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Influence of *Lachancea thermotolerans* on cv. Emir wine fermentation

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Abstract

The present paper describes the behaviour of *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in pure, co-cultured and sequential fermentations in cv. Emir grape must. Faster fermentation rates were observed in wine made with a pure culture of *S. cerevisiae* and wine produced with simultaneously inoculated cultures of *L. thermotolerans* and *S. cerevisiae*. Both *L. thermotolerans* and *S. cerevisiae* gave high population numbers. The use of *L. thermotolerans* in mixed and sequential cultures led to an increase in final total acidity content in the wines, varying in the range 5.40–6.28 g/l (as tartaric acid), compared to pure culture *S. cerevisiae*, which gave the lowest level of total acidity (5 g/l). The increase was in the order of 1.18–2.06 g/l total acidity. Increase in final acidity by the use of *L. thermotolerans* might be useful to improve wines with low acidity due to global climate change. Volatile acidity levels (as acetic acid) were in the range 0.53–0.73 g/l, while the concentration of ethyl alcohol varied in the range 10.76–11.62% v/v. Sequential fermentations of wines and pure culture fermentation of *L. thermotolerans* resulted in reduction in the concentrations of acetaldehyde and higher alcohols, with exception of *N*-propanol and esters. According to the sensory analysis, wine obtained with sequential inoculation of *L. thermotolerans* followed by inoculation of *S. cerevisiae* after 24 h, and simultaneous inoculation of these yeasts, was the most preferred.

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Keywords: ***Lachancea thermotolerans*; non-*Saccharomyces* yeast; *Saccharomyces cerevisiae*; simultaneously inoculated and sequential cultures; wine**

Introduction

The fermentation of grape juice into wine is a complex biological and biochemical interaction which is affected by wine-making practices. The microorganisms involved in fermentation include yeasts, fungi, lactic and acetic acid bacteria and the viruses and bacteriophages affecting them. The yeasts, the key microorganisms of wine making, are responsible for the transformation of grape sugars into ethanol, CO_2 and also various flavour compounds, such as esters, acids, higher alcohols, polyols, carbonyl compounds and volatile and non-volatile sulphur compounds (Fleet, 2003; Fleet and Heard, 2003; Jolly *et al*., 2006).

It is well known that *Saccharomyces cerevisiae*, and to a lesser extent the related *S. bayanus* species, are the main wine yeasts due to their ability to rapidly conduct alcoholic fermentations (Fleet, 2008; Dashko *et al*., 2014). Apart from this principal wine yeast, the significance of non-*Saccharomyces* yeasts for wine making has also been well established. It has been shown that some non-*Saccharomyces* yeasts can contribute positively to the chemical and sensory characteristics of wines (Fleet, 2008; Ciani and Comitini, 2011; Jolly et al., 2006, 2014). Of the non-*Saccharomyces* yeasts, *Torulaspora delbrueckii* (formerly *Saccharomyces rosei*, anamorph *Candida colliculosa)*, *Metschnikowia pulcherrima* (anamorph *Candida*

pulcherrima) and *Lachancea thermotolerans* (formerly *Kluyveromyces thermotolerans*; Kurtzman, 2003) are commercially available (Jolly et al., 2014). However, the effects of these yeasts on the wine profile may not always be fully understood by the end-user.

L. thermotolerans is one of the non-*Saccharomyces* yeasts that can be naturally found in wine fermentations (Mora *et al*., 1988; Torija *et al*., 2001; Kapsopoulou *et al*., 2005; Nurgel *et al*., 2005) and this yeast has been reported to increase the total acidity of wines by producing l-lactic acid (Mora *et al*., 1990; Kapsopoulou *et al*., 2005, 2007; Gobbi *et al*., 2013; Zara *et al*., 2014; Benito *et al*., 2015a). This attribute could be advantageous in addressing the problems of increased alcohol content and a reduction in the total acidity of wines associated with global climate change and variations in viticulture and wine-making practices (Gobbi *et al*., 2013; Jolly *et al*., 2014; Benito *et al*., 2015b). In addition to the biological acidification of wine, the use of *L. thermotolerans* in combination with *S. cerevisiae* could result in enhancement of floral and tropical fruit aromas in white wines and more complex and rounded flavours in red wines (Jolly *et al*., 2006, 2014). Sequential fermentation between *L. thermotolerans* and *S. cerevisiae* showed better sensorial impression and general acidity with higher final concentrations of ethyl lactate, 2-phenyl ethanol and 2-phenyl ethyl acetate compared to mixed fermentation of both these yeasts and single-culture fermentation of *S. cerevisiae* in low acidity Airen grape wine from the south of Spain (Benito *et al*., 2016). The present paper describes the behaviour of *L. thermotolerans* and *S. cerevisiae* in pure, mixed and sequentially inoculated grape must fermentations in order to improve cv. Emir wine quality.

Materials and methods

Yeast cultures

The *S. cerevisiae* strain used in this study was previously isolated from *Vitis vinifera* L. cv. Emir fermentations (Nurgel *et al*., 2005) and *L. thermotolerans* CBS 2860 was obtained from the CBS Yeast Collection (Utrecht, The Netherlands: http://www.cbs.knaw.nl). The yeasts were maintained on malt extract agar (Merck, Germany) slants.

Fermentation conditions

White grape must cv. Emir (pH 3.37; total acidity, 4.22 g/l as tartaric acid; sugar, 20.52°Brix) was used for fermentations. Healthy grapes were obtained from the Nevşehir-Ürgüp region (ancient Cappadocia) of Turkey. The grapes were crushed and pressed. The must was left to settle at 15 °C for 24 h and then racked. All fermentations were conducted under static conditions in 1 l sterile Erlenmeyer flasks containing 800 ml sterile grape must, which was sterilized by autoclaving at 105 °C for 5 min. The flasks, fitted with foam bungs and covered with aluminium foil, were incubated at 18 °C. Fermentation was followed by measuring the specific gravity. Two replicates of each fermentation were performed. Yeast cultures were propagated aerobically in the sterile grape must with orbital shaking at 160 rpm at 25 °C for 48 h. The yeast cells were centrifuged at 4000 rpm for 10 min at 4 °C, washed with cold sterile water and the pellet was resuspended in 5 ml sterile grape must. After counting by haemocytometer, 5×10^6 cells/ml each yeast was added into fermentation medium (Erten and Campbell, 2001).

The inoculations were carried out as follows: wine A, pure culture fermentation of *L. thermotolerans*; wine B, pure culture fermentation of *S. cerevisiae*; wine C, co-culture (simultaneous inoculation) of *S. cerevisiae* and *L. thermotolerans*; wine D, sequential inoculation of *L. thermotolerans* followed by inoculation of *S. cerevisiae* 24 h later; wine E, sequential inoculation of *L. thermotolerans* followed by inoculation of *S. cerevisiae* 48 h later; wine F, sequential inoculation of *L. thermotolerans* followed by inoculation of *S. cerevisiae* 72 h later.

Enumeration of yeasts

Samples were taken under aseptic conditions for counting yeasts during fermentations. The samples were diluted in physiological water as necessary and spread-inoculated (0.1 ml) onto plates of malt extract agar and l-lysine agar (Oxoid, UK). l-Lysine agar was used to enumerate *L. thermotolerans*. The plates were incubated for 3–5 days at 25 °C before yeast colony counting (Fleet, 1993). The yeast counts were done in triplicate.

Analytic determinations

Specific gravity was determined using a density meter (Mettler Toledo, Switzerland). Total acidity,

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pH, ethanol and volatile acidity were analysed according to the methods described by Ough and Amerine (1988).

Glucose and fructose were analysed by high-performance liquid chromatography (Shimadzu LC-20 AD, Shimadzu, Kyoto, Japan), using an Aminex HPX-87H column (Bio-Rad, Richmond, CA, USA) at 50 °C. The eluent was 5 mm H_2SO_4 in high-purity water at a flow rate of 0.6 ml/min. Concentrations of glucose and fructose were determined using a refractive index detector (Shimadzu, Japan) according to Erten (1998).

Volatile higher alcohols, esters and acetaldehyde were measured using a gas chromatograph (HP 5890, Hewlett-Packard, Stockport, UK). Samples were centrifuged in capped tubes at 4 °C to remove the yeast cells and diluted to 4% v/v ethanol; 5 ml diluted sample, 2 g sodium chloride and 50 µl internal standard (200 mg/l 3-heptanone) were added to the vials, which were sealed, and a 1 ml aliquot was injected into a 60 m long, 0.25 mm i.d. and 0.4 m thick column (Chrompac CP-Wax-57-CB, Middleburgh, The Netherlands), using a headspace auto sampler (Perkin-Elmer). The temperature was programmed at 43–180 °C. The stream from the column was split 1:1 to a flame ionization detector. The carrier gas was helium at a flow rate of 2.2 ml/min. The flavour compounds were tentatively determined by comparing the retention times with those from calibration standard curves on a data-handling system (Erten and Campbell, 2001).

Sensory analysis

After fermentation, the wines were racked off the lees and samples were taken for enological analyses. Then duplicate samples were combined and the wines were bottled in 250 ml sterile bottles. Sensory analysis was done 6 months after completion of fermentation. The sensory evaluation was performed using a ranking test (Barillere and Benard, 1986; Meilgaard *et al*., 2007), with a taste panel consisting of 13 staff of the Department of Food Engineering, under appropriate conditions. The wines were given code numbers and served in black tulip-shaped wine glasses at 15 °C in mixed order. Each panellist ranked the wines from the most to the least preferred and wine B, made with a pure culture fermentation of *S. cerevisiae*, was used as the control.

Statistical analysis

Data of the enological and volatile properties of wines were analysed for statistical significance by one-way analysis of variance (ANOVA). Sensory analysis was evaluated using Friedman analysis. Means were compared by Duncan test statistical analysis, using the software SPSS 18.0 for Windows.

Results and discussion

Fermentation kinetics

Specific gravity was used to monitor the fermentations and the patterns of sugar utilization in pure, simultaneous and sequential inoculations of *L. thermotolerans* and S. *cerevisiae* (Figure 1). Fermentation kinetics were affected by the different inoculum strategies. As expected, wines B (pure culture fermentation of *S. cerevisiae*) and C (simultaneous inoculation of *S. cerevisiae* and *L. thermotolerans*) resulted in the fastest rate of sugar consumption, reaching dryness by day 7. The time required to reach dryness for wine A (pure culture fermentation of *L. thermotolerans*) and the sequentially fermented wines D, E and F was 9 days. Similar results were

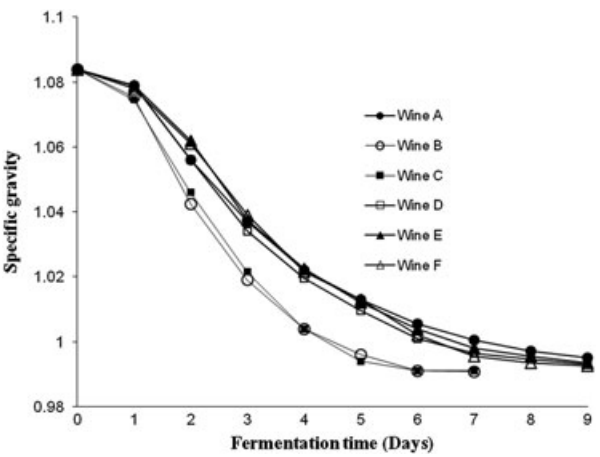

Figure 1. Decrease in specific gravity during fermentations. Wine A, pure culture fermentation of *L. thermotolerans*; wine B, pure culture fermentation of *S. cerevisiae*; wine C, co-culture (simultaneous inoculation) of *S. cerevisiae* and *L. thermotolerans*; wine D, sequential inoculation of *L. thermotolerans* followed by inoculation of *S. cerevisiae* after 24 h; wine E, sequential inoculation of *L. thermotolerans* followed by inoculation of *S. cerevisiae* after 48 h; wine F, sequential inoculation of *L. thermotolerans* followed by inoculation of *S. cerevisiae* after 72 h

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reported by Gobbi *et al.* (2013) on fermentations of pure, simultaneous and sequential inoculations using *L. thermotolerans* and *S. cerevisiae*.

Yeast population kinetics

Yeast population kinetics were followed by plate counting of *S. cerevisiae* on malt extract agar and *L. thermotolerans* on l-lysine agar, respectively. The growth of *S. cerevisiae* and *L. thermotolerans* during the fermentations of wines A (pure culture fermentation of *L. thermotolerans*), B (pure culture fermentation of *S. cerevisiae*) and C (simultaneous inoculation of *S. cerevisiae* and *L. thermotolerans*) is given in Figure 2. From an initial cell count of log 6.69 cfu/ml, *S. cerevisiae* and *L. thermotolerans* in pure culture fermentations achieved a maximum population of 8.02 log cfu/ml on day 5 and 7.90 log cfu/ml on day 6, respectively. After maximum growth, *S. cerevisiae* populations decreased to around 7.4 log cfu/ml and *L. thermotolerans* to 6.63 log cfu/ml at the end of fermentation. These growth patterns are in agreement with previous reports of pure culture fermentations with *S. cerevisiae* and *L. thermotolerans* (Kapsopoulou *et al.*, 2005; Cominiti *et al.*, 2011; Gobbi *et al.*, 2013).

When *S. cerevisiae* and *L. thermotolerans* were inoculated simultaneously (wine C), *S. cerevisiae* dominated over *L. thermotolerans* throughout the first day of fermentation and achieved a maximum population of 8.1 log cfu/ml by day 4. After a short stationary phase, the population decreased slightly to 7.41 log cfu/ml at the end of fermentation. Nevertheless, *L. thermotolerans* grew to a high of 7.24 log cfu/ml before showing a marked decrease to 4.96 log cfu/ml after day 7. This growth pattern for *S. cerevisiae* and *L. thermotolerans* was in agreement with a previous study (Gobbi *et al.*, 2013). In the co-culture studies by Kapsopoulou *et al.* (2007) and Cominiti *et al.* (2011), *S. cerevisiae* was also the dominant yeast, with the maximum population of up to 8 log cfu/ml, and L. *thermotolerans* showed proliferation to maximum levels of 6–7 log cfu/ml. However, in contrast, *L. thermotolerans* cell numbers showed a gradual decline before dying off by the end of alcoholic fermentation.

Figure 3 gives the growth of *S. cerevisiae* and *L. thermotolerans* during the sequential fermentations of wines D (sequential inoculation of *L. thermotolerans* followed by inoculation of *S. cerevisiae* after 24 h), E (sequential inoculation of *L. thermotolerans* followed by inoculation of *S. cerevisiae* after 48 h) and F (sequential inoculation of *L. thermotolerans* followed by inoculation of *S. cerevisiae* after 72 h). In the sequential fermentations (inoculation of *S. cerevisiae* delayed by 24, 48 and 72 h), *L. thermotolerans* reached maximum populations of approximately 7.8 log cfu/ml and competed better with *S. cerevisiae*, which exhibited reduced growth, reaching the highest levels of only 7.6–7.8 log cfu/ml in comparison to 8.1 log cfu/ml. In previous studies (Kapsopoulou

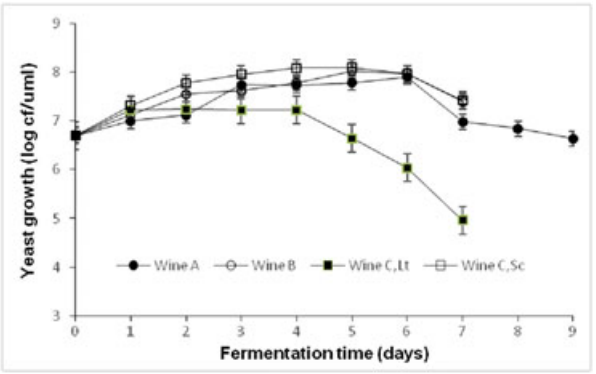

Figure 2. Growth of yeasts during pure culture and co-culture fermentations. Wine A, pure culture of *L. thermotolerans*; wine B, pure culture of *S. cerevisiae*; wine C, Lt and wine C; Sc, co-culture (simultaneous inoculation) of *L. thermotolerans and S. cerevisiae*

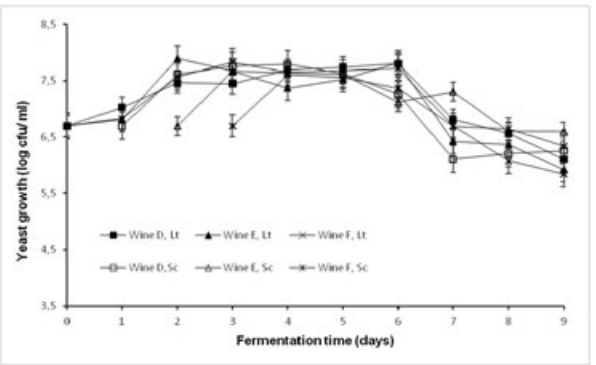

Figure 3. Growth of yeasts during sequential fermentations. Wine D, Lt and wine D; Sc, sequential culture of *L. thermotolerans* and *S. cerevisiae* (addition delayed 24 h). Wine E, Lt and wine E; Sc, sequential culture of *L. thermotolerans* and *S. cerevisiae* (addition delayed 48 h). Wine F, Lt and wine F; Sc, sequential culture of *L. thermotolerans* and *S. cerevisiae* (addition delayed 72 h)

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et al., 2007; Gobbi *et al*., 2013), delayed inoculation of *S. cerevisiae* also allowed better growth of *L. thermotolerans* and slightly higher maximum populations were reached. After this maximum growth, *L. thermotolerans* cell number showed a rapid decline and died off before fermentation was completed (Kapsopoulou *et al*., 2007). In the present study, the *Lachancea* yeasts survived until the end of fermentations, which is in an agreement with Gobbi *et al*. (2013).

Ethanol production

As can be seen from Table 1, the ethanol levels of the wines did not differ significantly from each other ($p > 0.05$), although sequential and pure culture fermentations of *L. thermotolerans* produced lower amounts of ethanol, varying in the range 10.76–11.44% v/v compared to a single culture of *S. cerevisiae* (11.50% v/v) and simultaneous inoculation of both yeasts (11.62% v/v). The final density values were < 1.000, indicating that the wines were, in general, fermented to dryness with < 3.91 g/l levels of glucose and fructose (Table 1). It therefore appears that the production of the wines was unaffected by pure, co-cultured and sequential fermentations. These final ethanol concentrations are similar to those reported in the literature (9.11–13.80% v/v) (Mora *et al*., 1990; Kapsopoulou *et al*., 2005, 2007; Cominiti *et al*., 2011; Gobbi *et al*., 2013), and this is due to the strong fermentative capacity and ethanol tolerance of *S. cerevisiae* in mixed and sequential fermentations. Several authors have reported the use of non-*Saccharomyces* yeasts to produce lower ethanol levels in wine (e.g. Kutyna *et al*., 2010; Contreras *et al*., 2014; Varela *et al*., 2015). Previous studies on the sequential fermentations of *L. thermotolerans* and *S. cerevisiae* also show lower ethanol concentrations than a control culture of *S. cerevisiae* (Gobbi *et al*., 2013; Benito *et al*., 2015a, 2015b, 2016) supporting the present study.

Total acidity

In agreement with previous studies (Mora *et al*., 1990; Kapsopoulou *et al*., 2005, 2007; Cominiti *et al*., 2011, Gobbi *et al*., 2013), the use of *L. thermotolerans* resulted in increased levels of total acidity compared to a pure culture of *S. cerevisiae.* In this study the total acidity in wines increased significantly, with the levels varying in the range 5.40–6.28 g/l (as tartaric acid), compared to a pure culture of *S. cerevisiae*, which gave the lowest level of total acidity (5 g/l). Therefore, *L. thermotolerans* has potential as a biological acidifier agent in wine making, which can be an advantage in warmer climates where acidity levels in grapes can be too low. As can be seen from Table 1, the wines had pH values of 3.28–3.36.

Table 1. Main enological properties of wines

	Wine A	Wine B	Wine C	Wine D	Wine E	Wine F	Significance
Density	0.994^{a}	0.990^{c}	0.990^{c}	0.992^{ab}	0.993^{ab}	0.991^{bc}	*
Ethanol % v/v	10.91 ± 0.15	11.5 ± 0.34	11.62 ± 0.18	11.34 ± 0.18	10.76 ± 0.84	11.44 ± 0.71	ns
Total acidity as tartaric acid g/l	6.29 ± 0.23^{a}	5.00 ± 0.01^{c}	5.40 ± 0.04^{b}	5.98 ± 0.17^{a}	6.28 ± 0.09^{a}	6.22 ± 0.12^{a}	**
Volatile acidity as acetic acid g/l	0.67 ± 0.03^{a}	0.53 ± 0.02^{b}	0.58 ± 0.01^{b}	0.70 ± 0.04^{a}	0.73 ± 0.01^{a}	0.69 ± 0.02^{a}	*
pH	3.37 ± 0.01^{a}	3.28 ± 0.01^{b}	3.28 ± 0.01^{b}	3.36 ± 0.01^{a}	3.36^{a}	3.36 ± 0.01^{a}	**
Glucose g/l	2.294 ± 0.89^{a}	0.756 ± 0.12^{b}	1.159 ± 0.09^{b}	0.812 ± 0.01^{b}	1.446 ± 0.13^{ab}	0.856 ± 0.06^{b}	*
Fructose g/l	3.915 ± 1.24^{a}	1.295 ± 0.12^{b}	1.292 ± 0.11^{b}	2.972 ± 0.21^{ab}	3.420 ± 1.24^{a}	2.028 ± 0.69^{ab}	ns
Total sugar g/l***	6.209 ± 0.35^{a}	2.052 ± 0.24^{d}	2.450 ± 0.02^{cd}	3.784 ± 0.21^{bc}	4.865 ± 1.1^{ab}	2.884 ± 0.63^{cd}	*

Wine A, pure culture fermentation of *L. thermotolerans*.
Wine B, pure culture fermentation of *S. cerevisiae*.
Wine C, co-culture (simultaneous inoculation) of *S. cerevisiae* and *L. thermotolerans*.
Wine D, sequential inoculation of *L. thermotolerans* followed by inoculation of *S. cerevisiae* after 24 h.
Wine E, sequential inoculation of *L. thermotolerans* followed by inoculation of *S. cerevisiae* after 48 h.
Wine F, sequential inoculation of *L. thermotolerans* followed by inoculation of *S. cerevisiae* after 72 h.
Significance,
*5% and
**1%, by LSD; values not sharing the same superscript letter within the horizontal line are different according to the Duncan test; ns, not significant.
***Total sugar equals the sum of the glucose and fructose values.

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Volatile acidity

Acetic acid, a by-product of yeast metabolism, has a sensory threshold value of 0.7–1.1 g/l (Henschke and Jiranek, 1993). In the present study, volatile acidity levels (as acetic acid) were in the range 0.53–0.73 g/l, with slightly higher levels in sequential fermentations. These amounts of volatile acidity are in disagreement with other studies (Mora *et al*., 1990; Kapsopoulou *et al*., 2005, 2007; Cominiti *et al*., 2011) that found lower levels of volatile acidity, in the range 0.27–0.41 g/l (as acetic acid). However, levels in the present study were in the accepted range of 0.2–0.7 g/l reported for wine (Lambrechts and Pretorius, 2000).

Volatile compounds of wines

Higher alcohols, esters, organic acids, carbonyl and sulphur compounds are the most important flavour compounds formed during the alcoholic fermentation of wines (Etiévant, 1991; Sarris *et al*., 2009). In addition to fermentation parameters and medium composition, volatile production is mainly strain-dependent. These flavour compounds give a wine its typical odour and taste.

Higher alcohols

Among the volatiles, higher alcohols (also called fusel oils) which are formed from the catabolic route (Ehrlich pathway) in the presence of amino acids, and from the anabolic route from sugars via biosynthesis, are quantitatively the most important group (Etiévant, 1991; Stewart and Russell, 1998). Information concerning the flavour compounds of *L. thermotolerans* together with wine yeast *S. cerevisiae* in co-cultured and sequential fermentations is scarce. In the previous work of Comitini *et al*. (2011), co-cultured fermentations of these two yeasts increased the total concentration of higher alcohols, compared to a pure culture of *S. cerevisiae*. Gobbi *et al*. (2013) investigated the influence of the addition of *L. thermotolerans* in simultaneously inoculated and sequential fermentations in association with the wine yeast *S. cerevisiae*. Their sequential fermentations resulted in a decrease in concentrations of 2-methyl butanol, 3-methyl butanol and iso-butanol (2-methyl-1-propanol) in micro- and industrial-scale fermentations. Sequential fermentations increased the content of *N*-propanol in microfermentations, but this was reduced in industrial fermentations (Gobbi *et al*., 2013).

In this study, as can be seen from Table 2, sequential fermentations showed significant reductions in the concentrations of 2-methyl butanol (23.94–25.47 mg/l), 3-methyl butanol (94.22–97.37 mg/l) and iso-butanol (28.43–33.04 mg/l) compared to the control wine B (pure culture of *S. cerevisiae*), which produced 32.42, 135.56 and 49.30 mg/l of 2-methyl butanol, 3-methyl butanol and iso-butanol, respectively. Similar results to those of the control wine were obtained with wine C (simultaneous inoculation of *S. cerevisiae* and *L. thermotolerans*). In contrast, *N*-propanol content significantly increased in sequential fermentations (57.32–58.43 mg/l), whereas control wine B formed 24.54 mg/l. Wine A, a pure culture of *L. thermotolerans*, was also characterized by lower formation of 2-methyl butanol (21.96 mg/l), 3-methyl butanol (82.22 mg/l) and isobutanol (26.43 mg/l) and higher production of *N*-propanol (54.50 mg/l), similar to sequential fermentations.

2-Methyl butanol has a flavour threshold of 300–330 mg/l, iso-butanol 75–500 mg/l and *N*-propanol 300–750 mg/l (Etiévant, 1991). In the present study, the concentrations of these higher alcohols were much lower than these threshold values, with the exception of 3-methyl butanol, which had a higher content than its threshold value of 14.5 mg/l. The results obtained in this study are generally similar to the observations of Comitini *et al*. (2011); Gobbi *et al*. (2013) and Benito *et al*. (2015a, 2016).

Esters

Esters, which are formed from a reaction between alcohols and fatty-acyl CoA molecules catalysed by alcohol acetyltransferase and other enzymes (Stewart and Russell, 1998), contribute fruity and floral aroma to wines (Etiévant, 1991). Wine B (pure culture of *S. cerevisiae*) and wine C (simultaneous inoculation of *S. cerevisiae* and *L. thermotolerans*) led to higher contents of isoamyl acetate (3-methyl butyl acetate) of 5.94 and 6.012 mg/l, respectively, exceeding the threshold level reported by Etiévant (1991) by 1 mg/l, compared to sequential fermentations of wines D (0.329 mg/l), E (0.183 mg/l) and F (0.172 mg/l) and wine B (pure culture fermentation of *L. thermotolerans*; 0.155 mg/l). This is in agreement

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Table 2. Volatile compounds of wines

Flavour compounds (mg/l)	Wine A	Wine B	Wine C	Wine D	Wine E	Wine F	Significance
Higher alcohols							
n-Propanol	54.50 ± 1.08^{b}	24.54 ± 0.25^{d}	28.15 ± 0.89^{c}	53.86 ± 1.14^{b}	58.43 ± 1.14^{a}	57.32 ± 2.64^{ab}	**
Isobutanol	26.43 ± 1.22^{b}	49.30 ± 7.05^{a}	54.72 ± 5.15^{a}	33.04 ± 0.23^{b}	29.90 ± 2.37^{b}	28.43 ± 5.9^{b}	**
2-Methyl butanol	21.96 ± 0.44^{b}	32.42 ± 3.59^{a}	34.34 ± 1.93^{a}	25.47 ± 0.07^{b}	23.94 ± 0.7^{b}	25.12 ± 4.87^{b}	*
3-Methyl butanol	82.22 ± 1.7^{b}	135.56 ± 15.46^{a}	142.7 ± 8.92^{a}	97.37 ± 1.11^{b}	90.48 ± 1.28^{b}	94.22 ± 19.37^{b}	**
Total higher alcohols	185.11 ± 4.42^{c}	241.82 ± 25.85^{ab}	259.90 ± 15.1^{a}	209.73 ± 2.08^{bc}	202.75 ± 3.21^{bc}	205.1 ± 32.78^{bc}	*
Esters							
Ethyl acetate	31.603 ± 3.7^{b}	46.403 ± 0.19^{a}	49.095 ± 1.96^{a}	35.303 ± 0.83^{b}	34.737 ± 1.79^{b}	33.048 ± 0.59^{b}	**
Ethyl butyrate	0.041 ± 0.009^{c}	0.265 ± 0.007^{a}	0.268 ± 0.01^{a}	0.101 ± 0.019^{b}	0.058 ± 0.001^{c}	0.059 ± 0.009^{c}	**
Isoamyl acetate	0.155 ± 0.03^{b}	5.940 ± 0.66^{a}	6.012 ± 0.06^{a}	0.329 ± 0.03^{b}	0.183 ± 0.04^{b}	0.172 ± 0.02^{b}	**
Isobutyl acetate	nd^{c}	0.145 ± 0.05^{b}	0.166 ± 0.07^{a}	nd^{c}	nd^{c}	nd^{c}	**
Ethyl hexonoate	nd^{c}	0.523 ± 0.05^{a}	0.482 ± 0.05^{a}	0.083 ± 0.002^{b}	0.047 ± 0.006^{b}	0.058 ± 0.007^{b}	**
Ethyl octanoate	nd^{c}	0.289 ± 0.03^{a}	0.198 ± 0.04^{b}	nd^{c}	nd^{c}	nd^{c}	**
Total esters	31.8 ± 3.76^{b}	53.57 ± 0.55^{a}	56.22 ± 2.11^{a}	35.82 ± 0.88^{b}	35.03 ± 1.84^{b}	33.34 ± 0.57^{b}	*
Carbonyl compounds							
Acetaldehyde	11.676 ± 0.6^{c}	12.579 ± 3.54^{c}	11.264 ± 1.2^{c}	17.703 ± 0.47^{ab}	18.702 ± 1.03^{a}	13.636 ± 1.32^{bc}	*
Total carbonyl compounds	11.676 ± 0.6^{c}	12.579 ± 3.54^{c}	11.264 ± 1.2^{c}	17.703 ± 0.47^{ab}	18.702 ± 1.03^{a}	13.636 ± 1.32^{bc}	*
Totals	228.58 ± 7.58^{b}	307.96 ± 22.86^{a}	327.39 ± 11.79^{a}	263.25 ± 2.49^{b}	256.48 ± 6.08^{b}	252.07 ± 33.53^{b}	*

Wine A, pure culture fermentation of *L. thermotolerans*.
Wine B, pure culture fermentation of *S. cerevisiae*.
Wine C, co-culture (simultaneous inoculation) of *S. cerevisiae* and *L. thermotolerans*.
Wine D, sequential inoculation of *L. thermotolerans* followed by inoculation of *S. cerevisiae* after 24 h.
Wine E, sequential inoculation of *L. thermotolerans* followed by inoculation of *S. cerevisiae* after 48 h.
Wine F, sequential inoculation of *L. thermotolerans* followed by inoculation of *S. cerevisiae* after 72 h.
nd, not determined.
Significance,
*5% and **1% by LSD; values not sharing the same superscript letter within the horizontal line are different according to Duncan test, ns, not significant.

with the previous studies of Gobbi *et al*. (2013) and Benito *et al*. (2016), that sequential fermentations resulted in reduced amounts of isoamyl acetate.

Ethyl acetate levels of Wine B (46.403 mg/l) and wine C (49.095 mg/l) were higher than the other wines (31.603-35.303 mg/l) in agreement with the results of Benito *et al*. (2015a) and Benito *et al*. (2016), but in disagreement with the results of Gobbi *et al*. (2013). However, in the present study, ethyl acetate levels of wines were above its threshold value of 12.7 mg/l (Etiévant, 1991).

Similar production patterns were observed for the contents of isobutyl acetate, ethyl butyrate, ethyl hexanoate and ethyl octanote where sequential fermentations and pure culture fermentation of *L. thermotolerans* were characterized by reduced formation of these flavour compounds (Table 2). Ethyl hexanoate has a threshold value of 0.08 mg/l, ethyl octanote 0.58 mg/l, isobutyl acetate 1.6 mg/l and ethyl butyrate 0.4 mg/l (Etiévant, 1991). In the present study, their levels did not exceed threshold values given by Etiévant (1991). However, the amounts of ethyl hexanoate were higher than its threshold value of 0.1 mg/l (Etiévant, 1991).

Acetaldehyde

Sequential fermentations of *L. thermotolerans* and *S. cerevisiae* formed relatively high amounts of acetaldehyde in the range 13.636–18.702 mg/l, in comparison with pure cultures (11.676–12.579 mg/l) and simultaneous fermentation (11.264 mg/l), as given in Table 2. Acetaldehyde levels in the present study were not in agreement with the studies of Benito *et al*. (2015a, 2016), who reported higher amounts of acetaldehyde with pure cultures of *S. cerevisiae*. Acetaldehyde is the important carbonyl compound in wine, derived from pyruvate during alcoholic fermentation, but in the present study its concentration did not exceed its threshold value of 100 mg/l (Etiévant, 1991).

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Table 3. Sensory evaluation of wines

	Wine A	Wine B	Wine C	Wine D	Wine E	Wine F
Difference between points of classification($p < 0.05$)	52^{c}	53^{c}	38^{a}	35^{a}	45^{b}	50^{bc}

Wine A, pure culture fermentation of *L. thermotolerans*.
Wine B, pure culture fermentation of *S. cerevisiae*.
Wine C, co-culture (simultaneous inoculation) of *S. cerevisiae* and *L. thermotolerans*.
Wine D, sequential inoculation of *L. thermotolerans* followed by inoculation of *S. cerevisiae* after 24 h.
Wine E, sequential inoculation of *L. thermotolerans* followed by inoculation of *S. cerevisiae* after 48 h.
Wine F, sequential inoculation of *L. thermotolerans* followed by inoculation of *S. cerevisiae* after 72 h.
Different superscripts (a, b, c) indicate statistically significant differences at 5%.

Sensory evaluation

Although sensory evaluation can be subjective, the human nose can detect aroma, flavour and other sensory nuances that may not be determined by current instrumental methods. Wine quality is often mainly determined by sensory evaluation and less by chemical analysis (Jolly *et al*., 2003). In the present study, wines were evaluated using ranking tests (Barillere and Benard, 1986; Meilgaard *et al*., 2007), with the wine produced by pure culture fermentation of *S. cerevisiae* (wine B) as the control; the results are given in Table 3. The most preferred was wine D, made with sequential inoculation of *L. thermotolerans* followed by *S. cerevisiae* after 24 h. The next-preferred was wine C, produced by co-culture fermentation, although statistically significant differences were not observed between wines D and C. However, a more in-depth sensory evaluation is needed to confirm findings in future studies.

Conclusions

The results of pure, simultaneously inoculated and sequential fermentations given in the present study showed that the *L. thermotolerans* yeast survived with high numbers in wine fermentations. This led to significant increases in the total acidity of wines, an attribute that can be used to improve low-acidity musts from warmer viticultural areas. Undesirable formation of volatile compounds was not observed. Wine D, produced by sequential inoculation of *L. thermotolerans* followed by inoculation of *S. cerevisiae* 24 h later, was the most preferred by the sensory panel. These features make the use of *L. thermotolerans* in wine making of technological interest. Further studies on various different strains and grape varieties other than cv. Emir are still required to supplement the limited knowledge of *L. thermotolerans*, as well as fermentation trials under industrial conditions.

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