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Combined Use of *Schizosaccharomyces pombe* and a *Lachancea thermotolerans* Strain with a High Malic Acid Consumption Ability for Wine Production

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Abstract: The development of new fermentative strategies exploiting the potential of different wine-related species is of great interest for new winemaking conditions and consumer preferences. One of the most promising non-conventional approaches to wine fermentation is the combined use of deacidifying and acidifying yeasts. *Lachancea thermotolerans* shows several other properties besides lactic acid production; among them, high malic acid consumption is of great interest in the production of red wines for avoiding undesirable refermentations once bottled. The combination of a *L. thermotolerans* strain that is able to consume malic acid with a *Schizosaccharomyces pombe* strain helps to ensure malic acid elimination during alcoholic fermentation while increasing the final acidity by lactic acid production. To properly assess the influence of this alternative strategy, we developed combined fermentations between specific strains of *L. thermotolerans* and *S. pombe* under sequential inoculation. Both species showed a great performance under the studied conditions, influencing not only the acidity but also the aromatic compound profiles of the resulting wines. The new proposed biotechnological strategy reduced the final concentrations of ethanol, malic acid and succinic acid, while it increased the concentrations of lactic acid and esters.

Keywords: wine; non-*Saccharomyces*; alternative fermentation strategies; biological acidity management; volatile compounds

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1. Introduction

The combined use of *Lachancea thermotolerans* and *Schizosaccharomyces pombe* has been recently described as an alternative strategy in winemaking for malolactic fermentation. Malolactic fermentation decreases acidity, since malic acid is more acidic than lactic acid. An excessive reduction in total acidity can lead to spoilage, so winemakers sometimes have to re-acidify wines by adding tartaric acid. The aim of the alternative strategy is to reduce the malic acid content through *S. pombe* metabolism in the red wine while maintaining the total acidity through lactic acid production by *L. thermotolerans* during the alcoholic fermentation [1]. This methodology should be considered for grape juices from warm viticulture areas that possess high sugar concentrations and high pH levels. Under those circumstances, it is difficult to perform classical malolactic fermentation without any deviation.

L. thermotolerans selection procedures have traditionally been focused on the ability to produce L-lactic acid to acidify wine [2–4]. Nevertheless, recent studies have focused on other secondary traits of *L. thermotolerans* that can also improve wine quality [5,6]. Among

those secondary objectives, malic acid consumption is one of the most important ones. The first studies that reported malic acid consumption in *L. thermotolerans* stated that most strains consumed 10% to 20% of it [6]. However, novel studies showed that specific *L. thermotolerans* strains may consume up to 50% of malic acid or even more in both single and mixed cultures [4,7]. The deacidification carried out by this non-*Saccharomyces* yeast is of great interest when wines require malic acid stabilization, since this reduction in the content of malic acid may reduce the risk of undesirable malolactic fermentation by lactic acid bacteria. However, any *L. thermotolerans* strain can totally consume all the malic acid present in standard grape musts.

S. pombe is noted for its malic acid consumption. The malic acid content reduction by this species varies from 60% to 100%, depending on the strain [8,9]. Nevertheless, the use of some strains of *S. pombe* is limited by the high production of acetic acid, over 0.8 g/L [8]. High acetic acid production is one of the main collateral effects of *S. pombe* strains, as only 5% of the isolated strains produce acceptable levels. Recent studies have reported characteristics of specific *S. pombe* strains other than malic acid deacidification, such as a high production of polysaccharides, mannoproteins, galactomannoproteins, pyruvic acid, glycerol and stable anthocyanins and a low production of higher alcohols and ethyl carbamate precursors [1,8,9]. Recent studies have started to pay attention to other yeast species besides those in the *Schizosaccharomyces* genus that are able to significantly reduce the initial malic acid concentration, such as *Pichia kudriavzevii* [10,11] and *Hanseniaspora occidentalis* [12]. Those species can reduce the initial malic acid content by about 50%, although their fermentative metabolism is less efficient.

This study proposes the use of an *L. thermotolerans* strain with high lactic acid production and high malic acid consumption and a commercial *S. pombe* strain to ensure proper alcoholic fermentation in order to produce a red wine of the Tempranillo grape variety that does not require classic malolactic fermentation.

2. Materials and Methods

2.1. Microorganisms

The study used the following yeast strains: *Lachancea thermotolerans* L1 (Complutense University of Madrid, Madrid, Spain), *Saccharomyces cerevisiae* AG006 (Agrovín S.L, Alcazar de San Juan, Spain) and *Schizosaccharomyces pombe* Atecrem 12H (Bioenologia, Oderzo, Italy).

2.2. Vinification

All fermentations used the grape juice of *Vitis vinifera* L. cultivar Tempranillo grapes grown at the Cuzcurrita vineyard (Rioja Alta, Spain). The grape juice was taken from a fermentation tank just after the grapes were destemmed, crushed and introduced into the tank, before any inoculation. The must was enriched with 0.15 g/L of di-ammonium phosphate and 0.30 g/L of Actimax Natura (Agrovin, Spain). Then, the must was pasteurized at 105 °C for 1 min in an autoclave: Presoclave 75 (J.P. Selecta, Barcelona, Spain).

All fermentations took place in 250 mL Pyrex™ borosilicate glass reagent bottles, each with a slightly open polypropylene cap and pouring ring, allowing for CO_2 release and preventing microbial contamination. Fermentations were carried out at 25 °C in triplicate.

The initial sum of glucose and fructose concentrations was 251.22 g/L, pH = 3.74, primary amino nitrogen = 172 mg/L, ammonia nitrogen = 26 mg/L, malic acid = 2.03 g/L. The initial lactic and acetic acid concentrations were below 0.1 g/L. Four treatments were used. Table 1 describes the strain combinations used in each treatment.

The preculturing of the strains of *L. thermotolerans* (LT), *S. pombe* (SP) and *S. cerevisiae* (SC) was carried out in 40 mL YMB medium for 24 h, with shaking, at 25 °C and 150 rpm in 100 mL borosilicate bottles. The optical density of the cultures was determined using a spectrophotometer (Genesys 2.0 Spectrophotometer, ThermoFisher, Waltham, MA, USA), and the lowest value was used. The fermentation cultures were inoculated at a concentration of 10^6 cells/mL (≈0.2 O.D.).

Table 1. Must inoculum compositions.

SC	*S. cerevisiae* (10^6 CFU/mL) alone.
LT . . . SC	*L. thermotolerans* (10^6 CFU/mL) followed by *S. cerevisiae* (10^6 CFU/mL) 5 days later.
LT . . . SP	*L. thermotolerans* (10^6 CFU/mL) followed by *S. pombe* (10^6 CFU/mL) 5 days later.
SP	*S. pombe* (10^6 CFU/mL) alone.

SC: *S. cerevisiae*, LT: *L. thermotolerans*, SP: *S. pombe*.

Fermentation monitoring was performed by measuring the weight loss every 24 h. Fermentation was considered complete when the weight loss was less than 0.01% for two consecutive days. The initial weight of each fermentation was considered as 100%.

In the sequential fermentations (LT . . . SC and LT . . . SP), the yeast of the most fermentative species (*S. cerevisiae* or *S. pombe*) was inoculated 5 days (96 h) after the initial inoculation of *L. thermotolerans*. The alcoholic fermentations took place at 25 °C in a thermostatic chamber.

2.3. Chemical Parameter Measurements

A Y15 Autoanalyzer and its commercial kits (Biosystems, Barcelona, Spain) were used in the determinations of glucose + fructose, L-malic acid, L-lactic acid, acetic acid, succinic acid and glycerol concentrations. The alcohol content was determined using the boiling method of GAB Microebu (http://shop.gabsystem.com (accessed on 20 December 2022)). A Crison pH Meter Basic 20 (Crison, Spain) measured the final pH.

2.4. Volatile Compounds

The volatile compounds of the fermentations were measured according to a previous methodology [13]. The samples were analyzed in triplicate using headspace solid-phase microextraction coupled with gas chromatography–mass spectrometry (HS-SPME-GC-MS). Two milliliters of each sample and 0.2 g of sodium chloride were placed in a 20 mL glass vial, and 10 µL of 4-methyl-2-pentanol solution (802 µg/mL in methanol) was added as an internal standard. The volatile compounds were extracted from the headspace with a 2 cm DVB/CAR/PDMS fiber (50/30 µm, Supelco, Bellefonte, PA, USA) at 45 °C for 30 min after 10 min of incubation. The fiber was conditioned at 250 °C prior to the sample extraction. After the extraction, the SPME fiber was immediately transferred to the injection port of a Trace 1310 gas chromatograph equipped with a TSQ 8000 EVO mass spectrometer (Thermo Fisher Scientific, MA, USA) to be thermally desorbed in splitless mode at 240 °C for 3 min. A DB-WAX polar capillary column (60 m × 0.25 mm i.d. × 0.25 µm film thickness, J&W Scientific, Folsom, CA, USA) was used to separate the volatile compounds of the samples. Helium was used as the carrier gas at a flow rate of 1.6 mL/min. The initial column temperature was set to 50 °C and held for 3 min. Afterwards, the temperature was increased to 220 °C at a rate of 5 °C/min and held at 220 °C for 8 min. Mass spectra were detected in electron impact (EI) mode at 70 eV, with a scan range from m/z 33 to m/z 300. The MS transfer line and the ionization source temperatures were 220 and 240 °C, respectively.

The RIs of the volatiles were calculated via co-injection with an alkane mixture (C7-C21, Sigma-Aldrich, St. Louis, MO, USA). Volatiles were identified by matching the obtained mass spectra with the standard NIST 08 library and by comparing the retention indices (RIs) to those of the compounds reported in the literature and the NIST Webbook (https://webbook.nist.gov/chemistry (accessed on 20 December 2022)). Moreover, the identification of a selected number of volatile compounds was confirmed by comparing the retention indices and mass spectra with those of the authentic reference compounds. Supplementary Table S1 shows the measured RIs and those reported in the literature.

2.5. Color Intensity

A Y350 diode array spectrophotometer (Biosystems, Spain) was used for the analysis. The samples were analyzed in a 1 mm path-length quartz cuvette with a range of

200–1100 nm. Absorbance at 420, 520 and 620 nm was measured. Color intensity was calculated as the sum of absorbance at the three wavelengths.

2.6. Statistical Analyses

All statistical analyses were performed using R software version 4.1.2 (R Development Core Team, Vienna, Austria, 2013). Analysis of variance (ANOVA) and Tukey post hoc tests were applied to compare the different groups and values.

3. Results and Discussion

3.1. Fermentation Kinetics

The fermentations lasted between 18 days (the fermentation conducted by *S. pombe*) and 27 days (the sequential fermentation conducted by the LT and SC strains) (Figure 1). A slowdown took place after 3–4 days of fermentation, and another took place after 9 days. The alcoholic fermentation of the combined fermentation between *L. thermotolerans* and *S. pombe* lasted for 22 days, which is the same time employed by the pure *S. cerevisiae* control. Three previous studies report delays that vary from 4 to 8 days for the new biotechnology compared to the regular *S. cerevisiae* control for alcoholic fermentation, while three other studies reported no differences, as is the case in this study [1]. However, regular *S. cerevisiae* fermentations made in red wines require performing additional malolactic fermentation before bottling to avoid possible refermentation problems. This additional process may require at least 21 additional days to obtain a stable wine from a microbiological point of view.

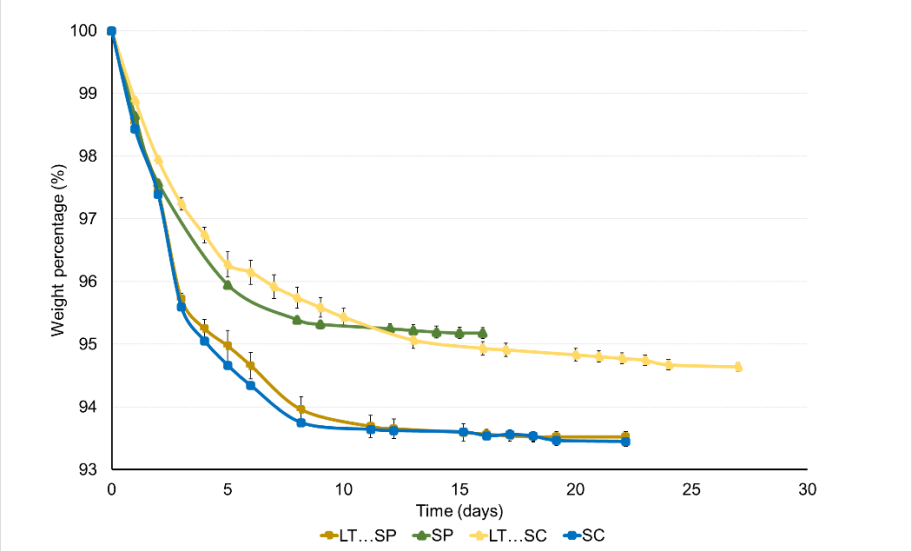

Figure 1. Fermentation kinetics of gravimetrically measured variants by total weight loss during fermentation. *S. cerevisiae* alone (SC); sequential fermentation with *S. cerevisiae* and *L. thermotolerans* (LT . . . SC); sequential fermentation with *S. pombe* and *L. thermotolerans* (LT . . . SP); *S. pombe* alone (SP). Data points represent the averages and standard deviation of the three replicates for each condition. SC fermentations are in blue; SP fermentations are in green; LC . . . SC fermentations are in yellow; LT . . . SP fermentations are in brown.

Previous studies have described that the initial consumption of nutrients by *L. thermotolerans* can compromise the availability of nutrients for the fermentative yeast strain used in the sequential mode to conclude the alcoholic fermentation [1,6,7]. The rapid fermentation by the *Schizosaccharomyces pombe* strain could have taken place due to the lower nutrient demands of this species, despite it normally showing slower kinetics due to its reproduction by bipartition, which requires more time than the budding of most yeasts [14].

3.2. Glucose and Fructose

Most of the fermentations showed final sugar concentrations of glucose and fructose below 2 g/L (Table 2). The only trial that showed a slightly higher concentration of 4.73 g/L was the sequential fermentation with *L. thermotolerans* and *S. cerevisiae*. Previous authors explained this effect by the higher nutrient demands of *L. thermotolerans* that can compromise the performance of *S. cerevisiae* in sequential fermentations. Most manufacturers recommend a second round of nutrient addition concurrently with *S. cerevisiae* inoculation to avoid this problem of a lack of nutrients that can slow down the last stages of alcoholic fermentation [7].

Table 2. Final chemical analysis of fermentations from Tempranillo red grapes: *S. cerevisiae* alone (SC); sequential fermentation with *S. cerevisiae* and *L. thermotolerans* (LT . . . SC); sequential fermentation with *S. pombe* and *L. thermotolerans* (LT . . . SP); *S. pombe* alone (SP).

	SC	LT . . . SC	LT . . . SP	SP
L-lactic acid (g/L)	0.11 ± 0.03 ^{a}	2.26 ± 0.64 ^{c}	1.52 ± 0.09 ^{b}	0.16 ± 0.02 ^{a}
L-malic acid (g/L)	1.41 ± 0.03 ^{d}	1.11 ± 0.10 ^{c}	0.11 ± 0.03 ^{a}	0.48 ± 0.07 ^{b}
Succinic acid (g/L)	1.47 ± 0.06 ^{c}	1.41 ± 0.07 ^{bc}	1.34 ± 0.03 ^{b}	1.26 ± 0.04 ^{a}
Acetic acid (g/L)	0.36 ± 0.05 ^{a}	0.51 ± 0.07 ^{b}	0.45 ± 0.03 ^{b}	0.45 ± 0.01 ^{b}
pH	3.77 ± 0.02 ^{b}	3.62 ± 0.02 ^{a}	3.78 ± 0.04 ^{b}	3.83 ± 0.01 ^{c}
Ethanol (g/L)	11.63 ± 0.06 ^{c}	11.21 ± 0.06 ^{a}	11.46 ± 0.06 ^{b}	11.72 ± 0.04 ^{c}
Glucose + Fructose (g/L)	1.24 ± 0.21 ^{a}	4.73 ± 0.06 ^{c}	1.85 ± 0.24 ^{b}	1.56 ± 0.14 ^{ab}
Glycerol (g/L)	9.25 ± 0.32 ^{b}	8.96 ± 0.47 ^{ab}	9.13 ± 0.26 ^{b}	8.26 ± 0.14 ^{a}

Results are mean ± SD of three replicates. Different letters indicate statistical significance between groups.

All the employed species employed in this study (*S. cerevisiae*, *S. pombe* and *L. thermotolerans*) are reported to show a preference to consume glucose against fructose [1,6,7,14], so, in demanding situations, significant amounts of fructose may remain in the wine. *S. cerevisiae* [1] and *S. pombe* [14] can completely metabolize glucose and fructose into ethanol for regular dry wines under no severe stress conditions. *L. thermotolerans* is widely reported to not be able to metabolize sugar concentrations over 170 g/L [6,7], which makes it impossible to produce regular wines using only strains of *L. thermotolerans*, although modern studies manage to produce beer or base sparkling wines [7]. In this study, the potential undesirable effect of residual sugars was diminished by combining *L. thermotolerans* with the more fermentative species *S. cerevisiae* and *S. pombe*.

3.3. Ethanol

Fermentations involving *L. thermotolerans* showed significantly lower concentrations of ethanol than fermentations involving *S. pombe* and *S. cerevisiae* (Table 2). The maximum difference was 0.65% (*v*/*v*) between the sequential fermentation involving *L. thermotolerans* and *S. cerevisiae* and the pure *S. pombe* fermentation. Although there were significant differences from a statistical point of view, the quantitative reduction represented about 1%, which is low compared to other, more effective technologies that are able to significantly reduce the final ethanol content at an industrial scale. *L. thermotolerans* fermentative metabolism is different, since part of the carbon flux is derived through lactic acid and glycerol production, together with the fact that this yeast is not a strongly fermentative one [7]. Previous authors reported differences of up to 3% (*v*/*v*), whereas others reported no significant differences regarding the ethanol content [1]. Additionally, *S. pombe* produces ethanol from malic acid metabolism and occasionally may increase the final ethanol concentration compared to *S. cerevisiae* controls when the initial concentration of malic acid is high [10].

3.4. L-Lactic Acid

Pure fermentations of *S. pombe* and *S. cerevisiae* did not show a significant production of lactic acid (Table 2). Fermentations involving *L. thermotolerans* produced significant final

concentrations of L-lactic acid that clearly influenced the final pH. The combined fermentation with *L. thermotolerans* and *S. cerevisiae* produced 2.26 g/L of lactic acid; this effect reduced the pH in 0.15 units compared to the *S. cerevisiae* control. The combined fermentation with *L. thermotolerans* and *S. pombe* produced 1.52 g/L of lactic acid, and it did not show differences in pH compared to the *S. cerevisiae* control due to the malic acid decrease that compensated the pH reduction by lactic acid. Previous studies reported the opposite effect, as all of them determined that the combined fermentations with *L. thermotolerans* and *S. pombe* produced higher final amounts of lactic acid than the combined fermentations with *L. thermotolerans* and *S. cerevisiae* [1]. These studies have indicated that the slower kinetics of *S. pombe* allow *L. thermotolerans* to survive longer. However, in this study, the combined fermentation using *L. thermotolerans* and *S. pombe* was faster than the combined fermentation using *L. thermotolerans* and *S. cerevisiae* (Figure 1). Recent studies indicate that the interactions between different strains of distinct species vary highly, indicating inhibition or symbiosis, depending on the specific strains [15]. The lactic acid production of *L. thermotolerans* under mixed fermentation conditions is extremely variable, ranging from 0 to 9 g/L; nevertheless, the most common value is around 3 g/L of lactic acid [7].

3.5. Malic Acid

The pure fermentation of *S. cerevisiae* reduced the initial concentration of malic acid by 30.5%. Previous studies reported a strain variability of malic acid production of up to 0.7 g/L and a variability of malic acid degradation of up to 50% [10]. The pure fermentation of *S. pombe* reduced malic acid significantly, by 78%, which clearly influenced the final pH. For *S. pombe*, the malic acid degradation varied from 60% to 100%, depending on the selected strain [16]. Although a 78% reduction is significant from a de-acidification point of view, it is not enough to stabilize a red wine from the malic acid point of view. Most previous studies have reported that combined fermentations with *L. thermotolerans* and *S. pombe* consume 95–100% of malic acid [1]. Similar studies have reported smaller reductions varying from 50% to 75% [1,17,18]. The main use of the commercial *S. pombe* strain employed in the study is to de-acidify wine, but not to stabilize it. The combined fermentation with the *L. thermotolerans* strain, which has a special ability to reduce malic acid, and *S. cerevisiae* reduced the initial concentration of malic acid by 45%. Finally, the combined fermentation with the malic-acid-metabolizing *L. thermotolerans* strain and *S. pombe* reduced the initial concentration of malic acid by almost 100%, achieving malic acid stabilization. The results show that the combined use of more than one malic-acid-consuming microorganism increases the stability of wines from a malic acid point of view.

3.6. Acetic Acid

S. cerevisiae produced the lowest final concentration of acetic acid: 0.36 g/L; the other fermentations produced 0.45 to 0.51 g/L (Table 2). All the final values were moderate and below the faulty threshold of 0.8 g/L [19]. Most studies reported that *Lachancea thermotolerans* species produce less acetic acid than *S. cerevisiae*, although others reported the opposite effect [7]. Most studies report that *S. pombe* produces higher acetic acid concentrations than *S. cerevisiae*, although recent studies reported that specific strains produce similar or lower amounts of acetic acid compared to *S. cerevisiae* [1,14]. Although joint fermentation with *L. thermotolerans* and *S. pombe* produced 0.09 g/L more acetic acid than pure *S. cerevisiae* fermentation, we must consider that this fermentation does not require stabilization from a malic acid point of view. During a regular controlled malolactic fermentation without any deviation, the volatile acidity usually increases in about 0.1 g/L [1,15].

3.7. Succinic Acid

Pure *S. pombe* fermentation produced the lowest concentration of succinic acid (1.26 g/L), and pure *S. cerevisiae* fermentations showed the highest concentration (up to 1.47 g/L) (Table 2). The literature reports the *S. cerevisiae* strain variability that results in succinic acid

contents from 0.3 to 1.8 g/L in wine [10]. No previous studies have reported data for *L. thermotolerans* or *S. pombe*.

3.8. Glycerol

S. pombe fermentation resulted in the lowest final concentration of glycerol, whereas the *S. cerevisiae* fermentation resulted in the highest (Table 2). The other trials resulted in intermediate concentrations.

A high glycerol concentration is often related to soft and mouthful sensory properties. The rise of the glycerol concentration in wine is one of the main contributions of some specific non-*Saccharomyces* species to the value of wine [5]. However, this study reports that the pure *S. cerevisiae* control produces higher final glycerol concentrations than its combination with *L. thermotolerans* and the pure *S. pombe* fermentation. This effect can be explained since, although several scientific articles report specific non-*Saccharomyces* as a higher glycerol producer, others report an additional high variability depending on the strain level. Previous studies report a strain variability for *L. thermotolerans* [6,7] and *S. pombe* [14] up to 50% regarding glycerol production. A similar strain variability has been previously reported for *S. cerevisiae*. The *S. cerevisiae* strain employed in this study was selected by the manufacturer, including the glycerol production as a selection parameter, while the employed *L. thermotolerans* strain was selected to produce high lactic acid concentrations, and the *S. pombe* strain was selected to reduce acidity.

The combination between *L. thermotolerans* and *S. pombe* did not show statistical differences compared to the *S. cerevisiae* control. Previous studies report changes in different parameters depending on the interactions between different strains and species that can promote specific metabolic routes or inhibit others. This phenomenon remains widely unknown, but most researchers recommend testing the interactions between different strains before using them at an industry scale to avoid undesirable effects [10].

3.9. Volatile Compounds

Pure fermentations of *S. cerevisiae* and *S. pombe* did not produce any ethyl lactate or isoamyl lactate, and fermentations involving *L. thermotolerans* showed significant final values (almost 90% higher) (Table 3). These results are related to the L-lactic acid production (around 95% higher) observed in *L. thermotolerans* fermentations, which favored the esterification. These lactic acid esters may increase the fruity profiles of the final wines by increasing the fruity and fatty odor series. Fermentations involving *L. thermotolerans* produced less ethyl hexanoate than the others.

The pure fermentations of *S. pombe* were the only ones that did not produce any detectable 3-methyl butanal. The pure *S. cerevisiae* fermentations resulted in the highest values. Additionally, the pure *S. pombe* fermentations produced the highest concentration of 2-nonanol (up to five times higher), and the fermentations involving *S. pombe* were the only ones that generated 1-(1-ethoxyethoxy)pentane. The production of compounds of these types increases the herbaceous and malt aromas in the final wines.

The pure fermentations of *S. pombe* had lower final levels of 3-methyl-1-butanol, ethyl phenylacetate and 2-phenylethyl acetate than pure *S. cerevisiae* fermentation, which can be related to reductions in several undesirable aromas of wines, mainly those related to chemical or synthetic products (Table 3). Previous studies reported that *S. pombe* is a lesser producer of alcohols and esters than *S. cerevisiae* [20,21]. This phenomenon is of interest in order to avoid varietal aroma masking [19]. Additionally, the pure *S. pombe* fermentation had the lowest concentrations of butyrolactone (50%) and phenylethyl alcohol (20%).

Fermentations involving *L. thermotolerans* reduced the final hexanol, dodecanal, ethyl octanoate, ethyl decanoate and hexanoic acid levels compared to the *S. cerevisiae* control by between 20% and 50%, reducing the fruity and floral profiles of the wines, which are usually related with lighter and fresher wines. Additionally, fermentations involving *L. thermotolerans* reduced the concentration of 3-(methylthio)-1-propanol in combined

fermentations with *S. cerevisiae*; the opposite effect took place for combined fermentations with *S. pombe* (three times higher).

Table 3. Final volatile compound profiles of fermentations from Tempranillo red grapes.

Compound (Area Units)	SC	SP	LT . . . SC	LT . . . SP
Ethyl acetate	1.42 ± 0.02 ^{a}	1.59 ± 0.2 ^{a}	0.77 ± 0.41 ^{b}	1.43 ± 0.14 ^{a}
3-Methyl butanal *	2.70 ± 0.62 ^{a}	0.00 ± 0.00 ^{a}	0.87 ± 0.70 ^{a}	1.88 ± 2.45 ^{a}
2-Methylpropyl acetate	0.03 ± 0.00 ^{a}	0.02 ± 0.00 ^{a}	0.01 ± 0.01 ^{b}	0.03 ± 0.00 ^{a}
Ethyl butanoate	0.05 ± 0.01 ^{a}	0.05 ± 0.01 ^{a}	0.01 ± 0.01 ^{b}	0.053 ± 0.01 ^{a}
Toluene	0.15 ± 0.14 ^{a}	0.11 ± 0.10 ^{a}	0.13 ± 0.11 ^{a}	0.10 ± 0.09 ^{a}
Ethyl 3-methylbutanoate *	5.56 ± 1.04 ^{a}	2.93 ± 0.28 ^{ab}	1.97 ± 1.89 ^{b}	4.95 ± 1.43 ^{ab}
1-(1-Ethoxyethoxy)pentane *	0.00 ± 0.00 ^{b}	3.24 ± 0.99 ^{a}	0.00 ± 0.00 ^{b}	3.17 ± 1.89 ^{a}
2-Methyl-1-propanol	0.89 ± 0.14 ^{a}	0.69 ± 0.09 ^{ab}	0.48 ± 0.08 ^{b}	0.92 ± 0.25 ^{a}
3-Methyl-1-butyl acetate	0.55 ± 0.07 ^{a}	0.39 ± 0.08 ^{a}	0.25 ± 0.20 ^{a}	0.26 ± 0.07 ^{a}
Butanol *	2.58 ± 2.65 ^{b}	3.73 ± 3.35 ^{b}	31.42 ± 13.54 ^{a}	7.88 ± 0.95 ^{b}
3-Methyl-1-butanol	6.65 ± 0.37 ^{a}	5.88 ± 0.43 ^{a}	5.06 ± 0.23 ^{a}	6.25 ± 1.07 ^{a}
Ethyl hexanoate	0.48 ± 0.10 ^{a}	0.51 ± 0.09 ^{a}	0.21 ± 0.08 ^{a}	0.42 ± 0.26 ^{a}
Ethyl lactate	0.03 ± 0.01 ^{c}	0.02 ± 0.00 ^{c}	0.19 ± 0.04 ^{a}	0.11 ± 0.01 ^{b}
Hexanol	0.29 ± 0.01 ^{a}	0.28 ± 0.02 ^{a}	0.26 ± 0.00 ^{a}	0.29 ± 0.02 ^{a}
cis-3-Hexen-1-ol *	20.35 ± 1.91 ^{a}	18.19 ± 1.51 ^{ab}	17.41 ± 1.23 ^{ab}	13.38 ± 3.91 ^{b}
Ethyl octanoate	0.11 ± 0.03 ^{b}	0.15 ± 0.02 ^{ab}	0.01 ± 0.00 ^{c}	0.19 ± 0.03 ^{a}
2-Nonanol *	4.82 ± 2.04 ^{b}	10.51 ± 1.95 ^{a}	2.71 ± 0.23 ^{b}	4.85 ± 1.33 ^{b}
Ethyl 3-hydroxybutyrate *	3.76 ± 1.12 ^{a}	4.44 ± 0.34 ^{a}	1.70 ± 0.08 ^{b}	2.99 ± 0.85 ^{ab}
Benzaldehyde	0.03 ± 0.00 ^{a}	0.02 ± 0.00 ^{b}	0.02 ± 0.00 ^{ab}	0.02 ± 0.00 ^{ab}
Ethyl nonanoate *	7.92 ± 1.35 ^{a}	7.96 ± 2.38 ^{a}	1.53 ± 0.62 ^{b}	8.51 ± 0.86 ^{a}
Ethyl 2-hydroxy-4-methylpentanoate *	14.75 ± 0.28 ^{a}	10.69 ± 0.67 ^{b}	11.09 ± 0.59 ^{b}	15.78 ± 1.04 ^{a}
Octanol *	11.93 ± 3.63 ^{a}	13.07 ± 1.85 ^{a}	3.66 ± 1.36 ^{b}	13.54 ± 2.62 ^{a}
2-Methyl propanoic acid	0.03 ± 0.00 ^{a}	0.02 ± 0.00 ^{a}	0.03 ± 0.01 ^{a}	0.02 ± 0.00 ^{a}
Isoamyl lactate	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{b}	0.03 ± 0.00 ^{a}	0.03 ± 0.00 ^{a}
Ethyl 2-furoate *	1.32 ± 0.14 ^{a}	1.01 ± 0.08 ^{b}	1.03 ± 0.11 ^{ab}	1.11 ± 0.12 ^{ab}
Methyl benzoate *	1.95 ± 0.27 ^{a}	0.98 ± 0.03 ^{c}	1.73 ± 0.11 ^{ab}	1.33 ± 0.15 ^{bc}
Butanoic acid *	2.04 ± 0.30 ^{a}	1.94 ± 0.31 ^{a}	1.62 ± 0.16 ^{a}	1.68 ± 0.52 ^{a}
Ethyl decanoate	0.01 ± 0.00 ^{a}	0.02 ± 0.00 ^{a}	0.00 ± 0.00 ^{b}	0.02 ± 0.04 ^{a}
Butyrolactone *	7.92 ± 0.11 ^{a}	4.08 ± 0.99 ^{b}	6.52 ± 0.71 ^{a}	8.26 ± 1.12 ^{a}
4-methylbenzaldehyde *	9.11 ± 1.74 ^{a}	6.36 ± 1.15 ^{a}	7.42 ± 1.12 ^{a}	7.30 ± 1.12 ^{a}
Acetophenone *	2.23 ± 0.90 ^{ab}	1.50 ± 0.36 ^{b}	2.17 ± 0.77 ^{ab}	3.79 ± 0.80 ^{a}
2-methyl butanoic acid	0.01 ± 0.00 ^{a}	0.01 ± 0.00 ^{a}	0.01 ± 0.00 ^{a}	0.01 ± 0.00 ^{a}
Diethyl succinate	0.08 ± 0.01 ^{b}	0.05 ± 0.01 ^{b}	0.04 ± 0.01 ^{b}	0.27 ± 0.11 ^{a}
Dodecanal *	4.52 ± 0.57 ^{ab}	4.68 ± 0.39 ^{a}	3.26 ± 0.15 ^{b}	4.21 ± 0.70 ^{ab}
3-(methylthio)-1-propanol *	7.06 ± 1.32 ^{b}	6.63 ± 1.27 ^{b}	3.70 ± 0.89 ^{c}	10.83 ± 0.72 ^{a}
Ethyl phenylacetate *	1.70 ± 0.13 ^{ab}	1.22 ± 0.12 ^{b}	1.76 ± 0.31 ^{a}	1.84 ± 0.14 ^{a}
2-phenylethyl acetate	0.02 ± 0.00 ^{a}	0.01 ± 0.00 ^{a}	0.02 ± 0.00 ^{a}	0.01 ± 0.00 ^{a}
β-damascenone *	4.75 ± 0.92 ^{a}	3.73 ± 0.58 ^{a}	4.61 ± 0.30 ^{a}	4.82 ± 0.68 ^{a}
Ethyl dodecanoate *	0.80 ± 0.12 ^{a}	1.12 ± 0.13 ^{a}	0.00 ± 0.00 ^{b}	1.26 ± 0.36 ^{a}
Hexanoic acid	0.07 ± 0.00 ^{a}	0.07 ± 0.01 ^{a}	0.04 ± 0.00 ^{b}	0.07 ± 0.01 ^{a}
N-(3-Methylbutyl)acetamide	0.06 ± 0.01 ^{b}	0.05 ± 0.00 ^{b}	0.05 ± 0.00 ^{b}	0.09 ± 0.01 ^{a}
Butanedioic acid, ethyl 3-methylbutyl ester *	2.30 ± 0.16 ^{b}	1.77 ± 0.19 ^{b}	1.22 ± 0.21 ^{b}	10.54 ± 4.61 ^{a}
Phenylethyl alcohol	1.44 ± 0.08 ^{a}	1.22 ± 0.11 ^{a}	1.43 ± 0.25 ^{a}	1.55 ± 0.13 ^{a}
Octanoic acid	0.20 ± 0.02 ^{a}	0.23 ± 0.01 ^{a}	0.08 ± 0.00 ^{b}	0.20 ± 0.01 ^{a}
Nonanoic acid	0.04 ± 0.00 ^{a}	0.05 ± 0.02 ^{a}	0.05 ± 0.01 ^{a}	0.04 ± 0.02 ^{a}
Decanoic acid *	25.98 ± 2.68 ^{a}	31.34 ± 2.19 ^{a}	28.92 ± 2.54 ^{a}	17.17 ± 1.96 ^{b}

S. cerevisiae control (SC); *S. pombe* control (SP); sequential fermentation with *S. cerevisiae* and *L. thermotolerans* (LT . . . SC) and *S. pombe* and *L. thermotolerans* (LT . . . SP). Compounds highlighted with an asterisk (*) have a 1000-times-lower area. Results are mean ± SD of three replicates. Different letters indicate statistical significance between groups.

The pure fermentations of *S. cerevisiae* produced the highest concentrations of benzaldehyde, diethyl succinate and 2-methyl butanoic acid (up to two times higher), which are usually related with the fruity, floral and roasted profiles of the final fermentation.

The combined fermentations between *S. pombe* and *L. thermotolerans* had the highest final amounts of N-(3-methylbutyl)acetamide and the lowest finals amounts of decanoic and octanoic acid.

3.10. Color Intensity

The color intensity results were minor and did not show any significant differences (Table 4). Previous studies reported eventual slight differences of up to 10% for *L. thermotolerans* fermentations due to the different colorations of anthocyanins at low pHs and the different yeast strain absorptions [7,17]. *S. pombe* fermentations did not show differences compared to the other trials, although previous studies usually reported higher color intensities due to the formation of highly stable anthocyanin compounds [22].

Table 4. Final color intensity analysis of fermentations from Tempranillo red grapes: *S. cerevisiae* alone (SC); sequential fermentation with *S. cerevisiae* and *L. thermotolerans* (LT . . . SC); sequential fermentation with *S. pombe* and *L. thermotolerans* (LT . . . SP); *S. pombe* alone (SP).

	SC	**LT . . . SC**	**LT . . . SP**	**SP**
420 nm	0.63 ± 0.02 [a]	0.65 ± 0.04 [a]	0.66 ± 0.03 [a]	0.69 ± 0.04 [a]
520 nm	1.22 ± 0.04 [a]	1.30 ± 0.06 [a]	1.26 ± 0.05 [a]	1.29 ± 0.06 [a]
620 nm	0.19 ± 0.01 [a]	0.21 ± 0.02 [a]	0.19 ± 0.02 [a]	0.21 ± 0.03 [a]
CI	2.04 ± 0.07 [a]	2.16 ± 0.12 [a]	2.11 ± 0.10 [a]	2.19 ± 0.13 [a]

Results are mean ± SD of three replicates. Different letters indicate statistical significance between groups.

4. Conclusions

The results show that the combined use of strongly malic-acid-consuming microorganisms increases the stabilization of wines from a malic acid point of view. The consideration of malic acid degradation in the selection of *L. thermotolerans* strains for fermenting red wine may be of great interest in facilitating future stabilization processes, such as malolactic fermentation. The results of the study showed that, when employing the biotechnology that combines *L. thermotolerans* and *S. pombe*, the *L. thermotolerans* strain should possess a great ability to consume malic acid in order to enhance *S. pombe's* malic acid consumption capacity. The proposed strategy reduces the final concentrations of other chemicals, such as ethanol, malic acid and succinic acid, while increasing the concentrations of lactic acid and esters.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9020165/s1, Table S1. Compounds identified using retention indices (RI) measured in the study and those reported in the literature, as well as using confirmation with reference compounds.

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