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# Investigating the biochemical and fermentation attributes of *Lachancea* species and strains: Deciphering the potential contribution to wine chemical composition

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ABSTRACT

Yeasts of various genera are increasingly used alongside *Saccharomyces cerevisiae* to drive wine fermentations owing to their positive contribution to the organoleptic profile of the resulting wines. One such yeast species is *Lachancea thermotolerans*. Other species of the genus *Lachancea*, namely, *L. fermentati* and *L. lanzarotensis* have also been isolated from the fermentation environment, but have not received the same degree of attention as *L. thermotolerans*. The aim of this study was to investigate the oenological potential of these three *Lachancea* species, regarding their expression of oenologically relevant enzymes, their fermentation attributes and the expression and location of β-glucosidase during fermentation of synthetic and real grape must (Muscat of Alexandria). In the current study we evaluated three species *viz. L. thermotolerans* (14 strains), *L. fermentati* (1 strain) and *L. lanzarotensis* (2 strains). Our data show that all the species and strains produced β-glucosidase but with different substrate specificities. Moreover, *L. theromotolerans* and *L. fermentati* also produced β-xylosidase. $H_2S$ production, $SO_2$ and ethanol tolerance was variable between species and strains, with the *L. lanzarotensis* and *L. fermentati* displaying considerably high $H_2S$ production while *L. thermotolerans* and *L. fermentati* displayed higher ethanol tolerance. Furthermore, *L. fermentati* showed higher $SO_2$ tolerance and could proliferate at 20 mg/L total $SO_2$. Interestingly, an increase in β-glucosidase activity during fermentation did not result in a significant increase in monoterpene concentrations. However, mixed-fermentations with *L. fermentati* and *L. thermotolerans* Concerto enhanced geraniol levels. The data show that this activity was mostly cell-associated and constitutively expressed. Sequential fermentations with the *Lachancea* spp. and *S. cerevisiae* resulted in wines with significantly altered chemical compositions compared to that obtained from *S. cerevisiae* inoculated alone. Wines produced from *L. thermotolerans* and *L. lanzarotensis* mixed culture fermentations exhibited similar volatile compound composition. Conversely, *L. fermentati* produced chemically distinct wines consistently associated with high isobutanol and isobutyric acid, and higher monoterpenes. In particular, linalool and geraniol had potential to make perceivable aroma contribution (OAV ≥ 1).

## 1. Introduction

Wine aroma is derived from a complex medley of compounds. This complexity is due to the formation of varietal, fermentation and ageing aroma compounds (Belda et al., 2017). Fermentative aromas form a large proportion of these compounds, and the yeast community plays a significant role in their production. Wine yeasts are however also able to release varietal aroma compounds, consisting of sulphur compounds, methoxypyrazines, C13-norisoprenoids and terpenes (Ebeler and Thorngate, 2009). Some of the varietal aromas, in particular monoterpenes, are present in varying amounts as conjugated non-volatile precursors, often linked to mono- and di-glycosides such as 6-O-β-L-arabinofuranosyl-β-D-glucopyranoside, 6-O-β-L-rhamnopyranosyl-β-D-glucopyranoside, 2-O-β-D-xylosyl-D-glucopyranoside and 6-O-β-L-apiofuranosil-β-D-glucopyranoside (Mateo and Di Stefano, 1997). Such glycosidically-bound monoterpenes are important precursors of the varietal aroma of wines from the Muscat family of *Vitis vinifera*. For instance, in Muscat of Alexandria grape must, the ratio between bound and free (aromatic) monoterpenes has been reported to be as high as 5:1 (Williams et al., 1982), leaving a large portion of potential aroma unavailable. Glycolytic enzymes can hydrolyse these complexes, where α-L-arabinofuranosidase, α-L-rhamnopyranosidase or β-D-apiofuranosidase (depending on the conjugated sugar moiety) and β-D-glucosidase work in succession to hydrolyse diglycoside complexes,

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and β-glucosidases act alone to release volatile monoterpenes from monoglucoside complexes (Günata et al., 1988).

β-Glucosidases occur widely in wine yeasts, including *Saccharomyces* and non-*Saccharomyces* species and strains (Belda et al., 2016; Manzanares et al., 1999; Maturano et al., 2012). However, non-*Saccharomyces* yeasts generally produce a broad range of enzymes, including β-glucosidases, β-xylosidases, proteases, pectinases, lyases and esterases in considerably higher amounts than *S. cerevisiae* (Albertin et al., 2016; Belda et al., 2016; Lopez et al., 2014; Maturano et al., 2012, 2015). Amongst the non-*Saccharomyces* yeasts, members of the genus *Lachancea*, in particular strains of *Lachancea thermotolerans* (formerly *Kluyveromyces thermotolerans*) and *Lachancea fermentati* (formerly *Zygosaccharomyces fermentati*), have been reported to produce β-glucosidases (Comitini et al., 2011; Cordero-Bueso et al., 2013; Romo-Sánchez et al., 2010; Rosi et al., 1994). Furthermore, strains of *L. thermotolerans* were shown to hydrolyse terpenoids, norisoprenoids and benzenoids glycosides in Muscat must and transform the non-volatile substrates into volatile monoterpenes (Fernández-González et al., 2003).

*Lachancea thermotolerans* is commonly associated with the wine fermentation environment and is the most extensively studied member of this genus. Recent population genetics analysis has revealed that *L. thermotolerans* isolates from grapes and wine are genetically close, but they currently occupy two domestic sub-populations with distant ancestries (Hranilovic et al., 2017). *L. thermotolerans* is one of the key non-*Saccharomyces* yeast species that are desired for their positive contribution to wine organoleptic characteristics. The most notable wine-relevant biochemical traits of *L. thermotolerans* strains, is their ability to produce less acetic acid, enhance wine acidity through the production of L-lactic acid, and enhance glycerol and 2-phenylethanol levels in mixed culture fermentations with *S. cerevisiae* (Balikci et al., 2016; Benito, 2018; Comitini et al., 2011; Kapsopoulou et al., 2005). However, members of this species display marked intra-specific diversity at a phenotypic level, particularly with regard to growth rates on different carbon substrates and under different physicochemical conditions (Hranilovic et al., 2017). Few studies on *L. fermentati* strains have compared this species to *Zygosaccharomyces baili* strains, and not *S. cerevisiae*, and have not been performed in a mixed fermentation setting. However, the comparisons found a higher fermentation vigour, and low acetic acid, low $H_2S$ and low $SO_2$ production (Cordero-Bueso et al., 2013; Romano and Suzzi, 1993). While a considerable amount of research has been done to explore the genotypic and phenotypic space of *L. thermotolerans*, the other *Lachancea* species remain untapped. The current study investigated the oenological behaviour of *Lachancea* spp. isolates and strains (isolated in the Stellenbosch region from different grape juices), in comparison with *S. cerevisiae*.

## 2. Materials and methods

### 2.1. Yeast strains and growth conditions

#### 2.1.1. Yeast strains

In the current study 14 strains of *Lachancea thermotolerans* comprising 13 strains isolated from different grape varietals and vineyards in South Africa, as well as 1 commercial strain, Concerto™ (from Chr. Hansen) were screened (Table 1). We also screened 6 South African isolates of *Lachancea lanzarotensis* as well as the type strain CBS 12615$^T$ (obtained from Central Bureau of Fungal Cultures (CBS) culture collection) and one *Lachancea fermentati* strain also isolated in South Africa (Table 1). The South African isolates and strains are routinely maintained as 20% (v/v) glycerol stock cultures at −80 °C in the culture collection of the Institute for Wine Biotechnology (Stellenbosch University). The South African isolates and strains with the exception of *L. thermotolerans* Y1240 have not previously been evaluated for their oenological characteristics. Positive controls used for hydrolytic enzyme activity screening included *Schwanniomyces polymorphus* var. *africanus* CBS 8047 (Cordero Otero et al., 2003), *Saccharomyces paradoxus* RO88 (Mocke, 2005), *Metschnikowia pulcherrima* IWBT Y1123 (Reid et al., 2012), *Saccharomyces cerevisiae* V517-5A (Unité de Recherche Œnologie, Institut des Sciences de la Vigne et du Vin, Villenave d'Ornon, France), *S. cerevisiae* VIN13car1::XYN4 (unpublished, courtesy of Dr. A Zietsman, IWBT) and *Saccharomyces bayanus* (Vivace, Renaissance Yeast, Vancouver, Canada). For comparative analysis, *Saccharomyces cerevisiae* VIN13 (Anchor Yeast, Cape Town, South Africa) was used as the control strain for enzyme activities, fermentations, and tolerance to ethanol and sulphur dioxide ($SO_2$).

#### 2.1.2. Growth conditions

All yeast strains were cultivated on Wallerstein Laboratory Nutrient (WLN) agar or in Yeast Extract Peptone Dextrose (YPD) broth at 25 °C for 5 days. For the *L. thermotolerans* and *L. fermentati* strains, flocculation was observed in YPD broth and therefore following harvesting of the cells, the pellets were treated with 50 mM Ethylenediaminetetraacetic acid (EDTA) (pH 8.0) and washed with physiological saline (0.9% (w/v) NaCl) in order to dislodge the flocs.

### 2.2. Screening for enzymatic activities and $H_2S$ production

The yeast cells were prepared as described in Section 2.1.2 above and the absorbance ($OD_{600nm}$) was determined after overnight growth in YPD broth. Five microlitres of a cell culture at an $OD_{600nm}$ of 0.1, was spotted, in triplicate, onto the selected agar plates prepared for enzyme activity screening. The medium was adjusted to pH 3.5 with 50 mM citrate phosphate buffer.

#### 2.2.1. β-Glucosidase and β-xylosidase activity

β-Glucosidase activity was determined on two substrates, namely arbutin and 4-methylumbelliferyl-β-D-glucoside (4-MUG, Sigma Aldrich, St Louis, MO). The arbutin medium contained per litre, 10 g yeast extract, 20 g peptone and 5 g arbutin (Sigma Aldrich), made up to 730 mL after the pH was adjusted to 3.5. The medium was autoclaved and subsequently mixed with 20 mL of a 1% (w/v) filter sterilised ammonium ferric citrate and 250 mL of a 4× bacteriological agar which had been autoclaved separately (Albertin et al., 2016). The 4-MUG medium contained in (w/v): 0.17% Yeast Nitrogen Base (YNB) -without amino acids and ammonium sulphate, 0.5% ammonium sulphate, 0.5% xylose and 2% bacteriological agar, adjusted to pH 5.5. This medium was prepared according to Manzanares et al. (1999), with the modification of the substrate addition (10% (w/v) filter sterilised 4-MUG). *Schwanniomyces polymorphus* var. *africanus* DSM 8047 was used as the positive control (Cordero Otero et al., 2003). Similarly, β-xylosidase activity was evaluated on 4-methylumbelliferyl-β-D-xyloside (4-MUX, Sigma Aldrich). *Saccharomyces cerevisiae* VIN13 Δ*car1*::*XYN4* was used as the positive control (unpublished, courtesy of Dr. A Zietsman, IWBT). For both assays, the plates were incubated at 25 °C for a minimum of 5 days. For arbutin, positive enzyme activity was seen as the formation of a brown colour in the colonies, while positive activity in the 4-MUG and 4-MUX assays was indicated by halos surrounding the colonies under UV light (Supplementary Table 1).

#### 2.2.2. Polygalacturonase activity

Polygalacturonases are pectin depolymerases that work synergistically with pectin methylesterases to breakdown pectin, thus enhancing grape juice extraction and reducing viscosity (Belda et al., 2016; Mostert, 2013; Strauss et al., 2001). Polygalacturonase activity was evaluated on differential agar plates containing 1.25% (w/v) polygalacturonic acid dissolved in 0.68% (w/v) potassium phosphate, together with 0.67% (w/v) YNB and 1% (w/v) glucose, adjusted to pH 3.5 and mixed with 2% (w/v) agar as described by Albertin et al. (2016), modified from McKay (1988). The plates were incubated at 25 °C for 5 days. The colonies were washed off with distilled water and the plates flooded with 6 M HCl (Mostert, 2013). The formation of a clear halo

**Table 1**
Yeast strains/isolates investigated in this study.

| Yeast species | Origin of isolation/manufacturer | Strain/Isolate |
|---|---|---|
| *Lachancea thermotolerans* | Sauvignon blanc, Somerset West, South Africa | Y1202 |
| | Sauvignon blanc, Somerset West, South Africa | Y1109 |
| | Chardonnay, Paarl, South Africa | Y1017 |
| | Muscat of Alexandria, Rawsonville, South Africa | Y1240 |
| | Chardonnay, Somerset West, South Africa | Y1326 |
| | Sauvignon blanc, Somerset West, South Africa | Y1206 |
| | Sauvignon blanc, Somerset West, South Africa | Y1197 |
| | Chardonnay, Sir Lowry's Pass, South Africa | Y1038 |
| | Sauvignon blanc – Chardonnay blend, Somerset West, South Africa | Y1295 |
| | Sauvignon blanc, Elgin Valley, South Africa | Y513 |
| | Chenin blanc, Riebeeck Casteel, South Africa | Y905 |
| | Shiraz, Stellenbosch, South Africa | Y940 |
| | Chardonnay, Sir Lowry's Pass, South Africa | Y1220 |
| | Chr. Hansen, Hørsholm, Denmark | Concerto™ |
| *Lachancea lanzarotensis* | Listán Negro and Malvasía Lanzarote, Canary Islands, Spain | CBS 12615$^T$ |
| | Sauvignon blanc, Elgin Valley, South Africa | Y992-1 |
| | | Y992-6 |
| | | Y992-4 |
| | | Y992-5 |
| | | Y992-2 |
| | | Y992-3 |
| *Lachancea fermentati* | Sauvignon blanc, Stellenbosch, South Africa | Y515 |
| *Saccharomyces cerevisiae* | Anchor Yeast | VIN13 |

surrounding the colonies was observed as positive polygalacturonase activity (Supplementary Table 1). *Saccharomyces paradoxus* RO88 was used as a positive control (Mocke, 2005).

### 2.2.3. Acid protease activity

Acid proteases or Aspartic proteases (EC 3.4.23) are increasingly gaining interest in winemaking due to their potential application in the removal of proteins to prevent haze formation in white wines (Reid et al., 2012). In the current study we screened *Lachancea* species for this activity on skim milk agar prepared according to Bilinski et al. (1988). Briefly, 480 mL of medium containing 4.8 g glucose, 3.36 g Difco™ Yeast Nitrogen Base (without amino acids and ammonium sulphate) (YNB) (BD, Sparks, MD) and 9.6 g bacteriological agar was prepared and autoclaved. The agar was mixed with a skim milk solution, which had been prepared by combining 70 mL of 10% (w/v) skim milk (in 0.05 M Citrate-Phosphate buffer, pH 3.5) with 60 mL of phosphate buffer (24 g/L $KH_2PO_4$ and 35 g/L $Na_2HPO_4.7H_2O$) and microwaving until it simmered. *Metschnikowia pulcherrima* Y1123 (Reid et al., 2012) was used as a positive control. The plates were incubated at 25 °C for a minimum of 5 days, after which, positive protease activity was visualised as a clear halo surrounding the colonies (Supplementary Table 1).

### 2.2.4. $H_2S$ production

The production of hydrogen sulphide ($H_2S$) was evaluated using Bismuth Sulfite Glucose Glycine Yeast (BiGGY) Agar (Sigma Aldrich). Individual colonies of the yeast strains were streaked onto the agar. Two strains, *S. cerevisiae* V517-5A (Unité de Recherche Œnologie, Institut des Sciences de la Vigne et du Vin, Villenave d'Ornon, France), and *Saccharomyces bayanus* Vivace (Renaissance Yeast, Vancouver, Canada), were used as high and low $H_2S$ producing controls, respectively. The plates were incubated at 25 °C for 5 days, after which $H_2S$ production was visually inspected. The formation of a brown colour in the colonies and/or surrounding the colonies was indicative of $H_2S$ production. The range of $H_2S$ production was subsequently based on the respective colour intensities of the positive and negative controls (Supplementary Table 2).

## 2.3. Screening ethanol and $SO_2$ tolerance

### 2.3.1. Ethanol and $SO_2$ tolerance

To evaluate tolerance to ethanol, cell solutions of each strain were prepared to $OD_{600nm}$ of 0.1 ($\approx 10^6$ cells/mL) and used as working cultures. A dilution series was prepared for each strain and 5 µL of each dilution was spotted onto YPD agar adjusted to pH 3.5 and supplemented with different concentrations of ethanol, namely; 0, 5, 7 and 10% (v/v). These plates were then incubated at 25 °C for 6 days, and growth observed – indicating relative sensitivity (Supplementary Table 3).

The $SO_2$ tolerance was evaluated in liquid cultures. Broth containing 0.67% (w/v) YNB and 2% (w/v) glucose (pH 3.5) was used as base medium to which potassium metabisulfite was added, to create total $SO_2$ concentrations of 4.22, 8.32, 10, 15, 20 and 25 mg/L, which corresponded to 0.05, 0.1, 0.12, 0.18 and 0.24 mg/L molecular $SO_2$, respectively, as determined in YNB according to (Usseglio-Tomasset, 1984). Yeast strains were inoculated at an $OD_{600nm}$ of 0.1 and incubated at 22 °C, with gentle agitation at 50 rpm. Optical density was measured at 24 and 48 h. *Saccharomyces cerevisiae* VIN13 was used as a positive control for both ethanol and $SO_2$ tolerance. Since, *L. fermentati* was found to be the most $SO_2$ tolerant, its growth was compared was compared to that of *S. cerevisiae* at 0, 10, 15, 20 and 25 mg/L, up to 72 h.

## 2.4. Sequential fermentation

### 2.4.1. Synthetic grape juice fermentations

Based on the initial enzyme activity screening and the respective tolerance levels, five strains were selected for further analysis. These included the *L. thermotolerans* strains, IWBT Y940 which was able to tolerate higher ethanol and $SO_2$ concentrations and the commercial strain, Concerto™ which is already well characterized for comparison, *L. lanzarotensis* type strain (CBS 12615) and only one South African isolate (IWBT Y992-5) as all the isolates displayed similar behaviour and could therefore be considered to belong to one strain, and lastly,

the *L. fermentati* strain, which was the only representative of the species during the course of the study. *Saccharomyces cerevisiae* VIN13 was used as a control. Fermentations were performed in 250 mL synthetic grape juice (pH 3.5), comprising (per litre) 100 g glucose, 100 g fructose, 1 g yeast extract (Oxoid), 2 g $(NH_4)_2SO_4$, 0.3 g citric acid, 5 g L-malic acid, 5 g L-tartaric acid, 0.4 g $MgSO_4$, 5 g $KH_2PO_4$, 0.2 g NaCl, 0.05 g $MnSO_4$, anaerobic factors (Ergosterol 10 mg, Tween 80 0.5 mL) prepared according to Henschke and Jiranek (1993), and 300 mg assimilable nitrogen provided as (460 mg $NH_4Cl$ and 180 mg amino acids) according to Bely et al. (1990). The flasks were fitted with S-shape airlocks partially filled with sterile distilled water and silicon caps. The fermentations were performed in triplicate at 25 °C with gently agitation at 60 rpm. The monoculture fermentations were inoculated with a final cell density of $\approx 1 \times 10^6$ CFU/mL and the mixed-culture (sequential) fermentations were inoculated with the *Lachancea* strains at $\approx 1 \times 10^7$ CFU/mL 48 h prior to the addition of *S. cerevisiae* VIN13 at $\approx 1 \times 10^6$ CFU/mL. Fermentation progress was monitored by weighing the flasks daily and the yeast growth by measuring optical density or determining viable counts on WLN agar (on which morphological differences between the species could be seen). The end of fermentation was assumed when the weight loss was constant over two days.

#### 2.4.2. Muscat of Alexandria grape juice fermentations

Frozen Muscat of Alexandria grape juice was thawed and thermovinified by heating at 70 °C for 15 min. A sample of the heated juice was plated onto YPD agar to assess viable yeast load. The juice had the following properties: pH 3.4, 187 g/L reducible sugars (glucose/fructose), 26 mg/L ammonia and 161 mg N/L primary amino nitrogen (as determined with the Megazyme K-FRUGL, K-AMIAR, K-PANOPA enzymatic kits (Megazyme International, Ireland), respectively). Sixty millilitres of the juice was dispensed into 100 mL cylindrical flasks and inoculated with monocultures or with mixed-cultures. The flasks were sealed with an S-shape airlock partially filled with sterile distilled water and closed with a silicon cap. The fermentations were performed and monitored as described for the synthetic grape juice.

### 2.5. β-Glucosidase activity throughout fermentations

#### 2.5.1. Protein extraction from synthetic grape juice fermentations

Samples were withdrawn at the beginning, middle and end stages of the fermentation in order to determine β-glucosidase activity. These stages correlated with days 1 and 2 (beginning), days 6 and 8 (middle), and day 14 (end), based on observed cumulative weight loss during fermentation. Intracellular and cell wall-associated proteins were extracted according to Dunn and Wobbe (1992) with modifications at the spheroplasts lysis step. Briefly, sterile microbeads (100 µL) were added to the solution containing the spheroplasts and very slowly vortexed for approximately 30 s. The solution was thereafter cooled down in ice for another 30 s and this vortex-cool cycle was repeated 3 times. The lysed spheroplasts were then centrifuged for 10 min at 1500 *g*. The supernatant provided the intracellular protein extract. Extracellular activity was determined from the cell free supernatant obtained after centrifugation at 1500 *g* for 5 min. Alternatively, cell-associated proteins were extracted using the yeast protein extraction reagent, Y-PER™ (Thermoscientific) and the lysate was used to quantify protein concentrations and β-glucosidase activity. The protein concentration was determined using the Bradford reagent (Sigma-Aldrich) as per the manufacturer's instructions; utilising bovine serum albumin (BSA) as the standard.

#### 2.5.2. β-Glucosidase activity assay

β-Glucosidase activity was determined according to Rättö and Poutanen (1988) using 1 mM 4-nitrophenyl-β-glucopyranoside (Sigma Aldrich) in 0.05 M citrate buffer pH 5.3 as a substrate. Reactions were performed by incubating 100 µL of sample with 900 µL of pre-warmed substrate for 10 min at 50 °C. The reaction was stopped by adding 500 µL of 1 M $Na_2CO_3$ and the liberated *p*-nitrophenol was measured spectrophotometrically at 400 nm. β-Glucosidase activity was calculated using a *p*-nitrophenol (Sigma Aldrich) standard curve and expressed in SI units (katals).

### 2.6. Chemical analysis of resulting wines

#### 2.6.1. Gas chromatography-flame ionisation detector (GC-FID) analysis

GC-FID was performed on the resulting wines (with 2× technical and 3× biological repeats) in order to analyse major esters, higher alcohols, and volatile acids. The wine, with internal standard 4-Methyl-2-Pentanol, (100 µL of 0.5 mg/mL solution in soaking solution), was extracted with diethyl ether. The injection volume was 3 µL and a DB-FFAP, 60 m × 0.32 mm × 0.5 µm f.t. column was utilised (Louw et al., 2009). To determine the potential contribution of individual major volatiles on wine aroma, odour activity values (OAVs) were calculated by dividing the mean concentration of each compound with its odour threshold (OTH, Supplementary Table 4) values previously determined in water/ethanol mixture (Guth, 1997; Peinado et al., 2004).

#### 2.6.2. High performance liquid chromatography (HPLC) analysis

HPLC was performed in order to determine the concentrations of the major sugars (glucose and fructose), organic acids (citric, tartaric, malic, succinic and acetic acid) in addition to glycerol and ethanol in the resulting wines (2× technical and 3× biological repeats). An argilent 1100 series HPLC system, Chemstation Rev. A10.02 software, an Aminex HPX-87 column (300 mm × 8.8 mm) and a Bio-Rad guard column (30 mm × 4.6 mm) was used (Eyéghé-Bickong et al., 2012).

#### 2.6.3. Head-space gas chromatography mass spectrometry (GC–MS) analysis

For the evaluation of monoterpenes in the Muscat juice-fermented wines (2× technical and 3× biological repeats), 10 mL of the wine samples, 2.5 mL 20% NaCl and 100 µL internal standard (3 octanol and Anisol-D8 1 ppm in methanol) were added to 20 mL screw cap GC vials, in triplicate, and vortexed. Solid phase micro-extraction (SPME) of the head-space was performed using a 50/30 µm grey divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibre (Supelco, Bellefonte, PA) which had previously undergone conditioning for 60 min at 270 °C in the GC injection port. The vials were incubated at 50 °C for 5 min in the autosampler heating chamber; which was rotated at 250 rpm to allow compounds in the sample and headspace equilibrium. While these conditions were maintained, the fibre was inserted through the septa and exposed to the headspace analytes for 20 min. In the injection port, the analytes were desorbed and the fibre maintained for 1 min to prevent carryover.

Analysis was performed utilising a Trace 1300 gas chromatograph (Thermo Scientific, Germany) system coupled to a Triplus RSH autosampler and a TSQ 8000 MSD mass spectrometer detector through a transfer line. A Zebron 7HG-G009-11 ZB-FFAP capillary column (30 m × 250 ID µm, 0.25 µm film thickness) (Phenomenex, USA) was also utilised. Analyte desorption took place at 250 °C for 5 min with a 50:1 split, and helium as the carrier gas (initial flow rate 1 mL/min). The oven temperature was initially 50 °C (3 min), which was then ramped at a rate of 10 °C/min to 240 °C and held for 2 min, with the total run of 24 min and the transfer line temperature of 250 °C.

### 2.7. Statistical analysis

The standard deviations of the biological and technical repeats for chemical and enzymatic analysis were calculated to analyse the variability of the experiments and technical processes, respectively. The data obtained was tested for normality and the statistical significance calculated through one-way analysis of variance (ANOVA). Tukey's test was utilised to calculate the significant differences between group

**Table 2**
Screening of extracellular enzyme activity and $H_2S$ production on substrate specific agarose plates. The strains chosen for further analysis are indicated with an asterisk.

| Yeast species | Strain/Isolate | β-glucosidase | | β-xylosidase | $H_2S$ production |
|---|---|---|---|---|---|
| | | Arbutin | 4-MUG | 4-MUX | Bismuth |
| *L. thermotolerans* | Y1202 | + | + | + | + + |
| | Y1109 | + | + | + | + + |
| | Y1017 | + | + | + | + + |
| | Y1240 | + | + | + | + |
| | Y1326 | + | + | + | + + |
| | Y1206 | + | + | + | + + |
| | Y1197 | + | + | + | + |
| | Y1038 | + | + | + | + + |
| | Y1295 | + | + | + | + + |
| | Y513 | + | + | − | + + |
| | Y905 | + | + | + | + + |
| | Y940* | + | + | + | + |
| | Y1220 | + | + | + | + |
| | Concerto* | + | + | − | + |
| *L. lanzarotensis* | CBS 12615* | + | − | − | + + + |
| | Y992-1 | + | − | − | + + + + |
| | Y992-6 | + | − | − | + + + + |
| | Y992-4 | + | − | − | + + + + |
| | Y992-5* | + | − | − | + + + + |
| | Y992-2 | + | − | − | + + + + |
| | Y992-3 | + | − | − | + + + + |
| *L. fermentati* | Y515* | + | + | + | + + + |
| *S. cerevisiae* | VIN13* | + | + | − | + |

+: Enzyme activity observed; −: No enzyme activity observed; $H_2S$ production: Low production (+) to high production (+ + + +) 4-MUG: 4-methylumbelliferyl-β-D-glucoside; 4-MUX: 4-methylumbelliferyl-β-D-xyloside. *: strains chosen for further analysis. Note: No polygalacturonase and pectinase activity observed (not shown).

means (for GC-FID, GCMS, HPLC and enzymatic quantitative data), with a significance level of 5%. Principal component analysis was utilised to discriminate between the means of various major volatiles present in wine and represented in correlation biplot graphs (coefficient = n/p).

## 3. Results

### 3.1. Screening for extracellular hydrolytic enzyme activity and $H_2S$ production

Strains of *L. thermotolerans*, *L. lanzarotensis*, *L. fermentati* as well as a commercial strain of *S. cerevisiae* (used as the control for fermentations) were screened for various extracellular enzyme activities including β-glucosidase, β-xylosidase, pectinase and protease activity. Overall, none of the strains exhibited pectinase or protease activity (not shown), while within the *Lachancea* genus, β-xylosidase activity varied between species and strains. For instance, only *L. fermentati* and *L. thermotolerans* exhibited β-xylosidase activity, however, two strains of *L. thermotolerans* did not display activity (Table 2). In contrast, β-glucosidase on arbutin was detected in all species and strains, but *L. lanzarotensis* did not display activity on 4-MUG. $H_2S$ production was detected for all yeast strains to varying levels, with *L. lanzarotensis* isolates producing notably higher levels than the other species tested (Table 2).

### 3.2. Ethanol and $SO_2$ tolerance

To evaluate how the yeast species respond to inhibitory compounds common in wine fermentation conditions, tolerance to ethanol and $SO_2$ was evaluated. The relative ethanol tolerance levels of the yeast strains were analysed and the results are displayed in Table 3. All but one of the *L. thermotolerans* strains, Y1240, displayed growth on agar plates with 10% (v/v) ethanol. *L. fermentati* Y515 and the *L. lanzarotensis* isolates tolerated up to 7% ethanol levels, with *L. lanzarotensis* displaying increased sensitivity at this concentration. The yeast strains' ability to tolerate (*i.e.* grow in the presence of) varying levels of $SO_2$ was analysed. Most of the *L. thermotolerans* strains could only tolerate 4.22 mg/L total $SO_2$ with the exception of Y940, which tolerated exposure to 8.32 mg/L total $SO_2$ (data not shown). The same level of tolerance was observed for the various *L. lanzarotensis* isolates, while *L. fermentati* exhibited growth at higher $SO_2$ concentrations. Consequently, its growth dynamics were monitored for a longer period. The data show that, *L. fermentati* Y515 was able to proliferate when exposed to 10, 15, and 20 mg/L total $SO_2$ although a longer lag phase was observed (Supplementary Fig. 1A). In contrast, *S. cerevisiae*, exhibited considerable growth at all levels of $SO_2$ and its lag phase was only protracted at 25 mg/L $SO_2$ (Supplementary Fig. 1B).

### 3.3. Synthetic grape juice fermentations

Selected strains, *viz L. thermotolerans* Y940 (which exhibited high ethanol and $SO_2$ tolerance and low $H_2S$ production), *L. lanzarotensis* CBS 12615 (the type strain, which also showed low $H_2S$ production) *L. lanzarotensis* Y992-5 (to represent local South African strain, since all isolates were similar) and *L. fermentati* (only one strain was available) and *L. thermotolerans* Concerto (a well-characterized commercial strain) were further analysed regarding their fermentation abilities. In the current study, an ability to grow at 10% (v/v) ethanol was considered high tolerance, $H_2S$ colour intensity rating of (+) was considered as low production while growth at $SO_2$ levels ≥8.32 mg/L was used as a measure for tolerance amongst the *Lachancea* species.

In monoculture fermentations, *Lachancea* spp. strains displayed considerably lower fermentation rates than *S. cerevisiae* and became sluggish towards the middle of fermentation (Supplementary Fig. 2A). All the *Lachancea* spp. strains had residual sugars above 40 g/L (Table 4), with *L. thermotolerans* showing the lowest (≈47 g/L) and *L. lanzarotensis* CBS 12615 the highest (≈ 111 g/L) concentrations. The fermentation kinetics showed that *L. thermotolerans* Concerto released

**Table 3**
Respective ethanol tolerance levels for various yeast strains. The strains chosen for further analysis are indicated with an asterisk.

| | | Ethanol tolerance | | |
|---|---|---|---|---|
| Yeast species | Strain/Isolate | 5% | 7% | 10% |
| *L. thermotolerans* | Y1202 | ++++ | +++ | − |
| | Y1109 | ++++ | +++ | − |
| | Y1017 | +++ | +++ | − |
| | Y1240 | ++++ | ++ | − |
| | Y1326 | ++++ | ++++ | − |
| | Y1206 | ++++ | ++++ | − |
| | Y1197 | ++++ | ++++ | − |
| | Y1038 | ++++ | ++++ | − |
| | Y1295 | ++++ | ++++ | + |
| | Y513 | ++++ | ++++ | + |
| | Y905 | ++++ | ++++ | + |
| | Y940* | ++++ | ++++ | + |
| | Y1220 | ++++ | +++ | − |
| | Concerto* | ++++ | +++ | − |
| *L. lanzarotensis* | CBS 12615* | ++ | − | − |
| | Y992-1 | ++ | − | − |
| | Y992-6 | ++ | − | − |
| | Y992-4 | ++ | − | − |
| | Y992-5* | ++ | − | − |
| | Y992-2 | ++ | − | − |
| | Y992-3 | ++ | − | − |
| *L. fermentati* | Y515* | ++++ | +++ | − |
| *S. cerevisiae* | VIN13* | ++++ | +++ | ++ |

Ethanol tolerance: Growth at $10^6$ to $10^2$ cfu/mL; + to +++++, and no growth (−). *: strains chosen for further analysis.

27% less $CO_2$ than *S. cerevisiae*, while *L. lanzarotensis* CBS 12615 and Y992 released 46% and 55% less $CO_2$ than *S. cerevisiae*, respectively. In contrast, *L. thermotolerans* Y940 and *L. fermentati* Y515 displayed intermediary and very similar, fermentation rates, releasing 36% and 39% less $CO_2$ released than *S. cerevisiae*, respectively. Growth analysis revealed that *S. cerevisiae* grew faster and achieved the highest cell concentrations, while *L. thermotolerans* Concerto displayed rapid growth in the first 2 days but ultimately maintained similar cell concentrations as the other strains that only grew up to an OD of 6 (Supplementary Fig. 2B). Unfortunately, due to heavy flocculation by *L. fermentati* Y515, the growth of this yeast could not be monitored spectrophotometrically.

The sequential fermentations (Fig. 1A) exhibited slower fermentation rates than the *S. cerevisiae* monoculture fermentation. However, they all fermented to dryness and generated the same levels of ethanol as *S. cerevisiae* (Table 4). The growth of *S. cerevisiae* was similar in mono- and sequential fermentations, while amongst the *Lachancea* spp. strains, different growth trends were apparent. For instance, *L. thermotolerans* Concerto was able to survive with *S. cerevisiae* until the later stages of the fermentation, while the rest of the *Lachancea* spp. strains declined at different rates following the inoculation of *S. cerevisiae* and were no longer detectable by mid-fermentation (Fig. 1B).

### 3.4. Muscat grape juice fermentations

Overall, the fermentations of Muscat grape juice progressed at a slower rate than those of synthetic grape juice and took longer to complete. The monoculture fermentations revealed a considerable shift in the fermentation profiles of the *Lachancea* spp. strains. For instance, *L. fermentati* reached the same level of cumulative weight loss as *S. cerevisiae*, while *L. lanzarotensis* CBS 12615 and Y992, as well as *L. thermotolerans* Y940, displayed slight differences at the beginning of fermentation but overlapped towards the end of fermentation (Supplementary Fig. 2C). Conversely, the sequential fermentations displayed similar profiles for each of the inoculation modalities as observed in synthetic juice fermentation, despite the protracted fermentation time (Fig. 1C). Chemical analysis showed that *L. fermentati* fermented to dryness in monoculture while the other *Lachancea* spp. strains had significantly high residual sugars. All the sequential fermentations were dry and generated similar levels of ethanol (Table 4). Growth analysis revealed that *L. thermotolerans* Concerto remained viable until the end of fermentation in both monoculture (Supplementary Fig. 2D) and sequential fermentations (Fig. 1D). In contrast, *L. thermotolerans* Y940 and *L. lanzarotensis* CBS 12615 and Y992, showed a steady decline in monocultures (Supplementary Fig. 2C), while in mixed-cultures they declined rapidly after the addition of *S. cerevisiae* and could not be detected by day 8 of the fermentation (Fig. 1D).

### 3.5. β-Glucosidase activity during fermentations

The β-glucosidase activity in the different strains was determined at the beginning, middle and end stages of monoculture and sequential fermentations. The β-glucosidase activity in synthetic grape juice fermentation was determined in the extracellular, cell wall and

**Table 4**
Residual sugar and ethanol concentrations in the synthetic wine and Muscat wine at the end of fermentation with mono- and mixed cultures of *Lachancea* spp. strains and *S. cerevisiae*.

Synthetic grape juice fermentations

| | *S. cerevisiae* | *L. thermotolerans* | | | | *L. lanzarotensis* | | | | *L. fermentati* | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Compound | VIN13 | Y940 | Y940-Sc | Concerto | Concerto-Sc | CBS | CBS-Sc | Y992 | Y992-Sc | Y515 | Y515-Sc |
| Glucose (g/L) | 1.1 ± 0.1 | 44.9 ± 0.7 | 0 | 9.8 ± 1.6 | 0 | 44.4 ± 1.0 | 0 | 42.2 ± 0.8 | 0 | 22.7 ± 1.0 | 0 |
| Fructose (g/L) | 2.4 ± 0.4 | 56.7 ± 0.8 | 0 | 37.2 ± 2.5 | 0 | 66.8 ± 0.7 | 0 | 63.3 ± 0.8 | 0 | 49.8 ± 1.5 | 0.3 ± 0.1 |
| Total sugar (g/L) | 3.5 | 45.6 | 0 | 47.0 | 0 | 111.2 | 0 | 105.5 | 0 | 72.5 | 0.3 |
| Ethanol (% v/v) | 12.3 ± 0.3 | 8.9 ± 0.8 | 11.3 ± 0.8 | 10.6 ± 1.4 | 11.9 ± 0.9 | 7.6 ± 1.8 | 11.6 ± 0.3 | 8.1 ± 0.7 | 11.5 ± 1.1 | 9.8 ± 2.0 | 11.5 ± 0.7 |

Muscat grape juice fermentations

| | *S. cerevisiae* | *L. thermotolerans* | | | | *L. lanzarotensis* | | | | *L. fermentati* | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Compound | VIN13 | Y940 | Y940-Sc | Concerto | Concerto-Sc | CBS | CBS-Sc | Y992 | Y992-Sc | Y515 | Y515-Sc |
| Glucose (g/L) | 0 | 11.6 ± 1.6 | 0 | 6.7 ± 0.6 | 0 | 22.0 ± 1.2 | 0 | 22.0 ± 1.9 | 0.6 ± 0.7 | 0.3 ± 0.4 | 0 |
| Fructose (g/L) | 0 | 27.5 ± 4.1 | 0 | 21.5 ± 1.0 | 1.9 ± 1.2 | 49.9 ± 3.5 | 0.6 ± 0.1 | 48.1 ± 2.2 | 1.0 ± 1.0 | 3.6 ± 1.0 | 0 |
| Total sugar (g/L) | 0 | 39.1 | 0 | 28.2 | 1.9 | 71.9 | 0.6 | 70.1 | 1.6 | 3.9 | 0 |
| Ethanol (% v/v) | 11.5 ± 0.8 | 10.1 ± 0.6 | 11.3 ± 0.4 | 10.1 ± 1.7 | 11.5 ± 0.1 | 8.2 ± 1.6 | 11.1 ± 0.6 | 8.4 ± 1.7 | 11.9 ± 0.6 | 10.5 ± 1.4 | 11.7 ± 0.6 |

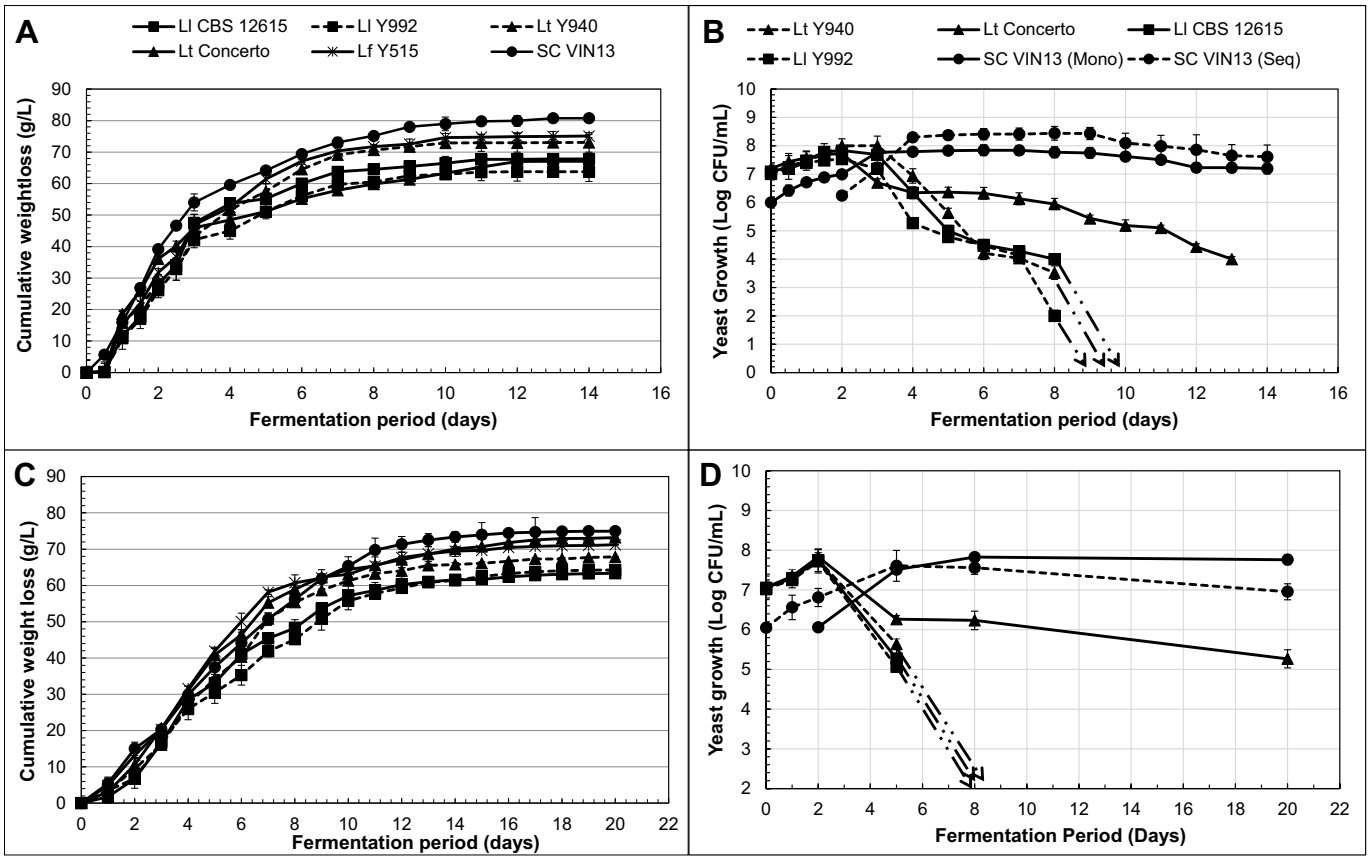


**Fig. 1.** Fermentation and growth kinetics during sequential fermentations of synthetic grape juice (A,B) and Muscat (C,D) with *S. cerevisiae* VIN13 (●) monoculture (solid line), mixed-culture VIN13 (Seq) (broken line) and *L. lanzarotensis* (■) CBS 12615 (solid line), Y992 (broken line); *L. thermotolerans* (▲) Y940 (broken line), Concerto™ (solid line); *L. fermentati* Y515 (✳) and *S. cerevisiae* VIN13 (monoculture). VIN13 (Seq) represents average viable counts for *S. cerevisiae* in mixed-culture fermentations. Viable counts for *L. fermentati* Y515 could not be obtained due to strong flocculation which resulted in erroneous data.

intracellular fractions of the yeast cells (Fig. 2A & B). No activity was detected in the extracellular matrix. The data show that, in all the strains studied, the highest β-glucosidase activity was detected in the cell wall rather than intracellularly. Maximum activity during the synthetic grape juice fermentation was detected in monocultures by the second day of fermentation (Fig. 2A), while in the mixed cultures the highest activity was detected on day 6 (Fig. 2B). *Lachancea thermotolerans* Y940 displayed the highest activity in monocultures, reaching a maximum specific activity of 0.68 nkat/mg protein intracellularly and 1.4 nkat/mg protein in the cell wall (Fig. 2A). β-Glucosidase activity measurements in Muscat grape juice fermentation revealed similar trends as observed in synthetic grape juice fermentation (Fig. 2C). However, the maximum activity levels attained were generally higher than in synthetic grape juice fermentation, particularly in the mixed culture fermentations. For instance, the total (intracellular + cell wall-associated) maximum activity of the *L. thermotolerans* Y940 mixed culture fermentation in synthetic grape juice was ≈3.1 nkat/mg protein (Fig. 2B), while in the Muscat fermentation it was 4.6 nkat/mg protein (Fig. 2C). Furthermore, β-glucosidase activity was detectable until the end of fermentation, albeit at lower levels.

### 3.6. Chemical profiles of resulting wines

As majority of the monoculture fermentations resulted in high residual sugar levels, chemical analyses would have required high dilutions and, therefore, potential inaccuracy and loss of compounds. For this reason, chemical analyses could only be performed on the samples that had fermented to completion. Primary metabolites as well as major volatiles were measured.

#### 3.6.1. Organic acids and glycerol

In the wines produced from sequential fermentation of the synthetic medium and the *S. cerevisiae* fermentation, the range of citric acid was 0.2–0.4 g/L, succinic acid 1.0–1.5 g/L and glycerol 8.1–10.0 g/L (Table 5). The *L. fermentati* Y515 sequential fermentation produced the highest levels of acetic acid (1.06 ± 0.06 g/L), followed by the *S. cerevisiae* (0.63 ± 0.02 g/L), the *L. lanzarotensis* strains (0.42–0.51 g/L) and the lowest production by the *L. thermotolerans* strains (0.24 ± 0.02 g/L).

*L. thermotolerans*-Sc and *L. fermentati*-Sc mixed-culture as well as the *S. cerevisiae* monoculture Muscat wines displayed similar citric acid (0.25–0.49 g/L) and glycerol (8.9–10.1 g/L) levels. Furthermore, their succinic acid (0.56–0.7 g/L) levels were lower than those of their corresponding synthetic wines. Conversely, an increase in citric acid (0.78–0.83 g/L), succinic acid (0.83–0.87 g/L) and glycerol (11.1–11.7 g/L) was observed in the *L. lanzarotensis*-Sc mixed-culture Muscat wines compared to the synthetic wines (Table 5). The *L. fermentati* Y515 monoculture, which was able to complete fermentation in the Muscat grape juice, resulted in lower concentrations of citric acid (0.19 ± 0.04 g/L) compared to the *S. cerevisiae* monoculture. Overall, the *L. fermentati* Y515 and *L. fermentati*-Sc Muscat wines produced the highest acetic acid levels (0.93 ± 0.09 and 0.91 ± 0.04 g/L), respectively, followed by the sequential fermentations of the *L. lanzarotensis* strains (0.75–0.86 g/L), *L. thermotolerans* Y940-Sc (0.7 ± 0.02 g/L), *S.*

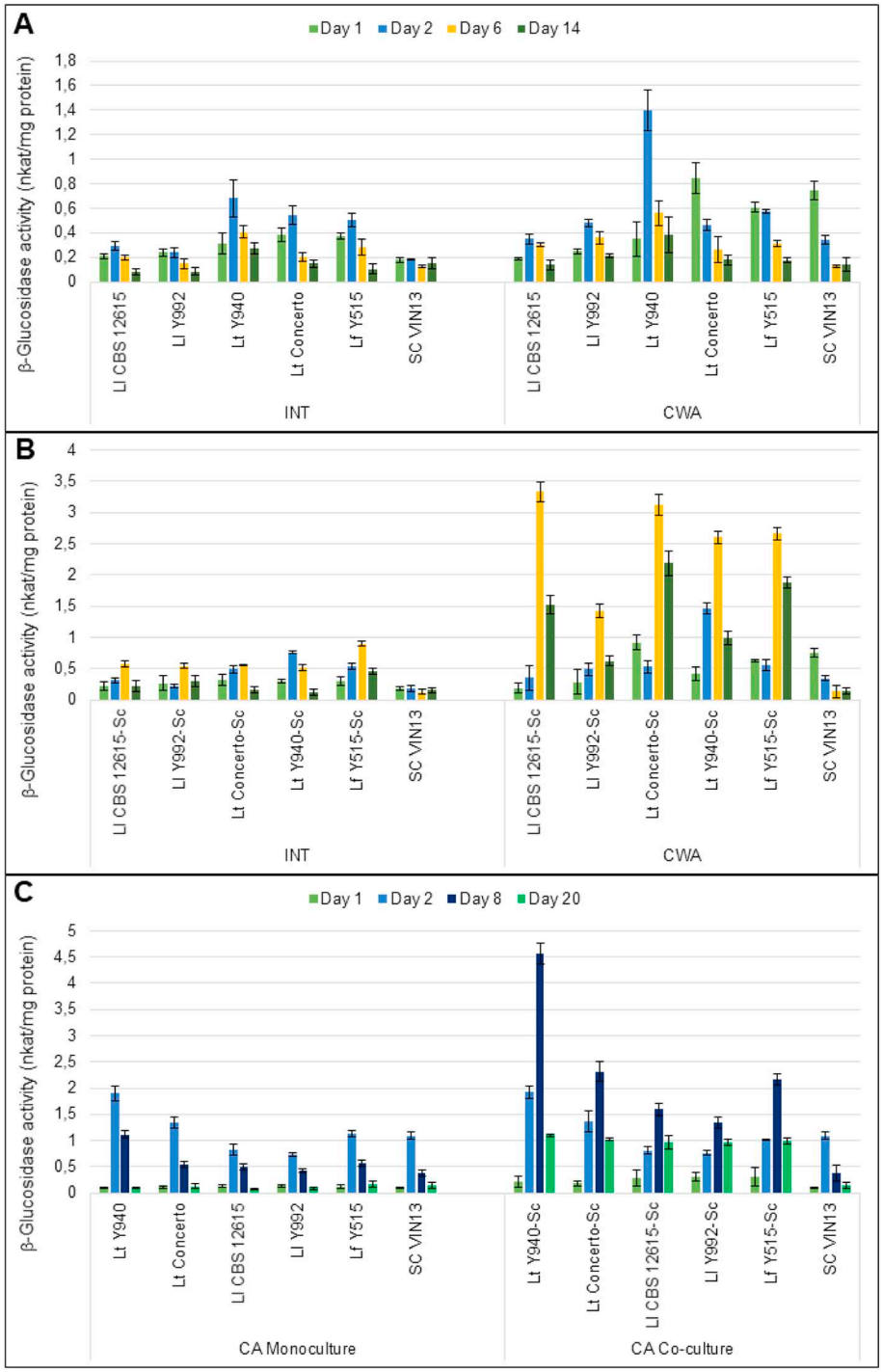


**Fig. 2.** A & B show β-Glucosidase activities measured in the intracellular matrix (INT) and in the cell wall (CWA) of monocultures of *Lachancea thermotolerans* (Lt Concerto & Lt Y940), *Lachancea lanzarotensis* (Ll CBS 12615 & Ll Y992), *Lachancea fermentati* (Lf Y515) and *Saccharomyces cerevisiae* (SC VIN13), as well as mixed-cultures (Lt Concerto-Sc, Lt Y940-Sc, Ll CBS-Sc, Ll Y992-Sc, Lf Y515—Sc) during fermentation of synthetic grape juice; **C** shows total cell associated (CA) β-glucosidase activity measured during fermentation of Muscat grape juice.

*cerevisiae* (0.65 ± 0.02 g/L) and lastly *L. thermotolerans* Concerto-Sc (producing only 0.41 ± 0.01 g/L).

### 3.6.2. Higher alcohols

Amongst the sequential fermentations in synthetic grape juice, the *L. fermentati*-Sc fermentation generated the highest levels of isobutanol and 1-propanol, while the *L. thermotolerans* Concerto-Sc and *L. lanzorotensis* CBS-Sc generated higher levels of phenyl ethanol and isoamyl alcohol (Table 6). However, in the Muscat wines, the *L. fermentati* mono- and sequential fermentations produced significantly higher levels of isobutanol and isoamyl alcohol; which contributed to total higher alcohol concentrations of 532 and 490 mg/L, respectively. *L. thermotolerans* Concerto remained the highest producer of 2-phenylethanol. The *L. lanzarotensis* CBS-Sc and Y992-Sc mixed-cultures produced Muscat wines with total higher alcohol levels similar to the *S. cerevisiae* monocultures. Significant differences between the contributions of the strains of *L. thermotolerans* and *L. lanzarotensis* were evident with regard to the production of higher alcohols in synthetic wines.

**Table 5**
Metabolites, above quantification/detection limits, present in wines following the completion of monoculture and mixed-culture synthetic grape juice-like and Muscat grape must fermentations by *S. cerevisiae* and *Lachancea* spp.

Synthetic grape juice-like fermentations

| | Sc VIN13 | *L. thermotolerans* | | *L. lanzarotensis* | | *L. fermentati* |
|---|---|---|---|---|---|---|
| Compound (g/L) | | Y940-Sc | Concerto-Sc | CBS-Sc | Y992-Sc | Y515-Sc |
| Citric acid | 0.4 ± 0.02$^{a}$ | 0.3 ± 0.01$^{b}$ | 0.4 ± 0.02$^{a}$ | 0.2 ± 0.03$^{b}$ | 0.3 ± 0.03$^{b}$ | 0.2 ± 0.01$^{b}$ |
| Malic acid | 0.9 ± 0.1$^{a}$ | 0.8 ± 0.1$^{a}$ | 1.02 ± 0.1$^{a}$ | 0.8 ± 0.1$^{a}$ | 0.8 ± 0.1$^{a}$ | 0.9 ± 0.04$^{a}$ |
| Succinic acid | 1.4 ± 0.2$^{a}$ | 1.3 ± 0.2$^{ab}$ | 1.5 ± 0.2$^{a}$ | 1.2 ± 0.2$^{ab}$ | 1.3 ± 0.1$^{ab}$ | 1.0 ± 0.1$^{b}$ |
| Acetic acid | 0.6 ± 0.01$^{b}$ | 0.2 ± 0.02$^{e}$ | 0.2 ± 0.02$^{e}$ | 0.5 ± 0.02$^{c}$ | 0.4 ± 0.01$^{d}$ | 1.1 ± 0.1$^{a}$ |
| Glycerol | 8.6 ± 1.3$^{ab}$ | 8.1 ± 0.3$^{b}$ | 9.4 ± 1.1$^{ab}$ | 9.9 ± 1.1$^{ab}$ | 8.9 ± 1.2$^{ab}$ | 9.4 ± 0.6$^{a}$ |

Muscat grape juice fermentations

| | Sc VIN13 | *L. thermotolerans* | | *L. lanzarotensis* | | *L. fermentati* | |
|---|---|---|---|---|---|---|---|
| Compound (g/L) | | Y940-Sc | Concerto-Sc | CBS-Sc | Y992-Sc | Y515-Sc | Y515 |
| Citric acid | 0.4 ± 0.02$^{b}$ | 0.4 ± 0.04$^{b}$ | 0.5 ± 0.03$^{b}$ | 0.8 ± 0.1$^{a}$ | 0.8 ± 0.1$^{a}$ | 0.3 ± 0.1$^{c}$ | 0.2 ± 0.04$^{c}$ |
| Malic acid | 2.1 ± 0.1$^{cd}$ | 2.2 ± 0.1$^{bc}$ | 2.3 ± 0.2$^{abc}$ | 2.5 ± 0.2$^{ab}$ | 2.5 ± 0.1$^{a}$ | 1.9 ± 0.1$^{d}$ | 1.7 ± 0.1$^{b}$ |
| Succinic acid | 0.6 ± 0.1$^{cd}$ | 0.7 ± 0.1$^{abc}$ | 0.6 ± 0.1$^{bcd}$ | 0.8 ± 0.1$^{a}$ | 0.9 ± 0.1$^{a}$ | 0.7 ± 0.1$^{abc}$ | 0.4 ± 0.1$^{d}$ |
| Acetic acid | 0.7 ± 0.02$^{c}$ | 0.7 ± 0.01$^{b}$ | 0.4 ± 0.01$^{d}$ | 0.9 ± 0.02$^{ab}$ | 0.8 ± 0.03$^{b}$ | 0.9 ± 0.04$^{a}$ | 0.9 ± 0.1$^{a}$ |
| Glycerol | 8.9 ± 0.5$^{c}$ | 9.3 ± 1.4$^{bc}$ | 9.5 ± 1.6$^{bc}$ | 11.1 ± 0.7$^{ab}$ | 11.7 ± 0.6$^{a}$ | 10.1 ± 0.9$^{abc}$ | 8.2 ± 0.8$^{c}$ |

Superscript letters indicate statistical significance based on Tukey's test ($p < 0.05$).

However, these differences diminished considerably in the Muscat wines.

### 3.6.3. Esters

Similar levels of acetate and ethyl esters were detected in the synthetic wine for all species and strains (Table 6), with all compounds generally over their respective odour thresholds. However, an increase of ethyl acetate in *L. thermotolerans* Y940-Sc (72 ± 3.7 mg/L) and *L. fermentati* Y515-Sc (128 ± 1.2 mg/L) sequential fermentations led to an increase in overall ester content in these wines, with the highest determined in *L. fermentati*. Conversely, in the Muscat wines the *L. lanzarotensis* CBS-Sc and Y992-Sc mixed-cultures generated the highest total esters - mainly due to higher ethyl acetate levels. Surprisingly, no ethyl acetate could be detected in the *L. fermentati* mono- and mixed-culture Muscat wines. *L. thermotolerans* Concerto-Sc Muscat wines exhibited the highest amounts of ethyl decanoate and ethyl phenylacetate, although in the synthetic wines the concentrations of both compounds were similar across the fermentation modalities.

### 3.6.4. Volatile acids (excluding acetic acid)

Overall, the different sequential fermentations produced similar levels of total volatile acids in synthetic wines except for the *L. fermentati* Y515-Sc sequential fermentation, which generated significantly higher levels in comparison to the other fermentations, including the *S. cerevisiae* monoculture control. This high level was attributed to the higher production of isobutyric acid. Similarly, in the Muscat wines, the *L. fermentati* mono- and mixed-culture wines contained 41.07 mg/L and 15.2 mg/L isobutyric acid, respectively, and had the highest levels of total volatile acids. In contrast, volatile acids were lowest for the *L. thermotolerans* Y940-Sc, *L. lanzarotensis* CBS-Sc and *L. lanzarotensis* Y992-Sc; corresponding to 7.5 ± 1.1, 8.2 ± 0.2, 7.0 ± 0.4 mg/L, respectively.

### 3.6.5. Monoterpenes

Monoterpene levels were evaluated in the monoculture and mixed-culture Muscat wine fermentations. Only the levels detected in the wines that fermented to dryness are reported (Table 7). The data show that mixed-culture fermentations generate lower levels of monoterpenes compared to the *S. cerevisiae* monoculture. Overall, amongst the wines produced from fermentations involving *Lachancea* species, the highest levels of total monoterpenes were found in the *L. fermentati* Y515-Sc wine which generated upto 271 μg/L, followed by the *L. fermentati* Y515 monoculture (Table 7). Linalool, α-terpineol and geraniol were the most abundant monoterpenes in all the wines, however, the *L. fermentati* Y515-Sc and *L. fermentati* Y515 wines produced the highest amounts. Eucalyptol was detectable only in the *L. thermotolerans* Y940-Sc wine at similar concentrations to the *S. cerevisiae* (Sc Vin13) monoculture wine and the *L. fermentati* Y515 at lower levels (Table 7).

### 3.6.6. Principal component analysis

All the quantified compounds present in the synthetic wine and Muscat, were subjected to Principal Component Analysis. The first two principal components derived from the major volatiles in the synthetic wines accounted for 72% of the total variation, with PC1 and PC2 explaining 40.5 and 31.5% variance, respectively (Fig. 3A). The synthetic wines from *L. thermotolerans* and *L. lanzarotensis* strains in co-fermentation with *S. cerevisiae* grouped together and were separated from the *L. fermentati* co-fermented wines along PC1. The *L. fermentati* wines were closely associated with low isoamyl acetate and high ethyl acetate, 2-phenylethyl acetate, isobutyric acid and isobutanol, while the *L. thermotolerans* and *L. lanzarotensis* wines were associated with isoamyl alcohol, propionic acid, 2-phenylethanol and ethyl decanoate (Fig. 3B). PC2 allowed for further differentiation of the wines according to the yeast inocula, resulting in the *S. cerevisiae* wine separating from the mixed-culture wines due to higher production of medium chain fatty acids (octanoic acid, hexanoic acid and decanoic acid) as well as isovaleric acid. In addition, PC2 separates the two *L. thermotolerans* strains (Concerto and Y940), with the separation mainly driven by ethyl phenylacetate and butanol.

The PCA of the Muscat wines revealed similar patterns as the synthetic wines (Fig. 3 C & D). PC1 and PC2, accounted for 36.5% and 18.3% of the variance, respectively (Fig. 3C). The wines were separated along PC1 based on the *Lachancea* co-inoculant, with the *L. fermentati* inoculated wines separated from the *L. thermotolerans* and *L. lanzarotensis*. The *L. fermentati* mono- and co-culture with *S. cerevisiae* was

**Table 6**
Major volatiles, above quantification/detection limits, present in wines following the completion of monoculture and mixed-culture synthetic grape juice-like and Muscat grape must fermentations by *S. cerevisiae* and *Lachancea* spp.

| Compound (mg/L) | Synthetic grape juice-like fermentations | | | | | |
|---|---|---|---|---|---|---|
| | Sc VIN13 | *L. thermotolerans* | | *L. lanzarotensis* | | *L. fermentati* |
| | | Y940-Sc | Concerto-Sc | CBS-Sc | Y992-Sc | Y515-Sc |
| Esters | | | | | | |
| Ethyl decanoate | 1.1 ± 0.01$^{a}$ | 1.2 ± 0.05$^{a}$ | 0.9 ± 0.02$^{a}$ | 1.2 ± 0.01$^{a}$ | 1.1 ± 0.03$^{a}$ | 0.46 ± 0.02$^{b}$ |
| Ethyl phenylacetate | – | 1.7 ± 0.18$^{a}$ | 1.4 ± 0.04$^{b}$ | 1.9 ± 0.32$^{a}$ | 1.4 ± 0.18$^{b}$ | 0.7 ± 0.06$^{a}$ |
| Ethyl acetate | 26.5 ± 2.60$^{a}$ | 72.5 ± 3.70$^{b}$ | 30.6 ± 1.59$^{d}$ | 27.0 ± 2.61$^{e}$ | 31.4 ± 1.01$^{c}$ | 127.69 ± 1.22$^{a}$ |
| Isoamyl acetate | 0.5 ± 0.13$^{b}$ | 0.2 ± 0.08$^{c}$ | 0.2 ± 0.01$^{bc}$ | 0.2 ± 0.03$^{c}$ | 0.2 ± 0.01$^{c}$ | 1.0 ± 0.51$^{a}$ |
| 2-Phenylethyl acetate | 0.5 ± 0.01$^{a}$ | 0.5 ± 0.03$^{b}$ | 0.5 ± 0.01$^{b}$ | 0.5 ± 0.14$^{ab}$ | 0.6 ± 0.01$^{ab}$ | 0.7 ± 0.05$^{a}$ |
| Σ Esters | 28.5 ± 3.19$^{c}$ | 74.3 ± 3.02$^{b}$ | 32.3 ± 5.72$^{c}$ | 28.9 ± 2.10$^{c}$ | 33.2 ± 1.01$^{c}$ | 129.8 ± 6.5$^{a}$ |
| Higher alcohols | | | | | | |
| Isobutanol | 23.7 ± 1.19$^{d}$ | 24.4 ± 0.29$^{cd}$ | 33.4 ± 0.4$^{b}$ | 32.4 ± 1.98$^{b}$ | 27.8 ± 2.34$^{d}$ | 54.64 ± 0.54$^{a}$ |
| 1-Butanol | 0.9 ± 0.09$^{e}$ | 5.8 ± 1.25$^{a}$ | 2.7 ± 0.28$^{b}$ | 1.5 ± 0.03$^{c}$ | 1.2 ± 0.03$^{d}$ | 0.5 ± 0.02$^{f}$ |
| 1-Propanol | 30.4 ± 1.79$^{e}$ | 33.8 ± 0.85$^{d}$ | 37.8 ± 1.46$^{b}$ | 34.6 ± 0.46$^{c}$ | 24.5 ± 4.75$^{f}$ | 53.8 ± 1.87$^{a}$ |
| Isoamyl alcohol | 165.3 ± 2.31$^{c}$ | 135.93 ± 2.4$^{e}$ | 188.9 ± 1.13$^{a}$ | 187.6 ± 2$^{a}$ | 168.31 ± 2$^{b}$ | 159.42 ± 0.99$^{d}$ |
| Hexanol | – | 0.3 ± 0.01$^{a}$ | 0.3 ± 0.01$^{a}$ | 0.3 ± 0.01$^{a}$ | 0.3 ± 0.01$^{a}$ | 0.3 ± 0.02$^{a}$ |
| 2-Phenylethanol | 54.3 ± 1.01$^{a}$ | 41.9 ± 0.42$^{e}$ | 50.5 ± 1.14$^{b}$ | 49.3 ± 0.58$^{c}$ | 43.4 ± 1.75$^{d}$ | 22.35 ± 0.6$^{f}$ |
| Σ Higher alcohols | 274.5 ± 3.02$^{c}$ | 242.1 ± 4.23$^{d}$ | 313.7 ± 5.02$^{a}$ | 305.7 ± 8.11$^{b}$ | 265.5 ± 7.98$^{c}$ | 291.1 ± 10.11$^{b}$ |
| Volatile acids | | | | | | |
| Propionic acid | 2.6 ± 0.04$^{bc}$ | 2.4 ± 0.17$^{cd}$ | 2.19 ± 0.06$^{e}$ | 3.15 ± 0.01$^{a}$ | 2.6 ± 0.04$^{b}$ | 2.2 ± 0.09$^{de}$ |
| Isobutyric acid | 1.9 ± 0.07$^{d}$ | 2.6 ± 0.02$^{c}$ | 3.5 ± 0.05$^{b}$ | 1.9 ± 0.02$^{d}$ | 1.6 ± 0.01$^{e}$ | 9.2 ± 0.57$^{a}$ |
| Isovaleric acid | 1.8 ± 0.11$^{a}$ | 0.8 ± 0.03$^{d}$ | 1.1 ± 0.04$^{c}$ | 1.2 ± 0.08$^{bc}$ | 1.0 ± 0.13$^{cd}$ | 1.3 ± 0.06$^{b}$ |
| Hexanoic acid | 1.5 ± 0.08$^{a}$ | 0.4 ± 0.07$^{b}$ | 0.3 ± 0.06$^{b}$ | 0.3 ± 0.02$^{b}$ | – | 0.3 ± 0.18$^{b}$ |
| Octanoic acid | 2.0 ± 0.06$^{a}$ | 0.6 ± 0.05$^{c}$ | 0.7 ± 0.03$^{bc}$ | 0.7 ± 0.04$^{bc}$ | 0.7 ± 0.03$^{bc}$ | 0.8 ± 0.09$^{b}$ |
| Decanoic acid | 1.2 ± 0.05$^{ab}$ | 0.9 ± 0.04$^{d}$ | 1.4 ± 0.02$^{a}$ | 0.9 ± 0.01$^{cd}$ | 0.9 ± 0.04$^{cd}$ | 1.1 ± 0.09$^{bc}$ |
| Σ Volatile acids | 11.1 ± 1.3$^{b}$ | 7.5 ± 1.06$^{bc}$ | 9.2 ± 1.06$^{b}$ | 8.2 ± 0.16$^{bc}$ | 7.0 ± 0.43$^{c}$ | 15.0 ± 2$^{a}$ |

| Compound (mg/L) | Muscat must fermentations | | | | | | |
|---|---|---|---|---|---|---|---|
| | Sc VIN13 | *L. thermotolerans* | | *L. lanzarotensis* | | *L. fermentati* | |
| | | Y940-Sc | Concerto-Sc | CBS-Sc | Y992-Sc | Y515-Sc | Y515 |
| Esters | | | | | | | |
| Ethyl decanoate | 0.2 ± 0.02$^{d}$ | 1.7 ± 0.98$^{b}$ | 2.3 ± 0.03$^{a}$ | 0.7 ± 0.02$^{c}$ | 1.2 ± 0.81$^{b}$ | 0.2 ± 0.04$^{d}$ | 0.1 ± 0.02$^{e}$ |
| Ethyl phenylacetate | 1.1 ± 0.06$^{c}$ | 0.8 ± 0.02$^{d}$ | 3.8 ± 0.11$^{a}$ | 0.7 ± 0.02$^{e}$ | 0.7 ± 0.02$^{e}$ | 1.6 ± 0.24$^{b}$ | 1.2 ± 0.17$^{c}$ |
| Ethyl acetate | 37.7 ± 1.24$^{e}$ | 46.9 ± 0.08$^{d}$ | 50.8 ± 0.38$^{c}$ | 63.5 ± 1.06$^{a}$ | 60.8 ± 0.52$^{b}$ | – | – |
| Isoamyl acetate | 0.3 ± 0.02$^{b}$ | 0.6 ± 0.13$^{a}$ | 0.7 ± 0.08$^{a}$ | 0.6 ± 0.08$^{a}$ | 0.7 ± 0.16$^{a}$ | 0.6 ± 0.13$^{a}$ | 0.6 ± 0.08$^{a}$ |
| 2-Phenylethyl acetate | 0.2 ± 0.02$^{c}$ | 0.5 ± 0.03$^{ab}$ | 0.4 ± 0.02$^{bc}$ | 0.5 ± 0.04$^{ab}$ | 0.7 ± 0.12$^{a}$ | 0.4 ± 0.04$^{bc}$ | 0.4 ± 0.01$^{b}$ |
| Σ Esters | 39.5 ± 2.67$^{d}$ | 50.6 ± 1.9$^{c}$ | 58.0 ± 1.06$^{b}$ | 66.1 ± 2.79$^{a}$ | 64.0 ± 0.56$^{a}$ | 2.8 ± 0.05$^{e}$ | 2.3 ± 0.11$^{f}$ |
| Higher alcohols | | | | | | | |
| Isobutanol | 65.7 ± 0.68$^{d}$ | 75.0 ± 2.08$^{c}$ | 62.3 ± 0.48$^{d}$ | 57.7 ± 1.11$^{f}$ | 61.7 ± 2.76$^{e}$ | 104.9 ± 1.52$^{b}$ | 183.4 ± 8.46$^{a}$ |
| 1-Butanol | 1.7 ± 0.01$^{c}$ | 2.3 ± 0.19$^{a}$ | 2.8 ± 0.03$^{a}$ | 1.7 ± 0.09$^{c}$ | 1.9 ± 0.09$^{b}$ | 1.2 ± 0.06$^{d}$ | 0.6 ± 0.03$^{e}$ |
| 1-Propanol | 37.6 ± 3.79$^{e}$ | 48.3 ± 1.78$^{a}$ | 47.7 ± 1.65$^{b}$ | 37.1 ± 1.46$^{e}$ | 38.9 ± 2.59$^{d}$ | 44.2 ± 1.71$^{c}$ | 33.7 ± 2.31$^{f}$ |
| Isoamyl alcohol | 200.6 ± 2.02$^{f}$ | 226.2 ± 1.95$^{c}$ | 233.6 ± 6.56$^{b}$ | 203.7 ± 2.09$^{e}$ | 213.2 ± 2.06$^{d}$ | 287.9 ± 1.16$^{a}$ | 283.7 ± 1.52$^{a}$ |
| Hexanol | 0.7 ± 0.04$^{a}$ | 0.6 ± 0.01$^{a}$ | 0.6 ± 0.01$^{a}$ | 0.6 ± 0.02$^{a}$ | 0.6 ± 0.01$^{a}$ | 0.6 ± 0.05$^{a}$ | 0.5 ± 0.04$^{a}$ |
| 2-Phenylethanol | 51.1 ± 1.59$^{c}$ | 51.7 ± 1.05$^{c}$ | 62.6 ± 1.19$^{a}$ | 51.5 ± 0.92$^{c}$ | 54.4 ± 1.69$^{b}$ | 51.6 ± 1.3$^{c}$ | 29.7 ± 1.12$^{d}$ |

(*continued on next page*)

**Table 6** (*continued*)

| Compound (mg/L) | Muscat must fermentations | | | | | | |
|---|---|---|---|---|---|---|---|
| | Sc VIN13 | *L. thermotolerans* | | *L. lanzarotensis* | | *L. fermentati* | |
| | | Y940-Sc | Concerto-Sc | CBS-Sc | Y992-Sc | Y515-Sc | Y515 |
| Σ Higher alcohols | 357.4 ± 6.33$^{d}$ | 404.0 ± 12.36$^{c}$ | 409.5 ± 10.61$^{c}$ | 352.2 ± 10.66$^{d}$ | 370.7 ± 9.53$^{d}$ | 490.5 ± 7.66$^{b}$ | 531.6 ± 8.62$^{a}$ |
| Volatile acids | | | | | | | |
| Propionic acid | 3.7 ± 1.21$^{bc}$ | 5.5 ± 0.31$^{a}$ | 5.4 ± 0.09$^{a}$ | 3.7 ± 1.45$^{bc}$ | 4.4 ± 1.73$^{ab}$ | 2.8 ± 0.34$^{c}$ | 2.5 ± 0.81$^{c}$ |
| Isobutyric acid | 3.0 ± 0.23$^{f}$ | 5.5 ± 0.52$^{e}$ | 8.4 ± 0.16$^{c}$ | 4.8 ± 0.22$^{e}$ | 4.5 ± 0.83$^{e}$ | 15.2 ± 1.24$^{b}$ | 41.1 ± 0.78$^{a}$ |
| Isovaleric acid | 2.0 ± 0.22$^{d}$ | 2.4 ± 0.20$^{b}$ | 2.3 ± 0.05$^{bc}$ | 2.3 ± 0.07$^{bc}$ | 2.1 ± 0.28$^{cd}$ | 2.7 ± 0.22$^{a}$ | 2.9 ± 0.37$^{a}$ |
| Hexanoic acid | 0.7 ± 0.03$^{d}$ | 1.0 ± 0.09$^{ab}$ | 0.9$^{bc}$ | 1.1 ± 0.03$^{a}$ | 1.1 ± 0.12$^{a}$ | 0.8 ± 0.06$^{cd}$ | 0.6 ± 0.01$^{b}$ |
| Octanoic acid | 0.8 ± 0.02$^{a}$ | 0.9 ± 0.12$^{ab}$ | – | 1.0 ± 0.01$^{ab}$ | 1.0 ± 0.18$^{a}$ | 0.6 ± 0.01$^{c}$ | – |
| Decanoic acid | 1.1 ± 0.01$^{a}$ | 0.7 ± 0.03$^{b}$ | 0.7 ± 0.05$^{b}$ | 0.7 ± 0.03$^{b}$ | 0.7 ± 0.02$^{b}$ | 0.7 ± 0.01$^{b}$ | 0.7$^{c}$ |
| Σ Volatile acids | 11.3 ± 1.11$^{f}$ | 16.0 ± 0.44$^{d}$ | 18.4 ± 0.12$^{c}$ | 13.6 ± 1.22$^{e}$ | 13.8 ± 1.19$^{e}$ | 22.9 ± 1.14$^{b}$ | 48.2 ± 1$^{a}$ |

Statistically significant differences for particular compounds based on Tukey's test ($p < 0.05$) are illustrated by superscript letters.

associated with isobutanol, isobutyric acid, isoamyl alcohol and isovaleric acid, as well as monoterpenes. However, the wines from the two fermentations were distinct mainly due to significant differences in the concentrations of these compounds. For instance, the Y515 produced 41 mg/L and 183 mg/L of isobutyric acid and isoamyl alcohol, respectively, while the Y515-Sc culture generated 15 mg/L and 105 mg/L, respectively (Table 6). In contrast, the Y515-Sc did not produce any detectable eucalyptol but produced significantly higher amounts of the other monoterpenes (Table 7). Within the *L. thermorolerans* and *L. lanzarotensis* the wines were associated with ethyl phenylacetate, 1-propanol, 2-phenylethanol, octanoic acid, 1-butanol and propionic acid (Fig. 3D). PC2 allowed for separation of the *S. cerevisiae* monoculture wines from the *Lachancea* spp. mixed-culture wines, with the separation mainly driven by significantly higher levels of decanoic acid and hexanol (Fig. 3D).

Most of the volatile compounds had odour activity values (OAVs) ≥ 1 (Supplementary Table 4), amongst them, ethyl phenylacetate which imparts a honey-like aroma to wine had the highest OAV followed by propionic acid which has a pungent, rancid sweaty odour (Louw et al., 2010; Du Plessis et al., 2017). Based on the OAVs, isobutanol, isoamyl alcohol and isovaleric acid could impart strong spiritous and cheesy aromas (Styger et al., 2011; Du Plessis et al., 2017) to the *L. fermentati* wines, while propionic acid could impart unpleasant aroma in the *L. thermotolerans* Y940-Sc and *L. thermotolerans* Concerto-Sc. However, these could be masked by the strong contribution of the ethyl phenylacetate, isoamyl acetate and 2-phenylethanol. The *L. thermotolerans* Concerto-Sc wine produced the highest OAV for ethyl phenylacetate (51.8) followed by *L. fermentati* Y515-Sc (22.3) (Supplementary Table 4). Amongst the monoterpenes, only linalool and geraniol had OAVs ≥1 (Table 7). The two compounds can impart floral character to wine.

## 4. Discussion

The current study evaluated the oenological potential of wine-related *Lachancea* species, and strains. The strains were screened for glycosidase activities, ethanol and $SO_2$ tolerance, $H_2S$ production as well as wine fermentation and wine aroma contribution. All the species and strains tested were devoid of acid protease or pectinase activity, but exhibited β-glucosidase activity with seemingly different substrate specificities. In contrast, xylosidase activity was only present in *L. thermotolerans* and *L. fermentati*. Pectinase activity is generally rare amongst wine yeasts including members of the genus *Lachancea*. Indeed, strains of *L. thermotolerans* and *L. fermentati* have previously been shown to lack this activity (Belda et al., 2016; Cordero-Bueso et al., 2013). Similarly, protease activity has never been reported in these species. To our knowledge, this is the first study to report β-glucosidase activity in *L. lanzarotensis*. The production of $H_2S$ and tolerance to $SO_2$ and ethanol were both species and strain dependent. *L. thermotolerans* and *L. fermentati* seemed to be more adapted to the increasing ethanol and $SO_2$ exposure, respectively, while *L. lanzarotensis* illustrated increased sensitivity when exposed to these inhibitory compounds. These results mostly confirmed existing literature for *L. thermotolerans* but provided novel findings for *L. lanzarotensis* and *L. fermentati*. Nevertheless, while previous studies evaluating β-glucosidase activity have made use of arbutin as a substrate and have shown strain differentiation for *L. thermotolerans* and *L. fermentati* (Belda et al., 2016; Comitini et al., 2011; Cordero-Bueso et al., 2013; Mostert, 2013; Romo-Sánchez et al., 2010; Rosi et al., 1994), our study showed that all the strains investigated were able to hydrolyse arbutin. The reported strain variability could be attributable to different β-glucosidases having different affinities for substrates, which could explain why *L. lanzarotensis* exhibited β-glucosidase activity against arbutin but not 4-MUG (van Rooyen et al., 2005).

These assays are qualitative in nature or semi-quantitative at best, and are performed under conditions dissimilar to that of a fermentation.

**Table 7**
Monoterpenes concentrations in the Muscat wines at the end of fermentation.

| Compound (μg/L) | OTHs (μg/L) | Aroma descriptor [a] | Sc VIN13 | *L. thermotolerans* | | *L. lanzarotensis* | | *L. fermentati* | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Y940-Sc | Concerto-Sc | CBS-Sc | Y992-Sc | Y515-Sc | Y515 |
| Eucalyptol | 3.2 | Eucalyptus, medicinal, mint | 0.7 ± 0.02$^{a}$ (0.21) | 0.8 ± 0.1$^{a}$ (0.24) | – | – | – | – | 0.54 ± 0.04$^{b}$ (0.27) |
| Linalool | 15 | Citrus, floral, lavender | 145.7 ± 3.1$^{a}$ (9.72) | 102.4 ± 3.1$^{b}$ (6.82) | 101.4 ± 5.8$^{b}$ (6.76) | 101.8 ± 5.4$^{b}$ (6.78) | 98.3 ± 6.9$^{b}$ (6.58) | 135.3 ± 3.4$^{a}$ (9.02) | 114.4 ± 3.7$^{c}$ (7.63) |
| α-Terpineol | 250 | Floral, lilac, pine | 85.9 ± 0.7$^{a}$ (0.34) | 52.8 ± 0.8$^{b}$ (0.21) | 55.2 ± 0.3$^{b}$ (0.22) | 54.8 ± 1.4$^{b}$ (0.22) | 54.1 ± 2.8$^{b}$(0.22) | 84.3 ± 3.1$^{a}$ (0.34) | 71.4 ± 2.1$^{c}$ (0.29) |
| Nerol | 15 | Rose, fruity, floral | 10.0 ± 0.3$^{a}$ (0.66) | 7.3 ± 0.6$^{b}$ (0.48) | 7.9 ± 0.2$^{b}$ (0.52) | 7.6 ± 0.3$^{b}$ (0.51) | 7.2 ± 0.4$^{b}$ (0.48) | 6.9 ± 0.1$^{b}$ (0.46) | 5.7 ± 0.4$^{c}$ (0.38) |
| Citronellol | 100 | Citronella, rose, green | 9.1 ± 0.2$^{a}$ (0.09) | 5.2 ± 1.2$^{b}$ (0.05) | 5.6 ± 0.2$^{b}$ (0.06) | 7.2 ± 0.2$^{cd}$ (0.07) | 6.9 ± 0.5$^{c}$ (0.07) | 7.8 ± 0.03$^{d}$ (0.08) | 5.1 ± 0.3$^{b}$ (0.05) |
| Geraniol | 30 | Rose, geranium | 33.4 ± 1.6$^{b}$ (1.11) | 32.9 ± 0.8$^{b}$ (1.10) | 37.6 ± 0.2$^{a}$ (1.25) | 35.7 ± 1.3$^{ab}$ (1.19) | 35.2 ± 1.3$^{ab}$ (1.17) | 36.8 ± 0.5$^{a}$ (1.23) | 34.3 ± 2.8$^{ab}$ (1.14) |
| Σ Terpenes | | | 284.8 ± 1.8$^{a}$ | 201.3 ± 2.9$^{c}$ | 207.6 ± 5.7$^{c}$ | 207.0 ± 8.5$^{c}$ | 201 ± 11.8$^{c}$ | 271.1 ± 6.7$^{a}$ | 231.7 ± 1.9$^{b}$ |

Statistically significant differences for particular compounds are illustrated by superscript letters, according to Tukey's test ($p < 0.05$). The numbers in parenthesis are the Odour Activity Values (OAVs), calculated using Odour Thresholds (OTHs) of the monoterpenes in model wine (Guth, 1997; Lukić et al., 2017).

[a] Black et al. (2015).

Rosi et al. (1994) in fact reported that high glucose and low pH (parameters common in wine fermentations) can have a repressive effect on β-glucosidase activity in yeasts. Despite the limitations of these assays, the resulting profiles such as higher tolerance to ethanol and $SO_2$ and positive β-glucosidase activity aided in selecting potential, oenologically beneficial, strains from each *Lachancea* species.

Fermentation rate varied between strains and species but none of the *Lachancea* strains could ferment to dryness. The faster fermentation rates corresponded to investigations previously performed for *L. thermotolerans* and *L. fermentati* (Ciani et al., 2006; Cordero-Bueso et al., 2013; Mora et al., 1990). In the synthetic medium, *L. fermentati* did not perform as well as *L. thermotolerans*, which contradicts the report by Cordero-Bueso et al. (2013), which saw *L. fermentati* demonstrate a faster fermentation rate; these fermentations were however carried out in real grape must (Malvar white variety). Indeed, when validating the *Lachancea* spp. fermentations in real (Muscat of Alexandria) grape must, *L. fermentati* demonstrated a higher fermentation rate, comparable to that of *S. cerevisiae*, suggesting that beyond the difference in strain between this study and that of Cordero-Bueso et al. (2013), the behaviour of this species is dependent on the composition of the grape must. The *L. thermotolerans* and *L. lanzarotensis* strains therefore required the inoculation of *S. cerevisiae* to complete the fermentation. However, the fermentation rate of the sequential fermentations was slower than that of *S. cerevisiae* monoculture. These findings correspond to previous reports for *L. thermotolerans* in mixed-culture fermentations (Balikci et al., 2016; Gobbi et al., 2013; Kapsopoulou et al., 2007).

Furthermore, during the synthetic and Muscat juice fermentations, the production of β-glucosidases as well as the cellular localisation of these enzymes was monitored. The enzyme activity accumulated at the beginning stages of the fermentations, linked to the exponential phase of growth or high metabolic activity of the yeasts after which a general decrease was observed; a pattern also reported by Fia et al. (2005) for other non-*Saccharomyces* yeasts. This subsequent decrease in activity could potentially be due to the inhibition of enzyme expression or activity by inhibitory compounds, such as the increasing ethanol levels (Mateo and Di Stefano, 1997; Maturano et al., 2012). Most of the enzyme activity was located in the cell wall while none was detected extracellularly, which corresponded to previous studies on other species (Cordero Otero et al., 2003; Fia et al., 2005; Manzanares et al., 2000; Mateo and Di Stefano, 1997; Rosi et al., 1994). The strong association to the cell wall suggests the enzyme to play a role in cell wall structure and maintenance. Indeed, glucosidases have been linked to the biosynthesis of cell wall β-1,6-glucan in *S. cerevisiae* (Abeijon and Chen, 1998). Intracellular enzyme activity was also present, albeit at lower levels. Nevertheless, intracellular enzymes have previously been shown to be able to hydrolyse natural glucosides (such as nerol, α-terpineol and geraniol) and therefore, following yeast cell lysis, their contribution cannot be disregarded (Rosi et al., 1994).

During fermentation in the synthetic medium, glycosidically-bound complexes, that are natural substrates for β-glucosidase activity were absent, however, activity was present in all *Lachancea* spp., suggesting that this enzyme is expressed constitutively in these yeasts. Indeed, similar enzyme levels were determined throughout the synthetic and Muscat grape must fermentations, wherein the glycosidically-bound complexes were present, confirming the constitutive expression observed. Previous studies have also observed constitutive expression of β-glucosidase activity by yeasts such as *S. cerevisiae*; however, the investigators have also proposed that the observed activity could possibly be due to *exo*-glucanases, which are able to hydrolyse the pNP substrate, and therefore result in false constitutive activity (Mateo and Di Stefano, 1997; Rodríguez et al., 2004). In both grape matrices, *L. thermotolerans* Y940 consistently showed high enzyme activity in comparison to the other *Lachancea* spp. and *S. cerevisiae*. *L. thermotolerans* Concerto and *L. fermentati* behaved similarly, followed by the *L. lanzarotensis* strains. Sequential fermentations all resulted in increased enzyme activity compared to the *S. cerevisiae* fermentation, suggesting a

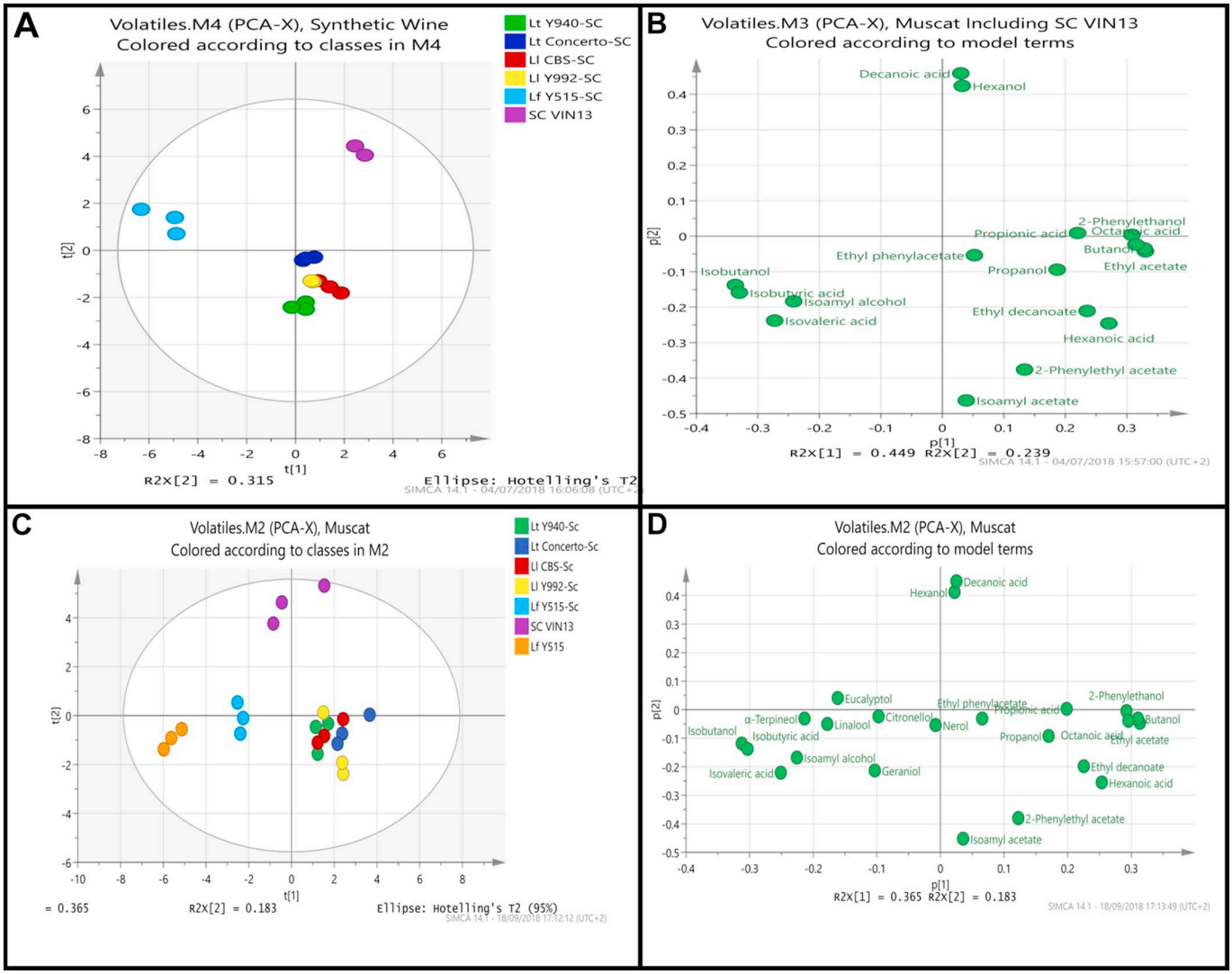


**Fig. 3.** Principal Component Analysis (PCA) scores plot (A and C) and loadings plot (B and D) based on all quantifiable major volatile compounds present in the synthetic wine (A and B) and Muscat wine (C and D) produced by *S. cerevisiae* monoculture (SC VIN13 Mono) and sequential mixed-cultures of *L. thermotolerans* Y940 and Concerto (Lt Y940-Sc and Lt Concerto-Sc), *L. lanzarotensis* CBS 12615 and Y992 (Ll CBS-Sc and Ll Y992-Sc) and *L. fermentati* Y515 (Lf Y515-Sc) together with *S. cerevisiae*.

compound/cumulative effect of both the *Lachancea* spp. and *S. cerevisiae*, which highlights the advantage of inoculating the *Lachancea* spp. Nevertheless, similar total monoterpene levels were present in the fermentations by *L. thermotolerans* Y940, *L. thermotolerans* Concerto and *S. cerevisiae*. Therefore, even though *L. thermotolerans* Y940 exhibited significantly higher β-glucosidase activity, this did not translate to significantly higher monoterpene levels. Most of the naturally present monoterpenol complexes exist as diglycosides as opposed to monoglucosides, and therefore require the action of additional enzymes, namely α-L-arabinofuranosidase, α-L-rhamnopyranosidase or β-D-apiofuranosidase prior to the action of β-glucosidase (Günata et al., 1988; Rosi et al., 1995). The absence of such enzyme activity would therefore hinder the ability for the β-glucosidase enzymes to release monoterpenols from a large portion of the available glycoside complexes. Indeed, Belda et al. (2016) reported the majority of *L. thermotolerans* strains, of which 88 strains were screened, and all the *S. cerevisiae* (11 strains), to lack α-L-arabinofuranosidase activity. This lack in activity could account for the reduced monoterpene accumulation, which has been reported before (Rosi et al., 1995). When analysing these results, it is also important to recognise the limitations of making use of an artificial substrate (nitrophenyl-glycosides), due to β-glucosidase activity previously being shown to have less activity against the natural glucoside substrates (Margolles-Clark et al., 1996). Following the sequential fermentations, lower levels and less variation were surprisingly observed for the monoterpene content, corresponding to literature (Cordero Otero et al., 2003; Garcia et al., 2002), despite the higher enzymatic activity measured during these fermentations. This could be due to the increased fermentation vigour of the sequential fermentations, leading to increased evaporation of the volatile monoterpenes due to enhanced $CO_2$ production, which was previously hypothesised by Günata et al. (1986). Additionally, during the sequential fermentations, the *Lachancea* spp. declined below detection much earlier than in the monoculture fermentations and upon lysis of the cells, the released enzymes could have resulted in the hydrolysis of the monoterpenes earlier in the fermentation process, subsequently leading to the biotransformation of the monoterpenes, into less fragrant compounds (such as monoterpene oxides and diols), not measured in this study (Günata et al., 1986; Vaudano et al., 2004). While this could also corroborate the decrease in total monoterpene content in the sequential fermentations, further investigation is required.

In addition to the monoterpene production, the chemical profiles of the completed fermentations demonstrated interesting results.

Fermentations that ran to dryness included the sequential fermentations, *S. cerevisiae* monoculture fermentations, and *L. fermentati* monoculture fermentation in Muscat grape must. Sequential fermentations, while fermenting at a slower rate than *S. cerevisiae*, resulted in minimal residual sugar and comparable ethanol levels to *S. cerevisiae*. The resulting acetic acid:ethanol ratio in the sequential fermentations were however lower than *S. cerevisiae*, with the exception of *L. fermentati*. While the *L. thermotolerans* and *L. lanzarotensis* sequential fermentation led to a reduction in the acetic acid levels, the levels produced during monoculture and sequential fermentations with *L. fermentati* were above what is considered acceptable in wine (0.7 g/L). This overproduction of acetic acid may disqualify the *L. fermentati* strain tested in this study as a potential starter culture. Regarding the volatile compounds formed in the fermentations, in both matrices increased acetic acid, isobutyric acid and isobutanol production led to the differentiation of *L. fermentati* from the other sequential fermentations. Interestingly, isobutanol was much higher in the Muscat grape must in comparison to that measured in the synthetic juice, which corresponds to a previous report on this species (Romano and Suzzi, 1993). The differentiation in the volatile compound profiles for the different yeasts could allude to potential differences in their respective metabolism and amino acid uptake. For instance, the increase in isobutanol and isobutyric acid could be due to better or preferential uptake of valine by *L. fermentati*, leading to the production of these compounds. Alternatively, it has recently been shown in *S. cerevisiae* that an increased flux from pyruvate can also account for the increase in isobutanol (Rollero et al., 2017). Sequential fermentations with *L. thermotolerans* and *L. lanzarotensis* strains also led to wines distinguishable from *S. cerevisiae*, due to increased production of various esters and higher alcohols, including butanol, 2-phenylethanol and phenylethyl acetate, features previously reported for *L. thermotolerans* (Beckner Whitener et al., 2015; Benito et al., 2016; Comitini et al., 2011; Gobbi et al., 2013). The increased production of 2-phenylethanol and phenylethyl acetate suggests to the increased uptake of phenylalanine by these species. An increase in 2-phenylethanol can also result from sugars *via* the pentose phosphate pathway (Rollero et al., 2017). Based on the OAVs of the volatile compounds, we could predict that co-inoculation with the *L. thermotolerans* and *L. lanzarotensis* strains would generate wines with high floral and fruity aromas and low solvent character while those from *L. fermentati* might present more spiritous wines with low floral notes. However, wine aroma is more complex and is dependent on the interactions of different compounds. Therefore, such a prediction would merely be speculative. Sensory analysis of the resulting wines utilising these *Lachancea* species will further elucidate whether the differences in the chemical composition of the wines are perceivable or not.

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