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Research Article

Influence of different *Lachancea thermotolerans* strains in the wine profile in the era of climate challenge

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Abstract

The study performed sequential fermentations of red grape juice using several strains of *Lachancea thermotolerans* and one strain of *Saccharomyces cerevisiae*. Due to the new conditions imposed by climate change, wine acidity must be affected as well as the volatile profile. Non-*Saccharomyces* yeasts such as *L. thermotolerans* are real alternatives to soften the impact of climate change in winemaking. The *L. thermotolerans* strains included three commercially available strains and two wine-related natural isolates. *L. thermotolerans* showed significant statistical differences in basic chemical parameters such as lactic acid, malic acid, or ethanol concentrations as well as in the volatile profile. *S. cerevisiae* clearly produced some volatile compounds in higher amounts than the studied *L. thermotolerans* strains while others showed the opposite effect. Sequential fermentations involving any of the studied strains of *L. thermotolerans* with *S. cerevisiae* showed an increased volatile profile compared to the *S. ceresisiae* single fermentation, highlighting the synergic effect between the studied species.

Keywords: *Lachancea thermotolerans*; *Saccharomyces cerevisiae*, climate change, wine fermentation, volatile compounds

Introduction

The popularity of non-*Saccharomyces* in modern winemaking and oenology research increased during the last decades (Jolly et al. 2003, 2014, 2017, de Celis et al. 2022) as some of those genera such as *Torulaspora*, *Metschnikowia*, *Pichia* or *Lachancea* possess species that can positively influence the quality of wine. However, we must not forget that other non-*Saccharomyces* genera such as *Brettanomyces/Dekkera* are commonly considered spoilage microorganisms in modern winemaking (Benito et al. 2009, Benito-Vazquez et al. 2021).

Climate change is affecting winemaking not only by reducing grapes acidity and increasing the fermentable sugars concentration (Vicente et al. 2022), but for reducing the volatile profile of the resultant wines too (Pons et al. 2017). The increases in temperatures are generally related with a reduction in the herbaceous vegetal notes of the final wines by affecting the content in pyrazines and carotenoid-derivates (such as norisoprenoids) (Pons et al. 2017). Non-*Saccharomyces* yeast are great aroma-producers mainly to the presence of different extracellular enzymes that can release the volatile molecules for its precursors (Belda et al. 2017). *Lachancea thermotolerans* is generally associated with an increase in the general volatile profiles of final wines by producing several odour-active compounds. Fermentations involving this species are generally associated with an increased fruity and floral profile if compared to the *Saccharomyces cerevisiae* single fermentation (Vicente et al. 2021).

Despite its influence in the aromatic profile, *L. thermotolerans* has focused the attention on it since it is the most favourite biological option to acidify wines during alcoholic fermentations in regions that suffer from lack of acidity due to its specific characteristics (Benito 2018, Vilela 2018, 2019, Porter et al. 2019, Vicente et al. 2022). The use of *L. thermotolerans* allows to increase the final lactic acid from 1 to 6 g/L, which may reduce the final pH down by 0.5 units. Some of the main advantages related to the acidification of *L. thermotolerans* are the stability of L-lactic acid compared to other possible unstable acids present in wine such as tartaric, malic, or citric acid that may suffer chemical or microbiological instabilities in winemaking. The use of *L. thermotolerans* is relatively easy to apply at industrial scale due to the availability of several commercial products that include *L. thermotolerans* strains. However, *L. thermotolerans* also shows significant limitations such as its moderate fermentative power or higher sensitivity to sulphur dioxide compared to *Saccharomyces* genus. For that reason, manufacturers recommend using *L. thermotolerans* in combination with specific recommended *S. cerevisiae* strains to avoid incompatibility problems and guarantee a proper alcoholic fermentation ending (Vicente et al. 2021).

The application of *L. thermotolerans* is mainly focused on fermentations carried out in warm viticultural locations such as Greece (Kapsopoulou et al. 2005, 2007), Italy (Comitini et al. 2011, Gobbi et al. 2013), Spain (Benito et al. 2016), Turkey (Balikci et al. 2016), South Africa (du Plessis et al. 2017, Minnaar et al.

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2017), China (YuHua et al. 2019) or Australia (Hranilovic et al. 2021).

Yeast industry is selecting and commercializing new strains, both from *Saccharomyces* and non-*Saccharomyces*. Initially (2012) there was only one commercial strain (Benito 2018) but now there are seven available ones (Vicente et al. 2022). The first selection processes focused on selecting *L. thermotolerans* strains with high ability to produce L-lactic acid and to reduce pH. Nevertheless, modern selection processes start to also pay attention to secondary selective parameters such as low production of acetic acid and ethanol, isovaleric acid or acetoin, while producing high concentrations of polysaccharides, glycerol, fruity esters, or terpenes (Benito 2018). Other interesting qualities are high sulphur dioxide resistance, ochratoxin A absorption or biofilm protection (Vicente et al. 2021).

The study tests for the first time three new commercial strains (Excellence X'Fresh, Levulia Alcomeno and EnartisFerm Qkappa) that can be easily bought in any viticultural country and other selected ones. Despite the use of industrial alternatives, natural strains are employed to verify its fermentative performance and its use as alternative against the commercial ones in order to exploit all the genomic diversity present in the species. The employed grape juice is Tempranillo as climate change is negatively influencing the cycle of this red grape variety that possess an early ripening time. Most studies related to *L. thermotolerans* in Spain focus on this variety that is the most planted red grape variety in Spain and that occasionally produce grape juices with low acidity, high sugar concentrations and low malic acid contents.

Materials and methods

Vinification assays

Different *Lachancea thermotolerans* yeast strains were employed. Two wine-related selected strains, L1 and L3 from the Complutense Yeast Collection (Complutense University of Madrid, Spain); and three commercial strains, Excellence X'Fresh (Lamothe-Abiet, France), Levulia Alcomeno (AEB Group, Italy) and EnartisFerm Qkappa (Enartis, Italy). *Saccharomyces cerevisiae* AG006 (Agrovín S.L, Spain) commercial strain was employed in the sequential fermentations as control.

Tempranillo (*Vitis vinifera* L. cultivar Tempranillo) grape must from Rioja Alta (Cuzcurrita, Spain) was employed in all the fermentation assays. Grapes were destemmed, crushed, introduced into a fermentation tank, and before any inoculation, free running grape juice was obtained. To reduce the microbial population, must was pasteurized at 105 °C for 1 minute. Grape must was supplemented with 0.15 g/L of di-ammonium phosphate and 0.30 g/L of Actimax Natura (Agrovin S.L, Spain) prior to pasteurization. The initial grape juice parameters were: 251.22 g/L of fermentable sugars, pH = 3.74, primary amino nitrogen = 172 mg/L, ammonia nitrogen = 26 mg/L and malic acid = 2.03 g/L. Lactic and acetic acid initial concentrations were below 0.1 g/L.

All assays were carried out in 250 mL borosilicate glass bottles with polypropylene cap, and slight open to allow CO_2 release and prevent microbial contamination. All conditions were assayed by triplicate at 25°C.

The preculture of the strains was carried out in YMB medium (5 g/L proteose peptone, 3 g/L malt extract, 3 g/L yeast extract and 10 g/L glucose) for 24 hours at 25ºC and shaking at 100 rpm. The optical density of the precultures was determined to calculate the final cell density (0.2 O.D. $\approx 1 \times 10^6$ cells/mL). *S. cerevisiae* was inoculated after 96 hours in the sequential fermentations.

Fermentation monitoring was performed by weight loss. Fermentation was considered complete when the weight loss was around 0.01% for two consecutive days.

Determination of basic oenological parameters

A Y15 Autoanalyzer and its commercial kits (Biosystems, Barcelona, Spain) were employed to conduct the determinations of L-malic acid and L-lactic acid. A FTIR autoanalyzer Bacchus 3 (TDI, Barcelona, Spain) was used to the determine the content of acetic acid, ethanol, glucose + fructose, succinic acid, pH and glycerol concentrations.

Analysis of volatile compounds

The analysis of the volatile compounds from the resulting wines was performed according to a previous methodology (Liu et al. 2019). The samples were analysed 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) at 45°C for 30 min after 10 min of incubation. The fiber was conditioned at 250°C prior to 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, Waltham, MA) to be thermally desorbed in the splitless mode at 240°C for 3 min. A DB-WAX polar capillary column (60m × 0.25 mm i.d.×0.25 μm film thickness, J&W Scientific, Folsom, CA) 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 at 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 temperatures of the MS transfer line and the ionization source 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). Volatiles were identified by matching the obtained mass spectra with the standard NIST 08 library and by comparing the retention indices (RI) to those of the compounds reported in the literature and the NIST Webbook (https://webbook.nist.gov/chemistry/). 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. Table S1 shows the measured retention index (RI) and those reported in the literature.

Colour intensity study

An Y350 diode array spectrophotometer (Biosystems, Spain) was used for the analysis. Samples were analysed in a 1 mm path length quartz cuvette and a range of 200–1100 nm. Absorbance at 420 nm, 520 nm, 620 nm was measured. Colour intensity was calculated as the sum of absorbance at the analysed wavelengths.

Statistical analyses

All statistical analyses were performed using R software version 4.1.2 (R Development Core Team, 2013). The significance level was

set at $P < 0.05$. Analysis of variance (ANOVA) and Tukey post-hoc tests were applied to compare the different groups and values.

Results and discussion

Fermentative kinetics

The fermentations lasted 22 days for the fermentation conducted by *S. cerevisiae* and it varied from 24 to 27 days for the sequential fermentations involving the different strains of *L. thermotolerans* (Fig. 1). A slowdown took place after 100 hours of fermentation and another after 300 hours for all fermentations involving *L. thermotolerans*. Pure fermentation of *S. cerevisiae* showed the slowdowns at 96 and 200 h. The results agree with previous works that observed sequential fermentations involving *L. thermotolerans* to be slightly slower and longer than the control fermentation with *S. cerevisiae* in a few days (Benito 2018, Vicente et al. 2021).

Basic oenological parameters determination

Saccharomyces cerevisiae pure fermentations showed the lowest final sugar concentration of 1.24 g/L, which correlates which the highest ethanol production (Table 1). The sequential fermentation between *L. thermotolerans* QKAPPA and *S. cerevisiae* showed also a low final sugar concentration and no statistical differences as ethanol production is concerned. The rest of the fermentations involving *L. thermotolerans* strains showed a similar behaviour between them, the final sugar concentration was around 3 g/L and the ethanol content was reduced in around 0.25%. *Lachancea thermotolerans* selected strain L1 showed the highest level of residual sugars, up to 5 g/L. Previous studies have reported sequential fermentations of *L. thermotolerans* to show occasionally slightly higher concentrations of residual sugars than the *S. cerevisiae* control due to the lack of nutrients that takes place after the inoculation of the second strain several days after the first inoculation (Vicente et al. 2021). This condition affects the ethanol concentration as well, being reduced from 0.2% to 1% compared to the *S. cerevisiae* control (Benito 2018, Vicente et al. 2021). By this reason, some manufacturers have developed specific nutrients for must enrichment to reduce these residual sugars.

The role of *L. thermotolerans* in lactic acid production and pH management has been deeply studied. The lactic acid concentration is extremely variable in the assays involving *L. thermotolerans,* L3 showed the highest concentration of L-lactic acid (3.56 g/L; pH reduction, 0.22) while that with QKAPPA produced the lowest concentration (0.8 g/L; pH reduction, 0.03). Previous studies reported contradictory results when comparing commercial strains of *L. thermotolerans* as far as this characteristic is concerned, but all of them conclude that it is possible to select better strains than the commercial offer (Vicente et al. 2021). In this study the selected strain L3 showed the highest concentration when compared with all the commercial strains, whereas the other one, L1, has a similar production to LEV, which has been previously assayed, producing from 1.0 (Hranilovic et al. 2021) to 2.8 g/L (Snyder et al. 2021). The variability in final lactic acid concentration observed in the different studies clearly indicate that there are several parameters influencing the metabolism of each *L. thermotolerans* strain, nutrient availability and interactions between different species may be some of the most important ones (Urbina et al. 2021).

Although the first selection criteria for *L. thermotolerans* strains was L-lactic acid production, the reduction in malic acid content was later considered as a tool to control the malic acid initial content. This characteristic is especially valuable in the production of red wines in viticultural areas that suffer from climate change and where to perform a classical malolactic fermentation can increase the risk of undesirable deviations (Benito 2020). All the fermentations showed a reduced concentration compared to the original must between 29 (L3 and AG006) and 44% (EXC and L1) (Table 1). Previous studies reported a big variability in malic acid reduction for both species, varying from malic acid production to 50% reduction (Blanco et al. 2020, Vicente et al. 2022).

The use of non-*Saccharomyces* yeast is sometimes limited by the increase of the volatile acidity. Usually, *S. cerevisiae* pure fermentations show lower values compared to those fermentations involving a non-conventional yeast. Accordingly, the single fermentations showed the lowest final value while sequential produced slightly higher concentrations up to 0.2 g/L (Table 1), although lower than the faulty threshold of acetic acid around 0.9 g/L (Ruiz et al. 2019).

Recent studies have focused on the production of several organic acids by yeast, succinic acid is the most sought. Sequential fermentations between *L. thermotolerans* strains and *S. cerevisiae* did not show significant differences in the final succinic acid concentration, varying from 1.22 to 1.41 g/L in comparison with 1.47 g/L for the *S. cerevisiae* control. This data suggests that *L. thermotolerans* may not have any role as far as succinic acid is concerned, being it only produced by *S. cerevisiae* at different concentration (Vicente et al. 2022).

Volatile compounds analysis

Wine is nothing without the flavour-active compounds produced by the microorganisms involved in the fermentation. In the resulted wines most of the volatile compounds are esters (22), higher alcohols (9), fatty acids (6), and aldehydes (4) (Table 2), many of them by-products of the fermentative process. Other compounds, such as terpenes, come from the grape and can be modified by the microbial activity during the process. Esters are generally related with fruity aromas and are a basic component of the wine bouquet. Higher alcohols, also called fusel alcohols, are produced by several routes and are the precursors of aged esters; and fatty acids, are generally associated with rancid or cheese aromas. Compared to *S. cerevisiae,* non-*Saccharomyces* yeast are great aroma producers, mainly due to a rich and diverse group of extracellular enzymes which use different precursors producing flavour-active compounds (Wei et al. 2022).

According to the results, the production of volatile compounds is an extremely strain dependent characteristic (Table 2). Overall, the presence of *L. thermotolerans* in sequential fermentation with *S. cerevisiae* increases the fruity profile of the final wines by its influence above esters as other studies have reported (Vicente et al. 2021). *L. thermotolerans* show an increased production of ethyl and isoamyl lactates, derived from the increased production of lactic acid. The production of ethyl lactate is increased in near 10 times, whereas the isoamyl lactate is exclusively produced by *L. thermotolerans*. These aromas are both generally described as fruity or sweet, making it desirable in wines up to a certain limit (Liu et al. 2019). Other esters such as ethyl decanoate are increased in around 50% by its presence. On the contrary, some minority esters are reduced to a half, like ethyl 3-hydroxybutyrate, ethyl nonanoate or ethyl 2-hydroxy-4-methyl-pentanoate.

Highers alcohols, on the contrary, have a double consideration since some of them are considered as pleasant, by producing rose/jasmine-related aromas, or as unpleasant, by producing solvent/polish-like aromas (Liu et al. 2019, Vicente et al. 2021). Some higher alcohols producing floral aromas are increased by the presence of *L. thermotolerans*, such as phenylethyl alcohol (rose

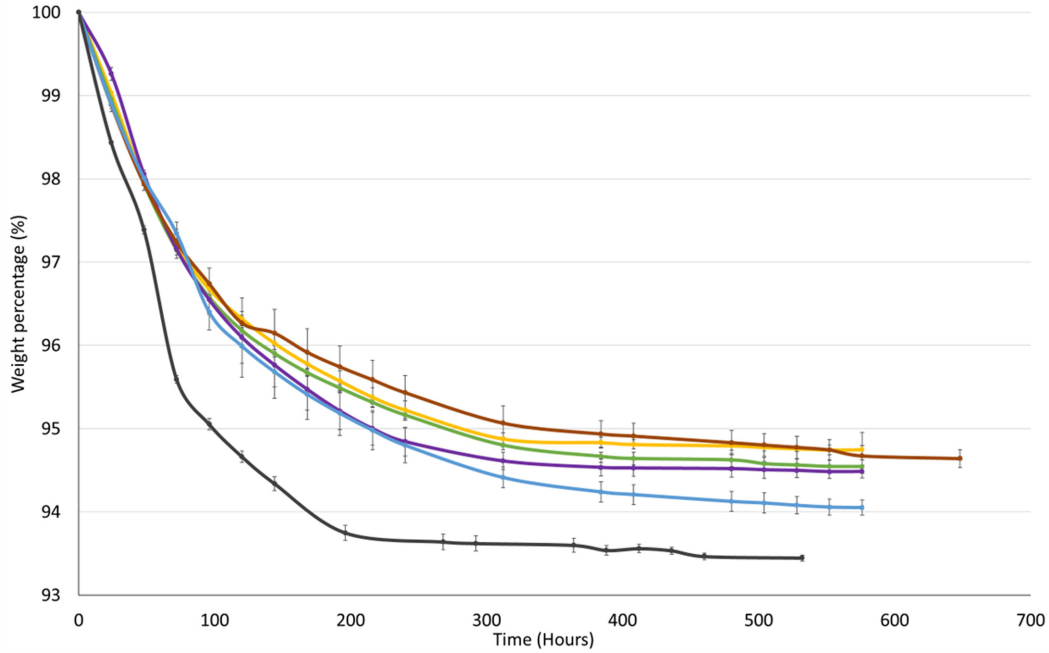

Figure 1. Fermentation kinetics of gravimetrically measured variants by total weight loss in course of fermentation. *S. cerevisiae* alone (AG006) in black; sequential fermentation with *S. cerevisiae* and *L. thermotolerans*: L1 (L1) in brown; L3 (L3) in blue; Excellence X'Fresh (EXC) in yellow; Levulia Alcomeno (LEV) in green and EnartisFerm Qkappa (QKAPPA) in purple.

Table 1. Basic oenological parameters of fermentations from Tempranillo red grapes.

	AG006	EXC	LEV	QKAPPA	L1	L3
Ethanol (%, v/v)	14.74 ± 0.08c	14.46 ± 0.06b	14.52 ± 0.07b	14.69 ± 0.07c	14.21 ± 0.08a	14.17 ± 0.09a
Glucose + Fructose (g/L)	1.24 ± 0.21a	2.62 ± 0.42b	2.25 ± 0.34b	1.31 ± 0.20a	4.73 ± 0.62c	3.69 ± 0.44c
Glycerol (g/L)	9.25 ± 0.32b	8.48 ± 0.33a	8.76 ± 0.24ab	8.87 ± 0.36ab	8.96 ± 0.47ab	8.82 ± 0.46ab
pH	3.77 ± 0.02d	3.65 ± 0.03b	3.69 ± 0.03bc	3.72 ± 0.02c	3.62 ± 0.02b	3.56 ± 0.02a
Lactic acid (g/L)	0.11 ± 0.03a	2.70 ± 0.18d	1.92 ± 0.26c	0.80 ± 0.11b	2.30 ± 0.64cd	3.19 ± 0.21e
Malic acid (g/L)	1.41 ± 0.12 b	1.16 ± 0.11a	1.26 ± 0.05ab	1.37 ± 0.11b	1.11 ± 0.10a	1.39 ± 0.15b
Acetic acid (g/L)	0.36 ± 0.05a	0.44 ± 0.06b	0.53 ± 0.07bc	0.46 ± 0.05b	0.45 ± 0.03b	0.56 ± 0.04c
Succinic acid (g/L)	1.47 ± 0.06b	1.26 ± 0.13a	1.41 ± 0.13ab	1.32 ± 0.04a	1.41 ± 0.17ab	1.22 ± 0.11a

Results are mean ± SD of three replicates. Different letters indicate statistical significance between groups. *S. cerevisiae* AG006 control (AG006) and sequential fermentations with *S. cerevisiae* (AG006) and *L. thermotolerans* Excellence X'Fresh (EXC); Levulia Alcomeno (LEV); EnartisFerm Qkappa (QKAPPA); L1 (L1) or L3 (L3).

or honey aromas) in around a 20% by QKAPPA; while others, described as nail polish, such as 2-methyl-1-propanol or 3-methyl-1-butanol are reduced in around a 50 and 25% respectively by L1. Despite this desirable effect, in some cases, some strains have a negative effect by increasing fusel alcohols associated with medicinal or alcohols descriptors, such as butanol (without significance).

Other molecules generally related to herbaceous aromas are aldehydes, nevertheless, some specific aldehydes can be considered as negative since it can be related to pungent descriptors (Liu et al. 2019, Wei et al. 2022). The presence of *L. thermotolerans* seem not to have a great influence in the final aldehyde content, apart from a slightly decrease of 3-methyl butanal (peach-like aroma). The ketones production is generally not influenced by the presence of *L. thermotolerans*.

Some other volatile compounds such as fatty acids are generally related to unpleasant aromas. The presence of *L. thermotolerans* may reduce the fatty acid profile of the fermentations by reducing some of the most abundant ones such as hexanoic (L1, up to 40%) or octanoic (L1, up to 60%), both related with sour or faint fruit, as well as those found in a smaller concentration such as butanoic and decanoic acid (du Plessis et al. 2017b). Nevertheless, in some cases, some strains may increase the presence of hexanoic (LEV) or decanoic acid (QKAPPA). These results agree with previous studies that report *L. thermotolerans* to produce fewer fatty acids than *Saccharomyces* yeasts (Vicente et al. 2021).

Colour intensity

Although there were not significant differences in final colour intensity values, the results showed some differences in absorbance at 520 nm that is related to red colour. *L. thermotolerans* strain L3 showed a slightly higher absorbance at 520 nm than the *S. cerevisiae* control (Table 3). Previous studies report some influence of *L. thermotolerans* on colour intensity that is related to anthocyanin changes in the coloration due to reductions in the pH units (Vicente et al. 2021).

Conclusions

The study observed differences between the different *L. thermotolerans* strains in basic wine quality parameters such as lactic acid, malic acid, and ethanol, while other parameters such as the production of acetic acid, succinic acid or glycerol were similar. This

Table 2. Volatile compounds organized by chemical families in each fermentation from Tempranillo red grapes.

Chemical family	Compound (mg/L)	AG006	EXC	LEV	QKAPPA	L1	L3
HIGHER ALCOHOLS	2-Methyl-1-propanol	0.89 ± 0.115a	0.685 ± 0.089ab	0.711 ± 0.004a	0.698 ± 0.004ab	0.481 ± 0.078b	0.921 ± 0.247ab
	Butanol*	2.583 ± 2.651a	6.402 ± 2.518a	5.66 ± 1.228a	2.809 ± 0.897a	3.417 ± 1.539a	6.797 ± 2.005a
	3-Methyl-1-butanol	6.651 ± 0.372a	5.88 ± 0.425a	5.367 ± 0.079a	5.13 ± 0.079a	5.058 ± 0.228a	6.254 ± 1.071a
	Hexanol	0.293 ± 0.01a	0.282 ± 0.017a	0.273 ± 0.001a	0.27 ± 0.001a	0.262 ± 0.003a	0.287 ± 0.015a
	cis-3-Hexen-1-ol*	20.352 ± 1.914a	18.187 ± 2.048a	19.508 ± 2.142a	17.684 ± 3.435a	17.411 ± 1.234a	17.033 ± 2.683a
	Octanol*	11.936 ± 3.639a	4.196 ± 1.849a	4.674 ± 1.499a	10.594 ± 6.85a	3.658 ± 1.363a	4.056 ± 1.123a
	3-(methylthio)-1-propanol*	7.056 ± 1.319a	4.543 ± 0.96a	5.145 ± 1.032a	3.792 ± 2.249a	3.697 ± 0.893a	4.924 ± 2.069a
	Phenylethyl alcohol	1.436 ± 0.075a	1.224 ± 0.108a	1.573 ± 0.022a	1.639 ± 0.022a	1.431 ± 0.251a	1.548 ± 0.125a
	2-Nonanol*	4.817 ± 2.041a	2.665 ± 0.184a	3.088 ± 0.367a	2.378 ± 0.162a	2.712 ± 0.23a	2.603 ± 0.689a
ESTERS	Ethyl acetate	1.419 ± 0.021a	1.182 ± 0.105ab	1.328 ± 0.089a	1.16 ± 0.069ab	0.771 ± 0.408b	1.271 ± 0.225ab
	2-Methylpropyl acetate	0.027 ± 0.002a	0.023 ± 0.003a	0.018 ± 0.001a	0.017 ± 0.001a	0.009 ± 0.009b	0.028 ± 0.004a
	Ethyl butanoate	0.049 ± 0.009a	0.05 ± 0.005abc	0.036 ± 0.001bc	0.034 ± 0.001ab	0.013 ± 0.011c	0.053 ± 0.012bc
	Ethyl 3-methylbutanoate*	5.557 ± 1.044a	4.858 ± 0.829a	5.292 ± 0.55a	3.65 ± 0.119ab	1.969 ± 1.888b	3.856 ± 0.651ab
	Ethyl hexanoate	0.483 ± 0.097a	0.51 ± 0.091bc	0.304 ± 0.013abc	0.264 ± 0.013ab	0.211 ± 0.076c	0.415 ± 0.26bc
	Ethyl lactate	0.028 ± 0.006c	0.017 ± 0.002ab	0.187 ± 0.013b	0.228 ± 0.013c	0.189 ± 0.043b	0.113 ± 0.006a
	Ethyl octanoate	0.108 ± 0.026a	0.15 ± 0.017c	0.139 ± 0.003c	0.149 ± 0.003b	0.013 ± 0.001c	0.185 ± 0.028c
	Ethyl 3-hydroxybutyrate*	3.76 ± 1.115a	2.018 ± 0.116b	1.933 ± 0.068b	2.47 ± 0.036ab	1.704 ± 0.077b	1.571 ± 0.319b
	Ethyl nonanoate*	7.924 ± 1.354a	3.145 ± 1.641ab	2.403 ± 0.545ab	6.566 ± 5.023ab	1.529 ± 0.624b	1.927 ± 0.45ab
	Ethyl 2-hydroxy-4-methylpentanoate*	14.752 ± 0.279a	12.793 ± 1.692a	13.838 ± 0.741a	10.672 ± 2.945a	11.094 ± 0.594a	11.049 ± 1.536a
	2-Methyl propanoic acid	0.025 ± 0.002a	0.02 ± 0.003a	0.023 ± 0a	0.022 ± 0a	0.027 ± 0.007a	0.021 ± 0.003a
	Isoamyl lactate	0 ± 0c	0 ± 0ab	0.039 ± 0.003ab	0.049 ± 0.003c	0.028 ± 0.002b	0.027 ± 0.002a
	Ethyl 2-furoate*	1.315 ± 0.143a	1.019 ± 0.121a	1.18 ± 0.118a	1.28 ± 0.547a	1.026 ± 0.111a	0.976 ± 0.217a
	Methyl benzoate*	1.952 ± 0.268a	1.711 ± 0.209a	2.108 ± 0.269a	1.713 ± 0.284a	1.732 ± 0.108a	1.753 ± 0.253a
	Ethyl decanoate	0.012 ± 0.003a	0.017 ± 0.002b	0.013 ± 0b	0.013 ± 0a	0.002 ± 0b	0.018 ± 0.004b
	Diethyl succinate	0.076 ± 0.008a	0.052 ± 0.006a	0.271 ± 0.021a	0.334 ± 0.021a	0.041 ± 0.005a	0.286 ± 0.113a
	Ethyl phenylacetate*	1.695 ± 0.132a	1.664 ± 0.234a	1.828 ± 0.249a	1.458 ± 0.181a	1.764 ± 0.306a	1.571 ± 0.321a
	2-phenylethyl acetate*	0.017 ± 0.002a	0.012 ± 0.002a	0.013 ± 0a	0.012 ± 0a	0.016 ± 0.002a	0.012 ± 0.002a
	Ethyl dodecanoate*	0.801 ± 0.119a	0 ± 0a	0 ± 0a	21.88 ± 37.026a	0 ± 0a	0 ± 0a
	3-Methyl-1-butyl acetate*	0.549 ± 0.069a	0.387 ± 0.077ab	0.116 ± 0.033a	0.018 ± 0.033ab	0.254 ± 0.205b	0.256 ± 0.071ab
	Ethyl 3-methylbutyl ester*	2.297 ± 0.156a	1.331 ± 0.081a	1.393 ± 0.086a	399.364 ± 687.166a	1.221 ± 0.211a	0.912 ± 0.107a

Table 2. Continued

Chemical family	Compound (mg/L)	AG006	EXC	LEV	QKAPPA	L1	L3
FATTY ACIDS	Butanoic acid*	2.044 ± 0.295a	1.467 ± 0.239a	1.576 ± 0.181a	4.079 ± 4.465a	1.624 ± 0.164a	1.186 ± 0.227a
	2-methyl butanoic acid	0.014 ± 0.001a	0.009 ± 0.003a	0.009 ± 0a	0.008 ± 0a	0.012 ± 0.003a	0.009 ± 0.003a
	Hexanoic acid	0.069 ± 0.003a	0.073 ± 0.006b	0.058 ± 0.001bc	0.056 ± 0.001a	0.043 ± 0.002bc	0.07 ± 0.005c
	Octanoic acid	0.202 ± 0.021a	0.228 ± 0.005ab	0.142 ± 0.005ab	0.128 ± 0.005ab	0.076 ± 0.004b	0.199 ± 0.012b
	Nonanoic acid	0.041 ± 0.004a	0.052 ± 0.018a	0.039 ± 0.001a	0.037 ± 0.001a	0.049 ± 0.014a	0.036 ± 0.017a
	Decanoic acid*	25.977 ± 2.678b	27.853 ± 2.029ab	17.868 ± 0.61c	33.404 ± 2.173a	28.916 ± 2.538ab	17.584 ± 2.178c
ALDEHYDES	3-Methyl butanal*	2.698 ± 0.618a	1.49 ± 0.73ab	1.711 ± 0.279ab	3.079 ± 0.642a	0.87 ± 0.703b	1.042 ± 0.413b
	Benzaldehyde	0.026 ± 0.001a	0.016 ± 0.002bc	0.016 ± 0.001ab	0.015 ± 0.001ab	0.02 ± 0.004ab	0.019 ± 0.003c
	Dodecanal*	4.518 ± 0.566a	3.222 ± 0.244a	3.363 ± 0.171a	4.828 ± 0.776a	3.264 ± 0.146a	3.065 ± 1.235a
	4-methylbenzaldehyde	9.111 ± 1.741a	7.677 ± 0.945a	8.706 ± 1.118a	5.021 ± 4.531a	7.415 ± 1.118a	6.907 ± 0.868a
KETONES	N-(3-Methylbutyl)acetamide	0.061 ± 0.011a	0.051 ± 0.003a	0.079 ± 0.002a	0.086 ± 0.002a	0.047 ± 0.004a	0.086 ± 0.009a
	β-damascenone*	4.75 ± 0.923a	4.613 ± 0.824a	4.532 ± 0.354a	2.904 ± 2.558a	4.609 ± 0.301a	4.744 ± 0.791a
	Acetophenone	2.233 ± 0.899a	1.306 ± 0.418a	2.26 ± 1.154a	6.511 ± 6.392a	2.171 ± 0.771a	1.502 ± 0.582a
	Butyrolactone	7.919 ± 0.112a	7.163 ± 0.447a	7.736 ± 1.23a	7.664 ± 1.893a	6.518 ± 0.705a	5.794 ± 0.79a
OTHERS	Toluene	0.15 ± 0.142a	0.11 ± 0.099a	0.107 ± 0.002a	0.101 ± 0.002a	0.128 ± 0.112a	0.103 ± 0.087a

Compounds highlighted with an asterisk (*) has been multiplied by 1000. Results are mean ± SD of three replicates. Different letters indicate statistical significance between groups. *S. cerevisiae* AG006 control fermentation (AG006) and sequential fermentations with *S. cerevisiae* (AG006) and *L. thermotolerans* Excellence X'Fresh (EXC); Levulia Alcomeno (LEV); EnartisFerm Qkappa (QKAPPA); L1 (L1) or L3 (L3).

Table 3. Final colour intensity analysis of fermentations from Tempranillo red grapes.

	AG006	EXC	LEV	QKAPPA	L1	L3
420 nm	0.63 ± 0.02 a	0.64 ± 0.03 a	0.61 ± 0.03 a	0.62 ± 0.03 a	0.65 ± 0.04 a	0.65 ± 0.03 a
520 nm	1.22 ± 0.04 a	1.31 ± 0.05 ab	1.27 ± 0.04 ab	1.21 ± 0.05 a	1.30 ± 0.06 ab	1.32 ± 0.05 b
620 nm	0.19 ± 0.01 a	0.21 ± 0.02 a	0.20 ± 0.01 a	0.19 ± 0.02 a	0.21 ± 0.02 a	0.21 ± 0.02 a
CI	2.04 ± 0.07 a	2.16 ± 0.10 a	2.08 ± 0.08 a	2.02 ± 0.10 a	2.16 ± 0.12 a	2.18 ± 0.10 a

Results are mean ± SD of three replicates. Different letters indicate statistical significance between groups. *S. cerevisiae* AG006 control (AG006) and sequential fermentations with *S. cerevisiae* (AG006) and *L. thermotolerans* Excellence X'Fresh (EXC); Levulia Alcomeno (LEV); EnartisFerm Qkappa (QKAPPA); L1 (L1) or L3 (L3).

specie may influence positively the volatile profile of the resultant wines. It increases the presence of some higher alcohols as well as esters. Some aldehydes with negative perception and several fatty acids are usually decreased in the mixed fermentations involving the studied strains. The results conclude that there is a great variability in the impact of *L. thermotolerans* on wine quality depending on the studied strain, therefore, a thorough characterization under different winemaking conditions is recommendable to assess the role of each strain. As remarked above, it is essential to increase the selection of the commercially available strains, which can be applied in wine fermentation. In our study, one of the selected strains has shown excellent results regarding the acid production (and pH control) as well as an optimum volatile profile, nevertheless, some compatibility studies between this strain and the selected *S. cerevisiae* should be performed to choose the best combination and eliminate residual sugars.

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Supplementary data

Supplementary data are available at FEMSYR online.

Conflict of interest. None declared.

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