

# Quality and Composition of Airén Wines Fermented by Sequential Inoculation of *Lachancea thermotolerans* and *Saccharomyces cerevisiae*

Ángel Benito, Fernando Calderón, Felipe Palomero and Santiago Benito\*

Department of Chemistry and Food Technology, Polytechnic University of Madrid, Ciudad Universitaria S/N, ES-28040 Madrid, Spain

Received: April 19, 2015

Accepted: November 30, 2015

## Summary

This study evaluates the influence of *Lachancea thermotolerans* on low-acidity Airén grape must from the south of Spain. For this purpose, combined fermentations with *Lachancea thermotolerans* and *Saccharomyces cerevisiae* were compared to a single fermentation by *S. cerevisiae*. Results of all developed analyses showed significant differences in several parameters including acidity, population growth kinetics, concentration of amino acids, volatile and non-volatile compounds, and sensorial parameters. The Airén wine quality increased mainly due to the acidification by *L. thermotolerans*. The acidification process caused a lactic acid increment of 3.18 g/L and a reduction of 0.22 in pH compared to the control fermentation, performed by *S. cerevisiae*.

**Key words:** Airén wine, *Lachancea thermotolerans*, *Saccharomyces cerevisiae*, L-lactic acid, pyruvic acid, glycerol, ethanol, amino acids, biogenic amines, combined fermentation

## Introduction

In recent years, global climate change has set a trend towards an increase in sugar content and a decrease in the acidity of grape juices. Microbiological acidification can play an essential role in satisfying the growing wine market demand for quality wines.

Traditionally, *Saccharomyces cerevisiae* is the yeast used widely for winemaking. However, grapes are not sterile media and there are many other yeast species with plenty of potential to solve new oenology challenges that must be studied. Several research groups have studied non-*Saccharomyces* yeast applications (1,2) in different grape varieties such as Sauvignon blanc (3,4), Chenin blanc (4), Chardonnay (4–6), Amarone (7), Muscat (8), Muscat d'Alexandrie (9), Debina (10), Macabeo (11,12), Folle blanche (13), Bobal (14), Alvarinho, Loureiro, Trajadura, Pedernã, Azal Branco, Avesso (15), Airén (16,17), Pedro Ximenez (18), Sangiovese (19), Pinot noir (20), Emir (21,22), Syrah (23–26), Tempranillo (27,28) and Riesling (29). In most cases improvements in wine quality were reported.

The presence of wild non-*Saccharomyces* yeasts in fermentations was traditionally associated with high levels of acetic acid and other off-flavours. Nevertheless, nowadays researchers and winemakers are aware of the positive influence of non-*Saccharomyces* yeasts on wine aroma complexity (1,2,29–40). The development of multistarter fermentation with *Saccharomyces cerevisiae* as a binding partner has been proposed to overcome the shortcomings of alcoholic fermentation with non-*Saccharomyces* yeasts. Mixed fermentations are of interest because of some enzymatic properties (glycosidases,  $\beta$ -lyase, etc.), ethanol reduction and the release of some interesting metabolites such as glycerol, pyruvic acid and mannoproteins, among others (41–44).

Some studies have analysed the use and influence of different non-*Saccharomyces* species on wine quality. In most cases sequential fermentation was reported to be the best option. These yeast species include *Kloeckera apiculata* (45), *Hanseniaspora uvarum* (46), *Hanseniaspora vineae* (6, 27), *Torulospira delbrueckii* (7,28,47), *Metschnikowia pulcher-*

\*Corresponding author: Phone: +34 913 363 710/+34 913 363 984; E-mail: santiago.benito@upm.es

rima (3,47,48), *Candida zemplinina* (49), *Zygosaccharomyces bailii* (50,51), *Schizosaccharomyces pombe* (44,52), *Hansenula anomala* (53), *Pichia guilliermondii* (54) and *Lachancea thermotolerans* (19,29,55–59). Among these species, *L. thermotolerans*, previously called *Kluyveromyces thermotolerans*, has been used specifically to increase the acidity of wines, causing increases of L-lactic acid concentration from 0.23 to 9.6 g/L depending on the different trial conditions (19,29,55–59).

This study aims to enhance Airén wine quality. This Spanish variety is considered as a neutral and very productive grape but is usually associated with low-quality wine. However, this variety is the most planted in Spain. Most Airén vineyards are located in the centre of the southern Spain. This area is considered to be a warm semi-desert region, where high sugar contents and lack of acidity in wine are the main problems. Therefore, *Lachancea thermotolerans* 617 was selected among other non-*Saccharomyces* yeasts in this study to perform combined fermentations with *S. cerevisiae* in order to increase the acidity and quality of Spanish Airén wine.

## Materials and Methods

### Microorganisms

The following yeast strains were used for the experimental fermentation of the studied Airén must: *Saccharomyces cerevisiae* 87 (CECT 12512; Spanish Type Culture Collection, Valencia, Spain) and *Lachancea thermotolerans* 617 (CECT 12672; Spanish Type Culture Collection).

### Vinification

Grapes of Airén cultivar (*Vitis vinifera* L.), grown in El Socorro experimental vineyard (Madrid, Spain) were used in the fermentations. Using a microvinification method similar to that described in scientific literature (60), 3.9 L of sterilised must (115 °C, 15 min) were placed in 4.9-litre glass fermentation vessels, leaving enough space for the emission of carbon dioxide. No sulphur dioxide was added to any vessel. Sugar concentration was 244.51 g/L, pH=3.68, primary amino nitrogen (PAN; Biosystems S.A., Barcelona, Spain) 177 mg/L, and lactic and acetic acids below 0.1 g/L. A concentration of 0.4 g/L of Actimax Natura (Agrovin S.A., Alcázar de San Juan, Spain), inactivated autolyzed yeasts naturally rich in amino acids, was added to provide nutrition for the media.

Three assays were performed (all in triplicate): (i) inoculation of the must with *S. cerevisiae* 87 alone (SC; 100 mL containing  $1.18 \cdot 10^7$  CFU/mL), (ii) inoculation with *S. cerevisiae* 87 ( $1.18 \cdot 10^7$  CFU/mL) and *L. thermotolerans* 617 (100 mL containing  $2.95 \cdot 10^7$  CFU/mL) together (mixed fermentation: LT×SC), and (iii) inoculation with *L. thermotolerans* 617 (100 mL containing  $2.27 \cdot 10^7$  CFU/mL) followed by *S. cerevisiae* 87 (100 mL containing  $10^7$  CFU/mL) 96 h later (sequential fermentation: LT...SC). Yeast inocula were produced using 100 mL of sterilised must with 1 mL of yeast extract peptone dextrose (YEPD; Pronadisa, Madrid, Spain) liquid medium (61), in the concentration of  $10^6$  CFU/mL (determined using a counting chamber). To reach this population, 100 µL of each yeast suspension were cultivated in 10 mL of YEPD at 25 °C for 24 h. This

procedure was repeated successively three times before the final inoculation of 1 mL of the suspension. All inocula were prepared in 250-mL flasks filled with 98 % H<sub>2</sub>SO<sub>4</sub> (Panreac, Barcelona, Spain), which allowed the release of CO<sub>2</sub> while avoiding microbial contamination (62), and sealed with a 14-cm Muller valve (Alamo, Madrid, Spain). The temperature was maintained at 25 °C for 48 h. The development of inocula proceeded without aeration, oxygen injection or agitation. All fermentation processes, which were done in triplicate, were carried out at 25 °C. When the sugar concentration fell below 3 g/L, the wines were racked and stabilised for 7 days at 4 °C. The wine was then bottled, and a concentration of 40 mg/L of sulphur dioxide in the form of potassium disulfite was added. Sealed bottles were placed horizontally in a climate chamber at 4 °C for three weeks until the sensory evaluation.

### Analytical determinations of non-volatile compounds

Glucose and fructose, L-lactic acid, acetic acid, glycerol, pyruvic acid, acetaldehyde, L-malic acid and primary amino nitrogen were all determined using a Y15 enzymatic autoanalyzer (Biosystems S.A.) with corresponding kits. Ethanol, pH, free SO<sub>2</sub> and total SO<sub>2</sub> profile were determined following the methods described in the Compendium of International Methods of Analysis of Wines and Musts (63).

### Growth kinetics during microvinification

During fermentations, aliquots were taken periodically under aseptic conditions and further tenfold dilutions were made serially. Growth kinetics was monitored by plating 100 µL of the appropriate dilution on lysine medium (Oxoid, Basingstoke, UK) for counting non-*Saccharomyces* yeasts (64) and YEPD medium (Pronadisa) for total yeast counts (61). Colonies were counted after growth at 30 °C for 48–72 h.

### Analytical determination of volatile compounds

The concentration of volatile compounds, all of which influence wine quality, was measured at the end of alcoholic fermentation by gas chromatography using an Agilent Technologies 6850 gas chromatograph with a flame ionisation detector (Hewlett Packard, Palo Alto, CA, USA) (65), calibrated with 4-methyl-2-pentanol (Fluka, Sigma-Aldrich Corp., Buchs, Switzerland) as an internal standard. Gas chromatography standards (Fluka, Sigma-Aldrich Corp.) were used to provide standard patterns. Higher alcohols were separated according to the Compendium of International Methods of Analysis of Wines and Musts (63), with the detection limit of 0.1 mg/L. Minor compounds were quantified using gas chromatography-mass spectrometry as described by Lopez *et al.* (66) with the modifications introduced by Loscos *et al.* (67).

### Analytical determination of amino acids

The amino acids were analysed using a Jasco (Tokyo, Japan) ultra-high-performance liquid chromatograph (UH-PLC) series X-LCTM, equipped with a fluorescence detector 3120-FP. Gradients of solvent A (methanol/acetonitrile 50:50, by volume) and B (sodium acetate/tetrahydrofuran 99:1, by volume) were used in a C18 (HALO®, Wilming-

ton, DE, USA) column (100 mm×2.1 mm; particle size 2.7 µm) as follows: 90 % B at 0.25 mL/min, from 0 to 6 min; 90–78 % linear gradient B at 0.2 mL/min, from 6 to 7.5 min; 78 % B from 7.5 to 8 min, 78–74 % linear gradient B at 0.2 mL/min, from 8 to 8.5 min, 74 % B at 0.2 mL/min, from 8.5 to 11 min, 74–50 % linear gradient B at 0.2 mL/min, from 11 to 15 min, 50 % B at 0.2 mL/min, from 15 to 17 min, 50–20 % linear gradient B at 0.2 mL/min, from 17 to 21 min, 20–90 % linear gradient B at 0.2 mL/min, from 21 to 25 min and re-equilibration of the column from 25 to 26 min to the initial gradient conditions. The scanning range for the detection of amino acids was 340–455 nm. Amino acids were quantified by comparison against their external standards, and different acids were identified by their retention times.

### Analytical determination of biogenic amines

The biogenic amines were analysed using a Jasco UHPLC chromatograph series X-LCTM, equipped with a fluorescence detector 3120-FP. Gradients of solvent A (methanol/acetonitrile, 50:50, by volume) and B (sodium acetate/tetrahydrofuran, 99:1, by volume) were used in a C18 (HALO®) column (100 mm×2.1 mm; particle size 2.7 µm) as follows: 60 % B at 0.25 mL/min, from 0 to 5 min; 60–50 % linear gradient B at 0.25 mL/min, from 5 to 8 min; 50 % B from 8 to 9 min, 50–20 % linear gradient B at 0.2 mL/min, from 9 to 12 min, 20 % B at 0.2 mL/min, from 12 to 13 min, 20–60 % linear gradient B at 0.2 mL/min, from 13 to 14.5 min, and re-equilibration of the column from 14.5 to 17 min to the initial gradient conditions. The scanning range for the detection of biogenic amines was 340–420 nm. Biogenic amines were quantified by comparison against their external standards, and different amines were identified by their retention times.

### Sensory analysis

The obtained wines were assessed using a blind test by a panel of 15 experienced wine tasters, all staff members of the Chemistry and Food Technology Department of the Polytechnic University of Madrid (Madrid, Spain) and the Accredited Oenology Laboratory of Haro (Haro, Spain). Following consistent terminology by consensus, five aromas and five taste attributes were chosen to describe the wines. The panellists used an unstructured scale, with scores ranging from 0 (no character) to 6 (very strong character), to rate the intensity of the 11 attributes.

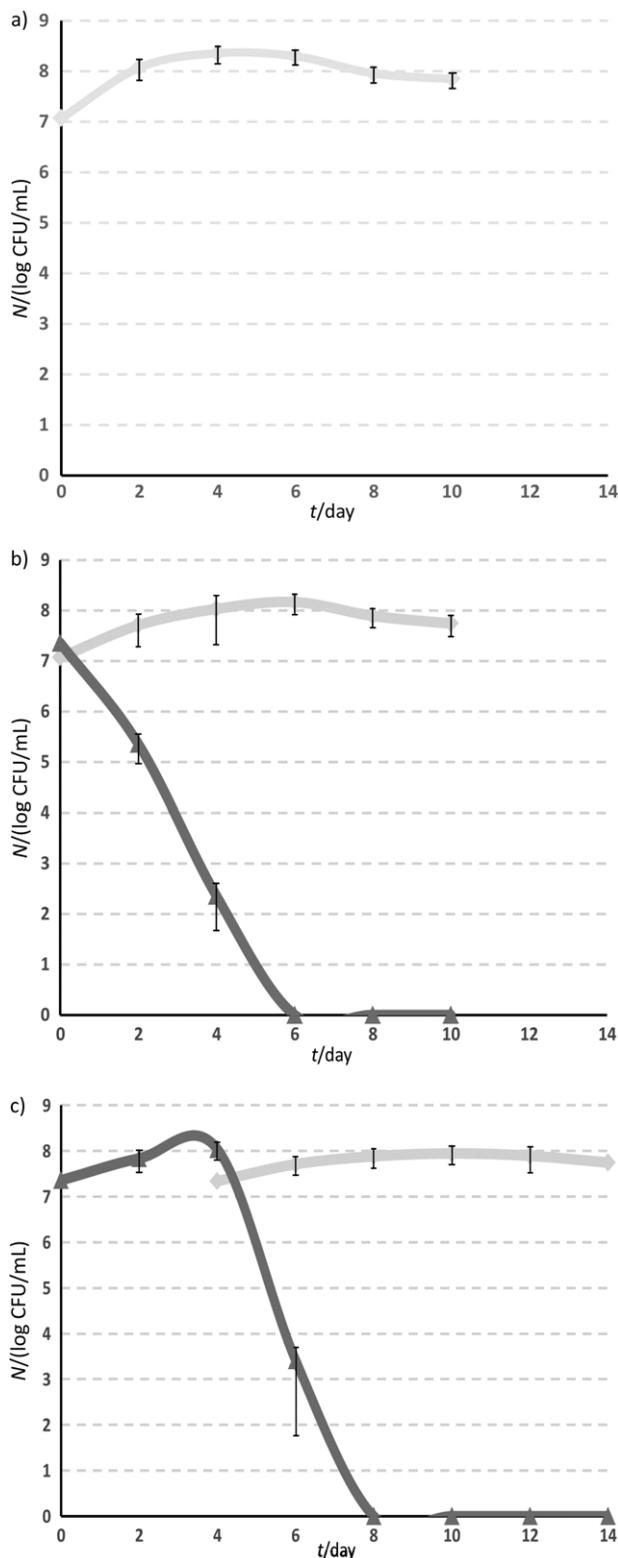
### Statistical analysis

PC Statgraphics v. 5 software (Graphics Software Systems, Rockville, MD, USA) was used for statistical analyses. The significance was set to  $p < 0.05$  for the ANOVA matrix F value. The mean values were compared using multiple range test.

## Results and Discussion

### Fermentation kinetics of the yeast population

Fig. 1 shows the development of different yeast strains during fermentation. Fermentation time varied



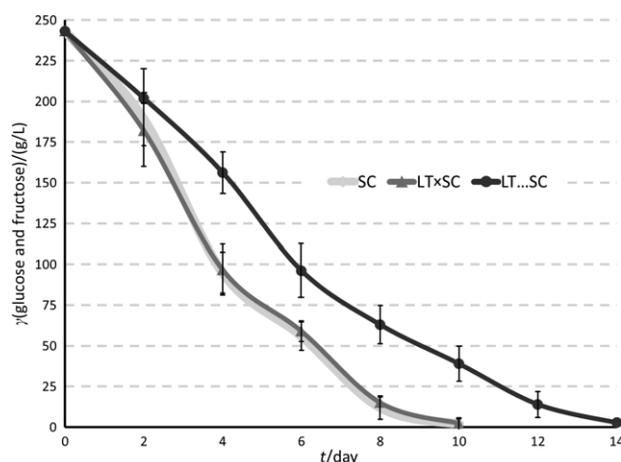
**Fig. 1.** Yeast cell counts during: a) fermentation with *Saccharomyces cerevisiae* 87 alone (SC;  $\diamond$ ), b) mixed fermentation with *Lachancea thermotolerans* 617 ( $\blacktriangle$ ) and *S. cerevisiae* 87 (LT×SC), and c) sequential fermentation with *L. thermotolerans* 617 followed by *S. cerevisiae* 87 (LT...SC)

from 10 to 14 days. In all mixed fermentations (LT×SC or LT...SC) when *Saccharomyces cerevisiae* 87 was inoculated,

the number of *L. thermotolerans* 617 cells started to decline fast. Other authors reported previously fermentation kinetics of other non-*Saccharomyces* strains, in which the presence of non-*Saccharomyces* strains was also observed during the early stages of fermentation. In this trial *L. thermotolerans* 617 strain disappeared on day 8 in the sequential (LT...SC) fermentation (Fig. 1). This can be explained by the higher fermentation activity of this species compared to other low-fermenting non-*Saccharomyces* strains. Some *S. cerevisiae* strains were also reported to secrete antimicrobial peptides that inhibit non-*Saccharomyces* yeast growth (68). This could explain the early disappearance of *L. thermotolerans* once *S. cerevisiae* was inoculated, even though it has been reported to tolerate up to 9 % (by volume) of ethanol when it ferments on its own (55). In this trial, the LT...SC fermentation was the best option. In the case of the LT×SC fermentation, *L. thermotolerans* disappeared fast so acidification was not completed. Cell flocculation or loss of viability can explain the observed reduction in cell numbers during fermentation.

### Sugar consumption kinetics and alcohol production

The *Saccharomyces cerevisiae* 87 fermenting on its own (SC) and in the LT×SC fermentation consumed the sugar the fastest (Fig. 2). Fermentation time varied from 10 to 14 days and final alcohol content varied from 13.91 to 14.36 % (by volume). The ethanol content was lower in the LT...SC fermentation (Table 1). The sugar consumption results analysed in this work (Fig. 2) are in agreement with the lower fermentation activity of *Lachancea* spp. compared with *S. cerevisiae* (55), due to the fact that in the last stages of fermentation only *S. cerevisiae* was detected. Several authors question the usefulness of non-*Saccharomyces* yeast in the production of lower volume fractions of alcohol in wines (43,69). These previous results are in agreement with the lower final alcohol content of the wines produced in the sequential fermentations involving *Lachancea thermotolerans* 617 (Table 1). However, in our case the alcohol reduction was about 0.4 % (Table 1).



**Fig. 2.** Change in the glucose and fructose concentration in the studied Airén wines during fermentation with *Saccharomyces cerevisiae* 87 alone (SC), mixed fermentation with *Lachancea thermotolerans* 617 and *S. cerevisiae* 87 (LT×SC), and sequential fermentation with *L. thermotolerans* 617 followed by *S. cerevisiae* 87 (LT...SC)

**Table 1.** Analytical results for the wines produced by different fermentations

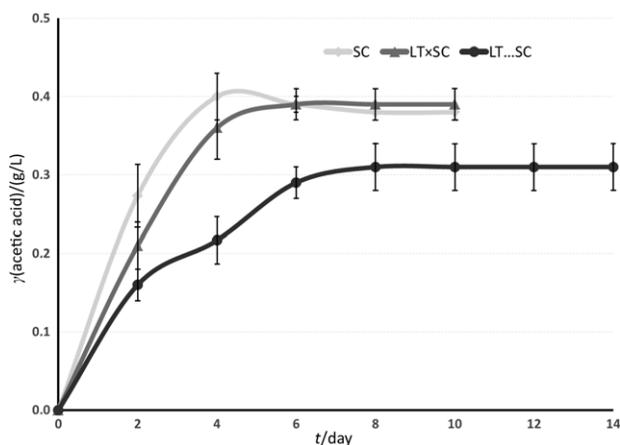
Compound	SC	LT×SC	LT...SC
$\gamma$ (L-lactic acid)/(g/L)	(0.02±0.01) <sup>a</sup>	(0.24±0.04) <sup>b</sup>	(3.18±0.19) <sup>c</sup>
$\gamma$ (L-malic acid)/(g/L)	(0.98±0.02) <sup>a</sup>	(1.02±0.03) <sup>ab</sup>	(1.04±0.03) <sup>b</sup>
$\gamma$ (acetic acid)/(g/L)	(0.38±0.02) <sup>a</sup>	(0.39±0.02) <sup>a</sup>	(0.31±0.03) <sup>b</sup>
$\gamma$ (glucose+fructose)/(g/L)	(1.88±0.42) <sup>a</sup>	(2.32±0.48) <sup>a</sup>	(2.77±0.56) <sup>a</sup>
$\gamma$ (glycerol)/(g/L)	(7.11±0.05) <sup>a</sup>	(7.18±0.08) <sup>a</sup>	(7.55±0.16) <sup>b</sup>
$\gamma$ (free SO <sub>2</sub> )/(mg/L)	(21.12±2.72) <sup>a</sup>	(19.99±3.26) <sup>a</sup>	(17.82±3.42) <sup>a</sup>
$\gamma$ (total SO <sub>2</sub> )/(mg/L)	(48.11±1.12) <sup>a</sup>	(46.28±2.46) <sup>a</sup>	(41.32±2.21) <sup>b</sup>
$\phi$ (alcohol)%	(14.36±0.02) <sup>a</sup>	(14.29±0.04) <sup>a</sup>	(13.91±0.08) <sup>b</sup>
$\gamma$ (acetaldehyde)/(mg/L)	(39.00±3.02) <sup>a</sup>	(35.00±2.01) <sup>b</sup>	(27.00±4.02) <sup>c</sup>
pH	(3.74±0.01) <sup>a</sup>	(3.71±0.02) <sup>a</sup>	(3.52±0.06) <sup>b</sup>

Results represent the mean value±S.D. of three replicates. Mean values in the same row with the same letter are not significantly different ( $p < 0.05$ )

SC=fermentation with *Saccharomyces cerevisiae* 87 alone, LT×SC=mixed fermentation with *Lachancea thermotolerans* 617 and *S. cerevisiae* 87, LT...SC=sequential fermentation with *L. thermotolerans* 617 followed by *S. cerevisiae* 87

### Acetic acid metabolism

Fig. 3 shows the kinetics of acetic acid release. Acetic acid concentration varied from 0.31 to 0.39 g/L (Table 1). LT...SC fermentation produced the lowest final acetic acid concentration. SC and LT×SC fermentations had similar final acetic acid content of about 0.38 g/L (Fig. 3). One of the problems raised by winemakers is the excessive increase of acetic acid in wines with high presence of non-*Saccharomyces* yeasts (1). However, previous experiments with *L. thermotolerans* reported significant reduction in final volatile acidity in sequential fermentations of 0.25 (19), 0.06 (56), 0.2 (42) and 0.08 g/L (70). Our results confirm an additional decrease in this compound related to the presence of *L. thermotolerans* (Fig. 3; Table 1). Never-

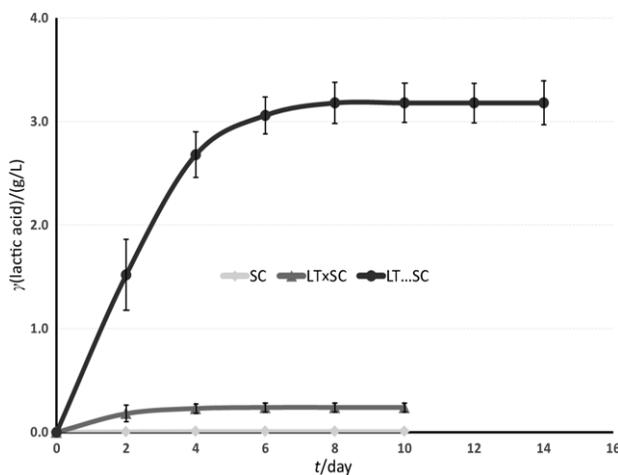


**Fig. 3.** Change in acetic acid concentration in the studied Airén wines during fermentation with *S. cerevisiae* 87 alone (SC), simultaneous fermentation with *Lachancea thermotolerans* 617 and *S. cerevisiae* 87 (LT×SC), and sequential fermentation with *L. thermotolerans* 617 followed by *S. cerevisiae* 87 (LT...SC)

theless, acetic acid concentration in all fermentations was not excessive and it did not affect wine quality negatively. The results show that a controlled use of *L. thermotolerans* in sequential fermentations can cause a decrease of acetic acid production.

#### L-lactic acid metabolism

Fig. 4 reports that only the fermentations involving *Lachancea thermotolerans* 617 produced L-lactic acid. The results varied from 0.24 g/L in LT×SC to 3.18 g/L in LT...SC (Table 1). Other authors obtained significant acidifications using combined microbiological cultures of *L. thermotolerans* and *S. cerevisiae* with the main objective of acidifying musts that were low in titratable acidity. Previously obtained values such as 3.42 g/L (19) were similar to the ones reported in this work. In other cases, acidification was higher; up to 5.13 g/L (56) has been reported depending on different trial conditions. The production of L-lactic acid is linked to the viable cell content (70). LT...SC fermentation proved to be the best option for acidifying wine in this study (Fig. 4; Table 1). In the case of LT×SC fermentation, the acidification was significantly lower due to the fast *Saccharomyces* growth, which impeded a higher acidification by *L. thermotolerans*.



**Fig. 4.** Change in L-lactic acid concentration in the studied Airén wines during fermentation with *Saccharomyces cerevisiae* 87 alone (SC), mixed fermentation with *Lachancea thermotolerans* 617 and *S. cerevisiae* 87 (LT×SC), and sequential fermentation with *L. thermotolerans* 617 followed by *S. cerevisiae* 87 (LT...SC)

#### L-malic acid metabolism

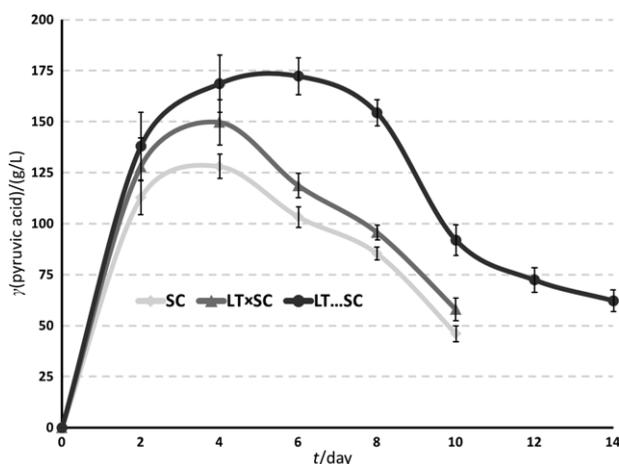
Only the final malic acid content in the SC fermentation was lower than in the other fermentations (Table 1); the maximum malic acid reduction rates of 17.65 % in SC, 14.29 % in LT×SC and 9.25 % in LT...SC fermentation from the initial concentration of 1.19 g/L were detected. The slight decrease in malic acid content observed in the fermentations (Table 1) is in agreement with other authors who confirmed that malic acid can be metabolised by several yeast species (44,52,57) at levels lower than 20 %, unless *Schizosaccharomyces* genus is involved.

#### Glycerol production

The glycerol content in LT...SC fermentation was higher than those observed in SC and LT×SC fermentations (Table 1). Final levels of glycerol varied from 7.11 to 7.55 g/L (Table 1). Increased glycerol content is described as one of the main contributions of non-*Saccharomyces* strains to wine quality (71) because it contributes positively to the mouthfeel. *L. thermotolerans* has been described before in literature as a higher glycerol producer than *S. cerevisiae*, reporting increases of about 0.69 (19) and 0.93 g/L (56). However, some authors have reported that an increase in glycerol production is usually related to an increase in acetic acid production (72), which can be detrimental to wine quality. Our results confirm that this fact seems to be irrelevant in the case of LT...SC fermentation.

#### Pyruvic acid production

Maximum pyruvic acid production was observed between the second and fourth day, reaching 128 and 149 mg/L, respectively (Fig. 5) during the fermentation of *Saccharomyces cerevisiae* 87 alone (SC) or LT×SC fermentation. LT...SC fermentation had higher values with a maximum concentration of pyruvic acid of 172.36 mg/L on day 6. Previous studies on the production of pyruvic acid by *S. cerevisiae* strains reported maximum values of 60–132 mg/L after 4 days of fermentation (52). Similar values were obtained in the present study in SC and slightly higher in LT×SC fermentation (Fig. 5). Nevertheless, the LT...SC fermentation obtained significantly higher levels, but not as high as those described for the genus *Schizosaccharomyces* (52). The concentrations of pyruvic acid and glycerol could indicate that *L. thermotolerans* possesses a highly active glyceropyruvic pathway (73).



**Fig. 5.** Change in pyruvic acid concentration in the studied Airén wines during fermentation with *S. cerevisiae* 87 alone (SC), mixed fermentation with *Lachancea thermotolerans* 617 and *S. cerevisiae* 87 (LT×SC), and sequential fermentation with *L. thermotolerans* 617 followed by *S. cerevisiae* 87 (LT...SC)

#### Acetaldehyde production

The fermentations involving *L. thermotolerans* 617 produced less acetaldehyde, with values that varied from 27 in LT...SC to 35 mg/L in LT×SC (Table 1). SC fermen-

tation produced more acetaldehyde than the others, with a final concentration of 39.00 mg/L (Table 1). Acetaldehyde is produced from the yeast metabolism of sugars and it is partly re-utilized (74). Although SC fermentation produced more acetaldehyde than the others (Table 1), all final values were under the sensory threshold of 100–125 mg/L (75).

### Volatile aroma

Isoamyl alcohol, ethyl octanoate and isoamyl acetate were formed in higher total concentrations during SC and LT×SC fermentations (Table 2). On the other hand, final concentrations of ethyl lactate, 2-phenylethanol and 2-phenylethyl acetate (Table 2) up to 5.98, 3.92 and 0.16 mg/L higher, respectively, were reported in LT...SC than in SC fermentation. Other authors have described non-*Saccharomyces* yeasts as weaker producers of higher alcohols than *Saccharomyces cerevisiae* (10,11,19,46,76). LT...DC fermentation produced the most 2-phenylethanol (Table 2). Other authors have reported higher production of 2-phenylethanol and ethyl lactate by *L. thermotolerans* than by *S. cerevisiae* (19), by up to 7.92 and 14.34 mg/L, respectively. *L. thermotolerans* has also been reported as a weaker ethyl acetate producer than *S. cerevisiae* (19).

Table 2. Concentrations of volatile compounds detected during different fermentations

$\gamma$ /(mg/L)	SC	LT×SC	LT...SC
Hexanol	(0.96±0.03) <sup>a</sup>	(1.02±0.04) <sup>a</sup>	(0.98±0.06) <sup>a</sup>
Isoamyl alcohol	(132.82±6.06) <sup>a</sup>	(126.92±9.11) <sup>a</sup>	(102.43±10.64) <sup>b</sup>
Isobutanol	(11.36±1.28) <sup>a</sup>	(12.56±1.86) <sup>a</sup>	(14.42±2.76) <sup>a</sup>
Ethyl acetate	(54.42±3.33) <sup>a</sup>	(53.61±3.42) <sup>a</sup>	(50.36±5.56) <sup>a</sup>
Ethyl decanoate	(0.11±0.02) <sup>a</sup>	(0.13±0.03) <sup>a</sup>	(0.15±0.06) <sup>a</sup>
Ethyl hexanoate	(0.32±0.02) <sup>a</sup>	(0.34±0.04) <sup>a</sup>	(0.29±0.06) <sup>a</sup>
Ethyl lactate	(7.16±0.21) <sup>a</sup>	(8.89±0.48) <sup>b</sup>	(13.14±2.18) <sup>c</sup>
Ethyl octanoate	(0.36±0.06) <sup>a</sup>	(0.39±0.09) <sup>a</sup>	(0.25±0.07) <sup>b</sup>
Isoamyl acetate	(1.62±0.03) <sup>a</sup>	(1.48±0.06) <sup>a</sup>	(0.98±0.11) <sup>b</sup>
Hexanoic acid	(8.24±0.54) <sup>a</sup>	(8.32±0.72) <sup>a</sup>	(8.18±1.16) <sup>a</sup>
Octanoic acid	(4.32±0.12) <sup>a</sup>	(4.44±0.18) <sup>a</sup>	(3.16±0.22) <sup>a</sup>
2-Phenylethanol	(18.16±0.15) <sup>a</sup>	(19.32±0.82) <sup>a</sup>	(22.08±0.93) <sup>b</sup>
2-Phenylethyl acetate	(0.36±0.01) <sup>a</sup>	(0.39±0.03) <sup>a</sup>	(0.52±0.06) <sup>b</sup>

Results represent the mean value±S.D. of three replicates. Mean values in the same row with the same letter are not significantly different ( $p < 0.05$ )

SC=fermentation with *Saccharomyces cerevisiae* 87 alone, LT×SC=mixed fermentation with *Lachancea thermotolerans* 617 and *S. cerevisiae* 87, LT...SC=sequential fermentation with *L. thermotolerans* 617 followed by *S. cerevisiae* 87

### Amino acids and biogenic amines

Higher final levels of histidine, glycine and leucine were obtained in SC and LT×SC fermentations than in LT...SC fermentation (Table 3). LT...SC fermentation had higher final levels of alanine, lysine and serine (Table 3). The final concentrations of each biogenic amine were always lower than 1 mg/L (Table 4). Differences in the amino acid patterns among the different fermentations were

Table 3. Concentrations of amino acids determined after different fermentations

$\gamma$ /(mg/L)	SC	LT×SC	LT...SC
Histidine	(6.42±0.87) <sup>a</sup>	(6.79±1.06) <sup>a</sup>	(4.15±1.21) <sup>b</sup>
Aspartic acid	(8.62±1.25) <sup>a</sup>	(9.13±1.60) <sup>a</sup>	(10.21±2.12) <sup>a</sup>
Alanine	(50.12±2.58) <sup>a</sup>	(52.27±2.89) <sup>ab</sup>	(58.14±3.12) <sup>b</sup>
Arginine	(26.06±1.86) <sup>a</sup>	(27.16±2.52) <sup>a</sup>	(29.42±3.06) <sup>a</sup>
Asparagine	(29.18±2.13) <sup>a</sup>	(28.42±2.82) <sup>a</sup>	(25.22±3.16) <sup>a</sup>
Phenylalanine	(8.52±0.63) <sup>a</sup>	(8.62±0.89) <sup>a</sup>	(8.76±1.62) <sup>a</sup>
Glycine	(28.43±1.08) <sup>a</sup>	(27.12±1.78) <sup>ab</sup>	(23.56±2.22) <sup>b</sup>
Tryptophan	(0.00±0.00) <sup>a</sup>	(0.00±0.00) <sup>a</sup>	(0.00±0.00) <sup>a</sup>
Isoleucine	(2.06±0.22) <sup>a</sup>	(2.18±0.42) <sup>a</sup>	(2.36±1.11) <sup>a</sup>
Lysine	(2.42±0.62) <sup>a</sup>	(2.82±0.86) <sup>a</sup>	(6.13±1.88) <sup>b</sup>
Leucine	(5.14±0.42) <sup>b</sup>	(4.92±0.91) <sup>a</sup>	(3.11±0.89) <sup>b</sup>
Ornithine	(25.17±0.16) <sup>a</sup>	(25.19±1.06) <sup>a</sup>	(23.18±1.18) <sup>a</sup>
Serine	(2.28±0.26) <sup>a</sup>	(2.36±0.76) <sup>a</sup>	(4.13±0.85) <sup>b</sup>
Tyrosine	(5.36±0.46) <sup>a</sup>	(5.39±0.68) <sup>a</sup>	(6.28±0.72) <sup>a</sup>
Threonine	(36.42±0.18) <sup>a</sup>	(35.43±0.68) <sup>a</sup>	(34.21±1.13) <sup>a</sup>

Results represent the mean value±S.D. of three replicates. Mean value in the same row with the same letter are not significantly different ( $p < 0.05$ )

SC=fermentation with *Saccharomyces cerevisiae* 87 alone, LT×SC=mixed fermentation with *Lachancea thermotolerans* 617 and *S. cerevisiae* 87, LT...SC=sequential fermentation with *L. thermotolerans* 617 followed by *S. cerevisiae* 87

Table 4. Biogenic amine concentration in the studied fermentations

$\gamma$ /(mg/L)	SC	LT×SC	LT...SC
Histamine	(0.37±0.02) <sup>a</sup>	(0.38±0.03) <sup>a</sup>	(0.40±0.04) <sup>a</sup>
Tyramine	(0.04±0.01) <sup>a</sup>	(0.03±0.02) <sup>a</sup>	(0.03±0.02) <sup>a</sup>
Phenylethylamine	n.d.	n.d.	n.d.
Putrescine	(0.76±0.03) <sup>a</sup>	(0.79±0.04) <sup>a</sup>	(0.75±0.05) <sup>a</sup>
Cadaverine	(0.22±0.01) <sup>a</sup>	(0.23±0.02) <sup>a</sup>	(0.21±0.04) <sup>a</sup>

Results represent the mean value±S.D. of three replicates. Mean values in the same row with the same letter are not significantly different ( $p < 0.05$ ). n.d.=not detected

SC=fermentation with *Saccharomyces cerevisiae* 87 alone, LT×SC=mixed fermentation with *Lachancea thermotolerans* 617 and *S. cerevisiae* 87, LT...SC=sequential fermentation with *L. thermotolerans* 617 followed by *S. cerevisiae* 87

found, but they could not be related to the aroma of the Airén wines. Different autolysis behaviour might be the reason for this. A histamine value of 2 mg/L is considered a limiting factor (77) in some countries due to food safety legislation. Our results prove that *L. thermotolerans* does not produce higher levels of biogenic amines than *S. cerevisiae*. However, most biogenic amines are produced during malolactic fermentation and wine ageing (78). Nevertheless, the lower concentration of histidine (precursor of histamine) found during LT...SC fermentation (Table 3) contributes to reducing the potential risk of histamine formation by bacterial metabolism. Even though no significant differences were found in final biogenic amine contents, other authors have reported reductions of histamine

of up to 2.2 mg/L during alcoholic fermentation with the non-*Saccharomyces* species *Hanseniaspora vineae* (6).

### Sensory evaluation

Wines produced in LT...SC fermentation trials had better sensorial properties and general acidity (Fig. 6). However, SC and LT...SC fermentations scored highest in sweetness (Fig. 6). This can be easily explained by the elevated L-lactic acid production by *L. thermotolerans*. Lack of acidity is a common fault described for Spanish Airén grape variety when compared to other European varieties. Although the wines obtained in SC and LT×SC fermentations were evaluated as sweeter than those in the LT...SC fermentation, all final wines were considered dry from a chemical point of view (Table 1). This perception could be explained due to the different balance between the acidity and sweetness.

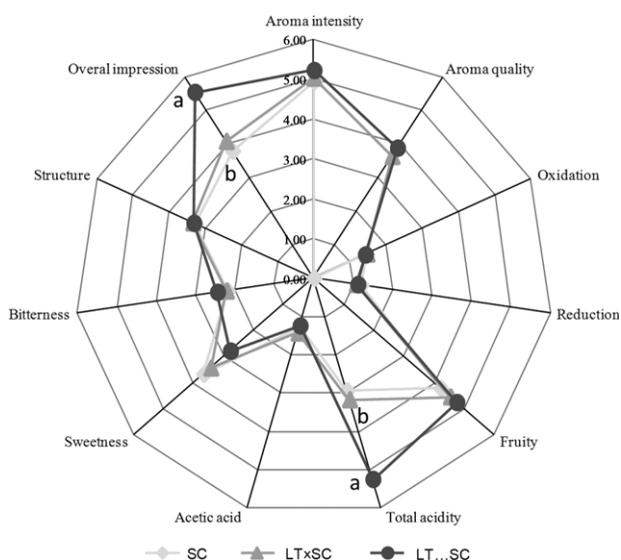


Fig. 6. Taste and olfactory attribute scores for the final wines

### Conclusions

The comparison of the results between the fermentation trials showed differences in several analysed parameters and the positive influence of the studied *Lachancea thermotolerans* yeast strain on Airén wine quality. Finally, sequential fermentation with *L. thermotolerans* and *Saccharomyces cerevisiae* remains the best option, as it considerably increased acidity and complexity of the studied neutral grape variety.

### Acknowledgements

The authors are very grateful to EUITA Agrícola oenology students for their help in performing the trials during the wine microbiology lessons and to the accredited laboratory Estación Enológica de Haro directors, Montserrat Iñiguez and Elena Meléndez (Haro, Spain) for performing the analyses of volatile aromas, amino acids and biogenic amines.

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