 2019). The concentration of ethyl lactate correlated with the lactic acid concentration (Table 2). Ethyl lactate traditionally appears during MLF because of an increase in lactic acid. The pH and ethanol concentration influence the production of ethyl lactate during MLF (Knoll *et al*., 2012). The lower the pH value and ethanol content, the higher the level of ethyl lactate. The *LT...SP* treatments showed the highest concentrations of ethyl lactate (Table 2) and lactic acid (Table 1). A previous study reported the weak ability of *L. thermotolerans* to produce esters, except for ethyl lactate (Gobbi *et al*., 2013).

A low production of ethyl isobutyrate (fruity, strawberry, lemon) (Sumby *et al*., 2010) was observed in the *LT...SP* treatments, whereas the concentration of this ester was not quantifiable in the *SP* treatments. The ethyl propionate concentration was higher in the *LT...SP* and *SC...OE* treatments (Table 2), while the *SP* treatments showed the lowest level of ethyl propionate. The *LT...SP* treatments reported the highest levels of 2-methylbutyl acetate and 3-methylbutyl acetate (isoamyl acetate), followed by the *SP* treatments. The *LT...SP* treatments showed the highest concentrations of phenyl ethyl acetate, followed by the *SCxOE* treatments. Other authors have reported *L. thermotolerans* as a high producer of phenyl ethyl acetate (Comitini *et al*., 2011; Gobbi *et al*., 2013; Benito, 2018; Porter *et al*., 2019b).

Fatty acids

Fatty acids have a low threshold level and therefore, depending on the concentration, can add complexity to or be detrimental to wine quality, imparting unpleasant characteristics such as rancid, cheesy, soy, pungent or fat-like (Ruiz *et al*., 2019). Both the *LT...SP* and *SP* treatments reported lower concentrations of all fatty acids compared to treatments involving *S. cerevisiae*. Regarding octanoic and decanoic acid, no significant differences were observed between the *S. cerevisiae* pure fermentation (*SC*) and wine that underwent MLF (Table 2).

CONCLUSIONS

This study shows interesting differences between the different biological deacidification strategies. The pure culture of *Sc. pombe* degraded all the malic acid and produced the lowest total acidity values. The results also showed that mixed cultures of *L. thermotolerans* and *Sc. pombe* can properly ferment musts that present adverse conditions for the correct performance of a classical malic acid fermentation. The *Sc. pombe* pure fermentation produced the lowest concentrations of acetic acid and higher alcohols, while yielding the highest ethanol production.

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