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Influence of sequential fermentation with *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* on wine quality

I. Loira , R. Vejarano , M.A. Bañuelos , A. Morata , W. Tesfaye , C. Uthurry , A. Villa , I. Cintora , J.A. Suárez-Lepe

A B S T R A C T

Torulaspora delbrueckii is a non-*Saccharomyces* yeast with interesting metabolic and physiological properties of potential use in oenology. This work examines the fermentative behaviour of five strains of *T. delbrueckii* in sequential fermentations with *Saccharomyces cerevisiae*, analysing the formation of aromatic compounds, polyalcohols and pigments. The fermentative power of these five strains ranged between 7.6 and 9.0% v/v ethanol; the associated volatile acidity was 0.2–0.7 g/l acetic acid. The production of glycerol was inferior to that of *S. cerevisiae* alone. The mean 2,3-butanediol concentration reached in single-culture *S. cerevisiae* fermentations was 73% higher than in the five sequential *T. delbrueckii/S. cerevisiae* fermentations. However, these fermentations produced larger quantities of diacetyl, ethyl lactate and 2-phenylethyl acetate than single-culture *S. cerevisiae* fermentation. 3-ethoxy propanol was produced only in the sequential fermentations. The five sequential fermentations produced smaller quantities of vitisin A and B than single-culture *S. cerevisiae* fermentation. In tests performed prior to the addition of the *S. cerevisiae* in the sequential fermentations, none of the *T. delbrueckii* strains showed any extracellular hydroxycinnamate decarboxylase activity. They therefore produced no vinyl phenolic pyranoanthocyanins.

1. Introduction

Sequential fermentation using two yeast species emulates natural, spontaneous fermentation in which several yeast species from grapes are active. In natural fermentations, species with low fermentative power are usually active in the initial phases (Romano, Suzzi, Domizio, & Fatichenti, 1997). *Saccharomyces cerevisiae* usually grows and ferments sugars later when the ethanol concentration produced by the initial fermenting species begins to inhibit its growth. Suitable non-*Saccharomyces* species can be used in sequential fermentations to improve the sensorial complexity of wines (Clemente-Jimenez, Mingorance-Cazorla, Martínez-Rodríguez, Las Heras-Vázquez, & Rodríguez-Vico, 2005). The role of such yeasts in oenology has been reviewed by several authors (Fleet, 2008; Jolly, Augustyn, & Pretorius, 2006; Suárez-Lepe & Morata, 2012).

Wine quality can be assessed by a mixture of sensory (colour, aroma and mouth feel) and chemical (pigments, aromatic profile

and flavour molecules) analyses. Yeasts may improve wine colour via the formation of pyranoanthocyanins during fermentation via the excretion of metabolites such as pyruvic acid and acetaldehyde – precursors of vitisins A and B respectively (Morata, Gómez-Cordovés, Colomo, & Suárez, 2003). Extracellular HCDC+ activity, if present, would allow the formation of vinylphenols – precursors of vinyl phenolic pyranoanthocyanins (VPAs) (Morata, Gómez-Cordovés, Calderón, & Suárez, 2006). Pyranoanthocyanins are more stable than grape pigments under oenological conditions since they are less sensitive to SO_2 bleaching, oxidation, and discolouration when the pH increases (Bakker & Timberlake, 1997; Romero & Bakker, 1999). The use of strains that adsorb few anthocyanins onto their cell walls may also be beneficial (Morata, Gómez-Cordovés, Suberviola, et al., 2003). Yeasts may also improve the body, weight and fullness of a wine, which can be sensed as it crosses the palate, via the release of polysaccharides from their cell walls (Giovani, Canuti, & Rosi, 2010) and by the production of glycerol (Kutyna et al., 2012). Aromatic quality can be improved via the production of esters and other aromatic compounds (Fleet, 2003). 2-phenyletyl acetate improves the perception of floral aromas in wine, ethyl lactate and isoamyl acetate provide enriching fruity notes (Lilly, Lambrechts, & Pretorius, 2000), and ethyl acetate increases wine complexity when at low concentration (too much produces an off-flavour) (Jackson, 2008). Different strains also produce different amounts of alcohol (Loira, Morata, González, & Suárez-Lepe, 2012). Non-*Saccharomyces* strains can be used in mixed or sequential fermentations with *S. cerevisiae* to help achieve the above ends. For example, the formation of pyranoanthocyanins can be increased by using *Pichia gillermondii* (Benito, Morata, Palomero, González, & Suárez-Lepe, 2011) or *Schizosaccharomyces pombe* (Morata et al., 2012), glycerol levels can be increased using *Candida stellata* (Ferraro, Fatichenti, & Ciani, 2000) or *Lachancea thermotolerans* (Gobbi et al., 2013), and polysaccharide (Giovani, Rosi, & Bertuccioli, 2012) and aromatic (Comitini et al., 2011) compound contents can be improved through the use of *Torulaspora delbrueckii*.

T. delbrueckii, anascomycetous yeast, was formerly known as *Saccharomyces delbrueckii* or *Saccharomyces rosei*. The anamorph form is known as *Candida colliculosa* (Kurtzman & Fell, 1998a). It has peculiar metabolic and physiological properties of potential use in oenology. Its low production of volatile acidity (Bely, Stoeckle, Masneuf-Pomarède, & Dubourdieu, 2008; Renault et al., 2009), ethyl acetate (Plata, Millan, Mauricio, & Ortega, 2003), acetaldehyde (Ciani & Picciotti, 1995) and acetoin (Ciani & Maccarelli, 1998), its medium production of H_2S (Comitini et al., 2011), its medium-high resistance to SO_2 (Comitini et al., 2011), its ability to biotransform terpenes (King & Dickinson, 2000), and its increased production of certain aromatic compounds (Azzolini et al., 2012) and molecules such as lactic (Valli et al., 2006) or succinic acid (Ciani & Maccarelli, 1998), render it a good complement to *Saccharomyces* when trying to improve wine quality. The fermentative power of *T. delbrueckii* is low, normally ranging from 7 to 10% v/v ethanol (Ciani & Maccarelli, 1998); it must therefore be used with *Saccharomyces* if all the sugars of a grape must are to be consumed. Modern oenological practices involve strains of *T. delbrueckii* produced by a European (ZYMAFLORE® ALPHA TD N. SACCH., Laffort) or a Canadian (LEVEL2™ TD, Lallemand, Canada) manufacturer under standard conditions, either in mixed or sequential cultures.

The aim of the present work was to study the effect on the sensorial properties of wines made from red musts fermented sequentially by different *T. delbrueckii* strains and *S. cerevisiae*. Special attention was paid to pigment formation, polyalcohol production and the formation of aromatic compounds. The growth of the different yeast populations was also recorded in order to examine the persistence of *T. delbrueckii* in the last stages of fermentation.

2. Materials and methods

2.1. Grape must, yeast strains, fermentations and sampling

Wine samples were produced from the must of Tempranillo grapes (*Vitis vinifera* L.) (sugar content 240 g/l, pH 3.6). To eliminate wild yeast populations, the must was pasteurised at 80 °C for 30 s. The effectiveness of this treatment was verified by plate counting (yeasts <10 cfu/ml). The initial anthocyanin content was 211 mg/l.

Either *T. delbrueckii* strain 7013, 717 (IFI, CSIC, Madrid, Spain), 291 (Lallemand, Danstard Ferment, Montreal, Canada), 1880 or 10558 (CECT, Valencia, Spain) was used in the initial stages of sequential fermentations. *S. cerevisiae* 7VA (Dept. of Food Science and Technology, UPM, Madrid, Spain) was used to complete all sequential fermentations; it was also used as a monospecific fermentation control. The interspecific hybrid *S. cerevisiae* × *Saccharomyces uvarum* S6U (hereinafter referred to as S6U, its commercial name) (Lallemand, Danstard Ferment, Montreal, Canada) was used as a monospecific control in tests of hydroxycinnamate decarboxylase (HCDC) activity (S6U has no such activity).

Fermentations were performed in fermentation flasks and involved 70 ml volumes of must. Those involving the five *T. delbrueckii* strains were initially inoculated with 1 ml of a 24 h pre-culture of the required *T. delbrueckii* strain (approximately 10^8 cfu/ml, as determined using a Neubauer chamber) produced in liquid YEPD (Kurtzman & Fell, 1998b). Fermentation then proceeded at 25 °C without agitation. On day 11, each flask was further inoculated with 1 ml of *S. cerevisiae* 7VA (10^8 cfu/ml) to ensure the complete fermentation of the sugars. Control fermentations with *S. cerevisiae* 7VA, and when needed with S6U, were performed in the same way, reinoculating with the corresponding *Saccharomyces* species on day 11. All fermentations were performed in triplicate.

The weight of each fermentation flask was recorded daily; weight reductions correspond to the loss of CO_2 formed during fermentation. Samples were taken every 3 days to test for anthocyanins and to determine yeast population sizes. Volatile compounds were analysed at the end of fermentation (day 21 in all cases). The ethanol content was determined on day 11 prior to inoculation with *S. cerevisiae*, and at the end of fermentation.

2.2. Selective media for plate counting of T. delbrueckii and S. cerevisiae

The growth and survival of the yeast cells was determined by plate counting. Single-culture or sequential fermentation samples were appropriately diluted using a medium composed of 8.5 g NaCl and 1 g peptone per litre. In the sequential fermentations, total yeasts (*Torulaspora* plus *Saccharomyces*) were determined on YPD agar after incubation at 25 °C for 2 days, at different intervals over the fermentation period. *Saccharomyces* cell numbers were determined by incubating plates at 39 °C for 2 days (at this temperature *Torulaspora* is unable to grow). The number of *Torulaspora* cells was then obtained by subtracting the number of *Saccharomyces* colonies obtained at 39 °C from the total number of yeast colonies obtained at 25 °C. Near the end of the fermentation period, when *Torulaspora* counts were very low, a synthetic lysine phosphate medium specific for *Torulaspora* was used to ensure its detection (Rodríguez-Navarro & Ramos, 1984). Preliminary experiments confirmed that *Saccharomyces* cells grew equally well (no significant difference [$p > 0.05$] as determined by the LSD test) in YPD at 25 °C and 39 °C.

Torulaspora cells grew equally well on YPD and the specific synthetic medium.

2.3. Determination of anthocyanins

Grape anthocyanins and pyranoanthocyanins were determined by HPLC-DAD-ESI/MS according to Morata et al. (2012). Briefly, solvent A (water/formic acid, 95:5, v/v) and B (methanol/formic acid, 95:5) gradients were used in an RP C18 column (100 × 4.6 mm; 2.6 μm) as follows: minutes 0–27, 20–50% B linear (0.8 ml/min); minutes 27–28, 50% B; minutes 28–29, 50–20% B linear; 29–30 min, re-equilibration. Detection was performed by scanning in the 500–600 nm range. Quantification was performed by comparison against an external standard at 525 nm and expressed as mg/l of malvidin-3-glucoside ($r^2 = 0.9999$). Anthocyanins were identified by their retention times, and by comparing their UV–visible and mass spectra with data in the literature. Mass spectrometry was performed in positive scanning mode (m/z 100–1000, fragmenter voltage 150 V from minute 0–23). One hundred microlitre samples of previously filtered (0.45 μm membrane) wines were injected into the HPLC apparatus. The detection limit was 0.1 mg/l.

2.4. Analysis of volatile compounds by gas chromatography with flame ionisation detection (GC-FID)

Volatile compounds were determined by GC-FID as described by Abalos, Vejarano, Morata, González, & Suárez-Lepe, (2011). A DB-624 column (60 m × 250 μm × 1.40 μm) was used to perform the separation. Volatile compounds were quantified using external calibration standards ($r^2 > 0.999$). 4-methyl-2-pentanol was used as an internal standard. The injector temperature was 250 °C and the detector temperature 300 °C. The column temperature was 40 °C (for 5 min), rising by 10 °C/min until reaching 250 °C (held for 5 min). Hydrogen was used as the carrier gas. The split ratio was 1:10, the flow rate in the column 2.2 l/min, and the detection limit 0.1 mg/l. One hundred microlitres of internal standard (500 mg/l) were added to 1 ml test samples and filtered (0.45 μm membrane). One microlitre was injected into the GC apparatus. 3-ethoxy propanol was identified by GC–MS under the same chromatographic conditions.

2.5. Analysis of glycerol, acetic acid and residual sugars

Glycerol, acetic acid and residual sugars were measured enzymatically using a Y15 Enzymatic Autoanalyzer (Biosystems, Barcelona, Spain).

2.6. Ethanol quantification

Ethanol was analysed by liquid chromatography with refractive index detection (LC-RI) using a Waters e2695 apparatus (Milford, Massachusetts, USA) equipped with a 2414 Refractive Index Detector. Analyses were performed using a reverse phase Phenosphere XDB C18 column (4.6 × 150 mm, 5 μm particle size) (Phenomenex, Torrance, CA, USA). The solvent was Milli-Q water (used in isocratic mode); the flow rate was 0.4 ml/min. The temperature was set at 30 °C in the column and detector. Calibration was performed using an external ethanol/glucose standard (Panreac, Barcelona, Spain). Samples were injected after filtration through 0.45 μm cellulose methyl ester membrane filters (Teknokroma, Barcelona, Spain). The injection volume was 2 μl.

2.7. Sensory analysis

Wines were evaluated by an experienced panel of six judges. Briefly, the panellists used a scale from 0 to 10 to rate the intensity of different attributes (0 = attribute not perceptible, 10 = attribute strongly perceptible). Each panellist also provided an overall impression of the wines produced, taking into account olfactory and taste features, including any defects.

2.8. Statistical analysis

Means and standard deviations were calculated and differences examined using ANOVA and the least significant difference (LSD) test. All calculations were made using PC Statgraphics v.5 software (Graphics Software Systems, Rockville, MD, USA). Significance was set at $p < 0.05$.

3. Results and discussion

3.1. Growth of the T. delbrueckii and Saccharomyces populations during fermentation

Growth curves were produced for single-culture fermentations of *S. cerevisiae* and S6U (Fig. 1A and B), and for the sequential fermentations (Fig. 1C–G) involving each of the five *T. delbrueckii* strains plus *S. cerevisiae*. In all fermentations, the initial must yeast concentration following inoculation with 10^8 cfu/ml ranged from 5.0 to 5.7 log cfu/ml. The single-culture *S. cerevisiae* and S6U populations reached a maximum size of 7.8–8.1 log cfu/ml (Fig. 1A and B) after 3 days of fermentation. After this time, the *S. cerevisiae* population began to slowly decline (Fig. 1A), probably due to the depletion of sugar from the growth medium (Cramer, Vlassides, & Block, 2002), while the S6U population remained stationary (Fig. 1B).

All the *T. delbrueckii* strains grew well over the first days of fermentation (Fig. 1C–G), reaching cell counts similar to those recorded for the *Saccharomyces* species after 3 days of fermentation. When *S. cerevisiae* was inoculated on day 11, the *T. delbrueckii* count began to fall slowly as the number of *S. cerevisiae* cells increased. On day 15, however, the *T. delbrueckii* populations fell markedly, except for strain 10558 (Fig. 1G). At the end of the fermentation period, *S. cerevisiae* had colonised the must. The early demise of *Torulaspora* (though not so evident for strain 10558) after the addition of *Saccharomyces* cannot be due to the ethanol concentration since *Torulaspora* yeasts can survive in concentrations of up to 16.5% v/v (Azzolini et al., 2012). The reduction in their numbers was likely due to their inability to compete with *S. cerevisiae* (Nissen & Arneborg, 2003).

3.2. Fermentations

On day 11, prior to the addition of the *S. cerevisiae* to the sequential fermentations, the ethanol content was 7.6–9.0% v/v (Fig. 2A). These values agree with others previously reported for *Torulaspora* (Ciani & Maccarelli, 1998). In single-culture fermentations, *S. cerevisiae* produced 14.7 ± 0.1% v/v ethanol, and S6U 11.7 ± 0.6% v/v. After 21 days of fermentation, all the sequential fermentations, as well as the single-culture fermentation with *S. cerevisiae*, finished with an ethanol content of 14.3–14.6% v/v; residual sugars were <3 g/l. The single-culture fermentation with S6U finished with 13.9 ± 0.1% v/v ethanol and a residual sugar content of 6.7 ± 2.2 g/l, probably a reflection of its more modest fermentative power.

T. delbrueckii has been described to produce little volatile acidity, and its production of acetic acid is reported to be unrelated to ethanol formation (Renault et al., 2009) – unlike in *S. cerevisiae* (Ciani & Maccarelli, 1998). Acetic acid production in the sequential fermentations was <0.7 g/l (Fig. 2B), with a mean of 0.4 g/l, similar to that reported by Bely et al. (2008). The *Saccharomyces* strains produced much less (<0.2 g/l).

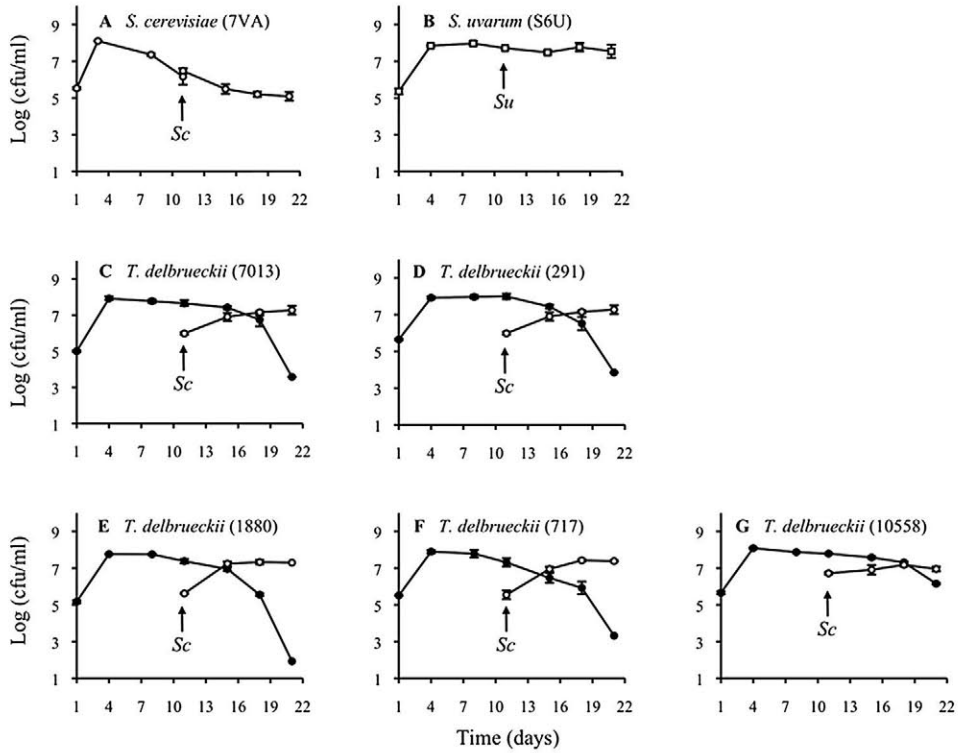

Fig. 1. Change in the populations of *T. delbrueckii*, *S. cerevisiae* 7VA and 6SU over fermentation (log cfu/ml). A. Single-culture fermentation with *S. cerevisiae* 7VA; B. single-culture fermentation with 6SU (on day 11 both fermentations were re-inoculated with either *S. cerevisiae* 7VA or 6SU respectively); C–G. sequential fermentations with *T. delbrucekii* and *S. cerevisiae* 7VA (the latter inoculated on day 11). Values are means ± standard deviations for three independent fermentations.

Glycerol is a by-product of the fermentation of sugar to ethanol, forming via a redox-neutral process (Taherzadeh, Adler, & Lidén, 2002). It is the third most common constituent of wine after water and ethanol, and it affects wine body and softness. Glycerol production in the sequential fermentations was in the range of 4.9–8.2 g/l (Fig. 2C), less than that produced by either *Saccharomyces* species alone (9.6 and 9.9 g/l for *cerevisiae* and 6SU). This supports the high fermentation purity of *T. delbrueckii* reported by other authors (Bely et al., 2008; Renault et al., 2009).

3.3. Pyranoanthocyanin formation

Vitisins are stable pigments formed during and after the fermentation of musts, a consequence of the condensation of grape anthocyanins (mainly malvidin-3-o-glucoside) and yeast metabolites such as acetaldehyde or pyruvate (to form vitisin B and A respectively). These pigments are more stable than naturally occurring grape pigments under oenological conditions (Bakker & Timberlake, 1997).

The production of vitisin B by the *T. delbrueckii* strains (i.e., in the sequential fermentations prior to the addition of *S. cerevisiae*) was low compared with either *Saccharomyces* species (Fig. 3A). The concentration of vitisin B in single-culture fermentation involving *S. cerevisiae* correlated with the excretion of acetaldehyde; condensation between this molecule and malvidin-3-o-glucoside is dependent on the concentration of both precursors (Morata, Gómez-Cordovés, Colomo, et al., 2003). The low acetaldehyde production shown by the *T. delbrueckii* strains (with the exception of strain 1880) in the first part of the sequential fermentations compared to *S. cerevisiae* in single-culture fermentation (Table 1) might explain their low vitisin B production. The change in the vitisin B concentration over fermentation shows a different pattern in *T. delbrueckii* compared to the *Saccharomyces* species (Fig. 3A), increasing with *S. cerevisiae* as previously reported (Morata, Gómez-Cordovés, Colomo, et al., 2003), but decreasing with *Torulaspora*.

The production of vitisin A in *Saccharomyces* during fermentation is correlated with the excretion of pyruvate – its precursor along with malvidin-3-o-glucoside. Pyruvate excretion is greater in the early-middle stages of fermentation; at the end it is consumed by the yeast as nutrients become scarce. Thus, the maximum concentration of vitisin A is reached in early-middle fermentation (Morata et al., 2006), and is then maintained. The formation kinetics of vitisin A were similar in the sequential fermentations (Fig. 3B), but the concentration reached was lower (0.2–0.3 mg/l) than in single-culture fermentations with *S. cerevisiae* (0.6–0.7 mg/l). This result probably indicates the reduced excretion of pyruvate by *T. delbrueckii* in agreement with its reduced production of other fermentative metabolites such as acetic acid (Bely et al., 2008), glycerol, and especially, acetaldehyde (Ciani & Picciotti, 1995).

Vinyl phenolic pyranoanthocyanins (VPAs) can be formed during fermentation via the condensation of vinylphenols (produced by yeasts with HCDC activity) and grape anthocyanins (Morata, González, & Suárez-Lepe, 2007). VPAs have properties similar to those of vitisins and behave as stable pigments. The total VPA concentration produced in single-culture fermentation with *S. cerevisiae* reached 0.2 mg/l. These pigments were not detected in the fermentations with S6U since it has no HCDC activity. The mean VPA concentrations reached in the sequential fermentations were only in the range of 0.0–0.1 mg/l (Fig. 3C). VPAs started to form after the inoculation of *S. cerevisiae* on day 11. Thus, they were formed by *S. cerevisiae*; the *T. delbrueckii* strains are therefore likely HCDC negative. Since only five strains were used, however, the entire species cannot yet be concluded as such.

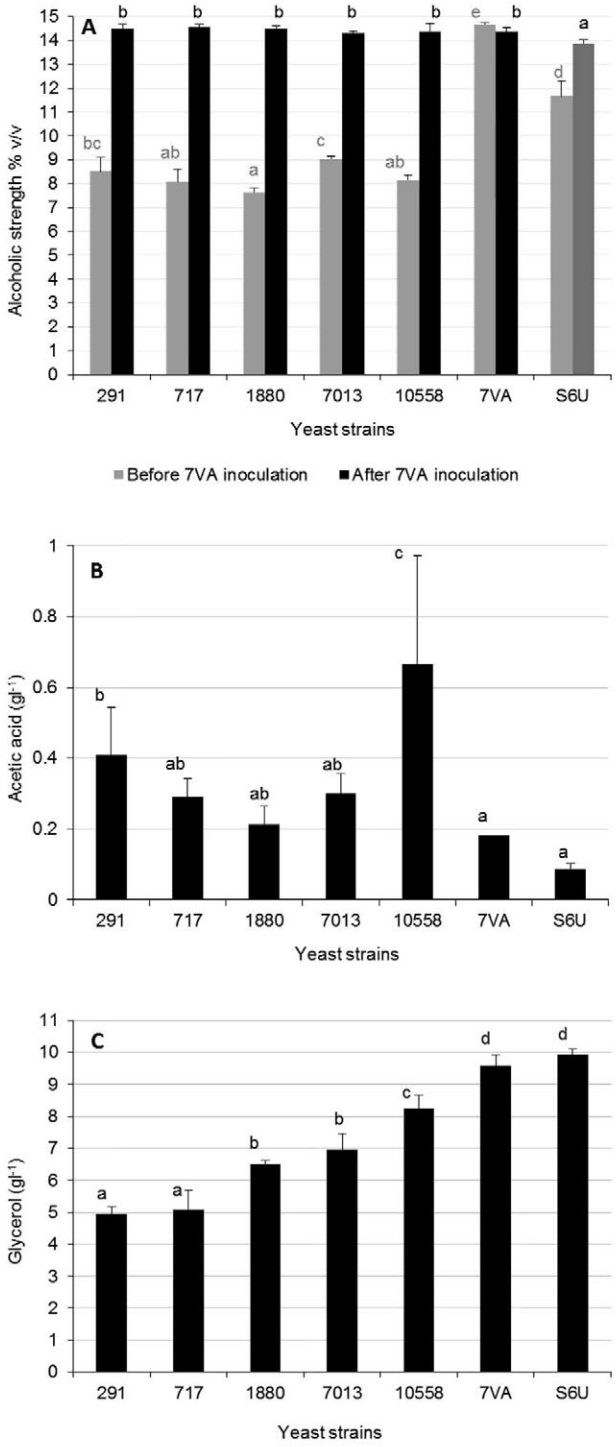

Fig. 2. A. Ethanol content expressed in % v/v: in sequential fermentations just before the inoculation of *S. cerevisiae* 7VA, and at the end of fermentation; and in single-culture fermentations just before and after re-inoculation with the corresponding *Saccharomyces* species. B. Acetic acid (g/l) at the end of sequential fermentation. C. Glycerol (g/l) at the end of sequential fermentation. Values are means ± standard deviations for three independent fermentations. Different letters in the same series indicate significant differences between means ($p < 0.05$).

The trend for total anthocyanins in the sequential fermentations was similar, with initial contents of 210 mg/l reduced to about 50 mg/l by the end of fermentation (Fig. 3D). Most of these fermentations produced less than the single-culture fermentation with *S. cerevisiae*. The anthocyanin concentration during fermentation is dependent on the polarity which is reduced with a rising ethanol content. In the fermentations with *Saccharomyces*, the maximum ethanol content was reached on day 11, and the polarity of the medium was probably stable from this time forward. However, in the *T. delbrueckii* fermentations, the ethanol concentration on day 11 was in the range 7.5–9% v/v, and started to increase again with the addition of *Saccharomyces*. This would lead to further reduced polarity and therefore a reduction in anthocyanin solubility. The addition of a large inoculum of *S. cerevisiae* can also lead to a reduction in anthocyanins via their adsorption onto the cell walls. This reduction might be due in part to the extra cell wall surface area made available or to differences in cell wall structure between *T. delbrueckii* and *S. cerevisiae*. Metabolites produced by *S. cerevisiae* in sequential culture might also affect the solubility of anthocyanins.

3.4. Formation of volatile compounds

The mean acetaldehyde concentration produced by the sequential fermentations was 14.1 mg/l (Table 1), smaller than that recorded in *S. cerevisiae* fermentations by other authors (Ciani & Picciotti, 1995; Romano, Fiore, Paraggio, Caruso, & Capece, 2003).

The sequential fermentations yielded smaller concentrations of 3-methyl-1-butanol than the single-culture *S. cerevisiae* fermentation, and similar or smaller amounts of 2-methyl-1-butanol depending on the strain. This finding is of some importance since excessive amounts of higher alcohols contribute to pungent aromas that can reduce wine quality (Rapp & Mandery, 1986; Rapp & Versini, 1991). However, the concentration of 1-propanol, which does not cause an over-pungent aroma and is associated with alcohol and ripe fruit descriptors (Peinado, Moreno, Bueno, Moreno, & Mauricio, 2004), was consistently higher in the sequential fermentations and always above its sensorial threshold (50 mg/l according to Tao & Zhang (2010)).

The concentrations of some positive esters, such as ethyl lactate (with descriptors of coffee and strawberry) or 2-phenylethyl acetate (rose petals) were higher in the sequential than in the single-culture *S. cerevisiae* fermentations (Table 1), in agreement with previous findings (Azzolini et al., 2012). The production of ethyl acetate in sequential fermentations was slightly greater than in the *S. cerevisiae* single cultures. This ester increases complexity at low concentration, and is associated with descriptors such as fruity, solvent and balsamic (Peinado, Moreno, Bueno, et al., 2004). However, it causes spoilage at 150 mg/l (Jackson, 2008). Its sensory threshold is 12 mg/l (Peinado, Moreno, Bueno, et al., 2004).

The diacetyl content (which increases the complexity of wines) was also higher in fermentations involving *T. delbrueckii* (5 mg/l on average). Diacetyl gives wine buttery and nutty aromas. Its sensorial threshold for diacetyl is dependent on the type of wine and ranges from 0.89 to 2.8 mg/l in red wines (Martineau, Acree, & Henick-Kling, 1995). The normal diacetyl concentration in red wines ranges from 0.3 to 2.5 mg/l (Bartowsky, Francis, Bellon, & Henschke, 2002).

A GC peak was observed only in the sequential fermentations (and was thus produced by *Torulaspora*); mass spectrometry tentatively identified it as corresponding to 3-ethoxy propanol. The sensorial threshold is 0.1 mg/l (Peinado, Moreno, Medina, & Mauricio, 2004), and it was detected at concentrations of >5 mg/l in the sequential fermentations (Table 1). This compound has a chemical odour typical of organic solvents, but it has been correlated with a blackcurrant aroma when in red wine (Tao & Zhang, 2010).

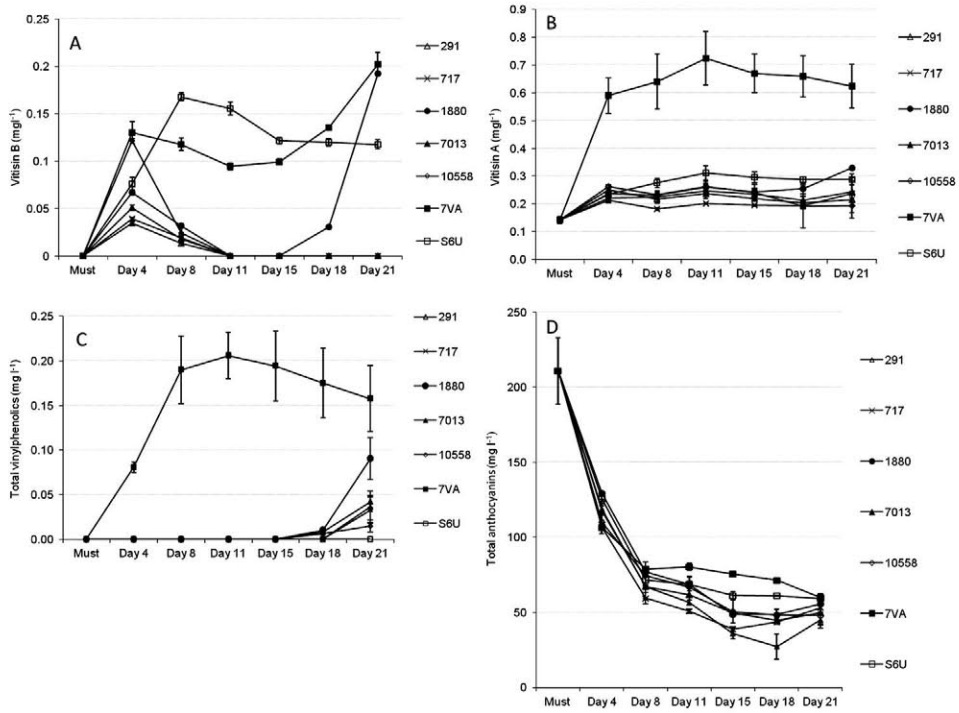

Fig. 3. Change in the concentration of pigments (mg/l) over sequential fermentation with each *T. delbrucekii* strain. *S. cerevisiae* 7VA was inoculated on day 11. Values are means ± standard deviations for three independent fermentations. A. Vitisin B. B. Vitisin A. C. Vinyl phenolic pyranoanthocyanins. D. Total anthocyanins.

2,3-butanediols are viscous polyalcohols with little repercussion on wine aroma (sensorial threshold 150 mg/l; Dubois, 1994); they mainly affect sweetness and softness in the mouth (Romano, Brandolini, Ansaloni, & Menziani, 1998). Concentrations in wines range from 0.5 to 3 g/l (average 0.57 g/l; Sponholz, Dittrich, & Muno, 1993). The trend of their production was similar to that of glycerol, with the sequential fermentations producing a mean of 580.7 mg/l, less than that produced by either *Saccharomyces* species (Table 1).

3.5. Sensory analysis

In sensory analysis (Fig. 4), overall perception was better for the wines produced by three of the sequential fermentations than by single-culture fermentation with *S. cerevisiae*. The aromatic quality was also better for most of the strains used in the sequential fermentations, although aromatic intensity was greater in single-culture *S. cerevisiae* fermentation. The existence of significant differences in aromatic quality at the sensorial level in several

Table 1
Concentration of volatile compounds produced at the end of all fermentation processes (mg l^{-1}), as determined by GC-FID.

Volatile compounds	Sequential fermentation involving *T. delbrueckii* strain:					Single-culture fermentation involving *S. cerevisiae*	Single-culture fermentation involving *S. uvarum*	*p*-value
	291	717	1880	7013	10558	7VA	S6U	
Acetaldehyde	12.3 ± 2.0^{ab}	14.0 ± 1.1^{ab}	19.6 ± 13.0^{bc}	14.5 ± 0.3^{ab}	10.2 ± 4.2^{a}	24.8 ± 3.5^{c}	12.9 ± 2.2^{ab}	0.07
Methanol	14.4 ± 0.6^{cd}	14.3 ± 0.6^{cd}	12.7 ± 0.4^{abc}	11.7 ± 1.0^{ab}	10.6 ± 0.3^{a}	13.4 ± 1.9^{bc}	12.4 ± 1.3^{abc}	0.00
1-propanol	64.0 ± 2.1^{e}	68.4 ± 5.4^{e}	65.4 ± 2.6^{e}	58.4 ± 2.8^{d}	46.8 ± 0.3^{c}	25.0 ± 1.5^{a}	31.7 ± 3.0^{b}	0.00
Diacetyl	5.1 ± 1.1^{bc}	5.5 ± 1.6^{c}	3.4 ± 0.2^{ab}	5.4 ± 1.3^{c}	6.0 ± 1.4^{c}	3.0 ± 0.3^{a}	2.2 ± 0.3^{a}	0.00
Ethyl acetate	61.7 ± 1.2^{ab}	63.7 ± 9.1^{ab}	67.5 ± 7.5^{b}	55.9 ± 9.2^{a}	71.7 ± 6.5^{b}	54.7 ± 4.9^{a}	70.0 ± 3.3^{b}	0.04
Isobutanol	50.8 ± 11.4^{bc}	58.4 ± 4.2^{c}	51.5 ± 2.2^{bc}	51.8 ± 5.0^{bc}	39.1 ± 6.8^{ab}	22.9 ± 3.4^{a}	65.6 ± 21.4^{c}	0.00
1-butanol	4.2 ± 0.1^{a}	4.4 ± 0.1^{a}	4.4 ± 0.1^{a}	4.2 ± 0.0^{a}	4.4 ± 0.3^{a}	4.2 ± 0.0^{a}	6.3 ± 0.6^{b}	0.00
Acetoin	12.1 ± 1.3^{ab}	11.9 ± 2.6^{ab}	10.4 ± 0.2^{ab}	12.8 ± 3.3^{b}	11.4 ± 1.9^{ab}	10.4 ± 1.1^{ab}	9.6 ± 0.8^{a}	0.40
2-methyl-1-butanol	206.0 ± 20.7^{ab}	232.0 ± 23.1^{b}	225.2 ± 4.6^{b}	209.8 ± 13.6^{ab}	178.0 ± 10.8^{a}	212.3 ± 28.2^{ab}	222.4 ± 45.7^{b}	0.23
3-methyl-1-butanol	40.3 ± 4.1^{ab}	51.4 ± 4.6^{bc}	44.2 ± 1.8^{ab}	36.4 ± 1.5^{a}	32.7 ± 1.2^{a}	60.3 ± 13.4^{cd}	66.4 ± 11.8^{d}	0.00
Ethyl lactate	17.2 ± 11.4^{ab}	9.4 ± 5.3^{a}	6.1 ± 0.1^{a}	10.4 ± 3.7^{ab}	28.3 ± 27.4^{b}	4.7 ± 4.2^{a}	6.5 ± 0.1^{a}	0.23
2–3 butanediol	660.6 ± 90.4^{b}	513.2 ± 11.6^{a}	494.7 ± 15.0^{a}	613.6 ± 24.9^{ab}	618.4 ± 81.7^{ab}	920.9 ± 110.9^{c}	1092.6 ± 118.7^{d}	0.00
3-ethoxy propanol	15.7 ± 1.4^{b}	15.3 ± 0.6^{b}	33.8 ± 1.0^{c}	15.1 ± 1.4^{b}	5.1 ± 8.8^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.00
Isoamyl acetate	3.5 ± 0.4^{a}	5.0 ± 1.2^{bc}	5.5 ± 1.2^{c}	3.6 ± 1.0^{a}	3.4 ± 0.3^{a}	4.1 ± 0.7^{ab}	4.3 ± 0.1^{abc}	0.03
Hexanol	8.1 ± 0.7^{c}	7.7 ± 0.3^{bc}	7.6 ± 0.2^{bc}	7.6 ± 0.4^{bc}	7.1 ± 0.2^{ab}	6.5 ± 0.3^{a}	6.2 ± 1.0^{a}	0.01
2-phenylethanol	34.5 ± 2.4^{a}	48.0 ± 6.1^{bcd}	52.3 ± 2.1^{cd}	43.1 ± 1.8^{abc}	37.1 ± 2.4^{ab}	101.8 ± 16.3^{e}	55.4 ± 2.0^{d}	0.00
2-phenylethyl acetate	19.1 ± 8.5^{ab}	33.2 ± 1.5^{d}	18.3 ± 5.5^{ab}	30.2 ± 4.1^{d}	28.7 ± 2.9^{cd}	15.2 ± 1.6^{ab}	11.9 ± 1.0^{a}	0.00
Σ Higher alcohols	423.5 ± 41.0^{ab}	485.5 ± 34.9^{b}	484.4 ± 8.1^{b}	426.4 ± 24.2^{ab}	350.3 ± 7.7^{a}	433.0 ± 61.4^{b}	453.94 ± 82.9^{b}	0.07
Σ Esters	101.6 ± 8.4^{ab}	111.3 ± 10.0^{bc}	97.4 ± 10.4^{ab}	100.0 ± 9.4^{ab}	132.1 ± 36.0^{c}	78.8 ± 5.7^{a}	92.7 ± 3.8^{ab}	0.03
Total	1229.5 ± 140.0^{a}	1155.8 ± 55.3^{a}	1122.6 ± 18.6^{a}	1184.5 ± 48.8^{a}	1138.9 ± 117.4^{a}	1484.2 ± 99.1^{b}	1676.3 ± 64.9^{c}	0.00

Values are means ± SD for three independent fermentations. Different letters in the same row indicate significant differences between means ($p < 0.05$).

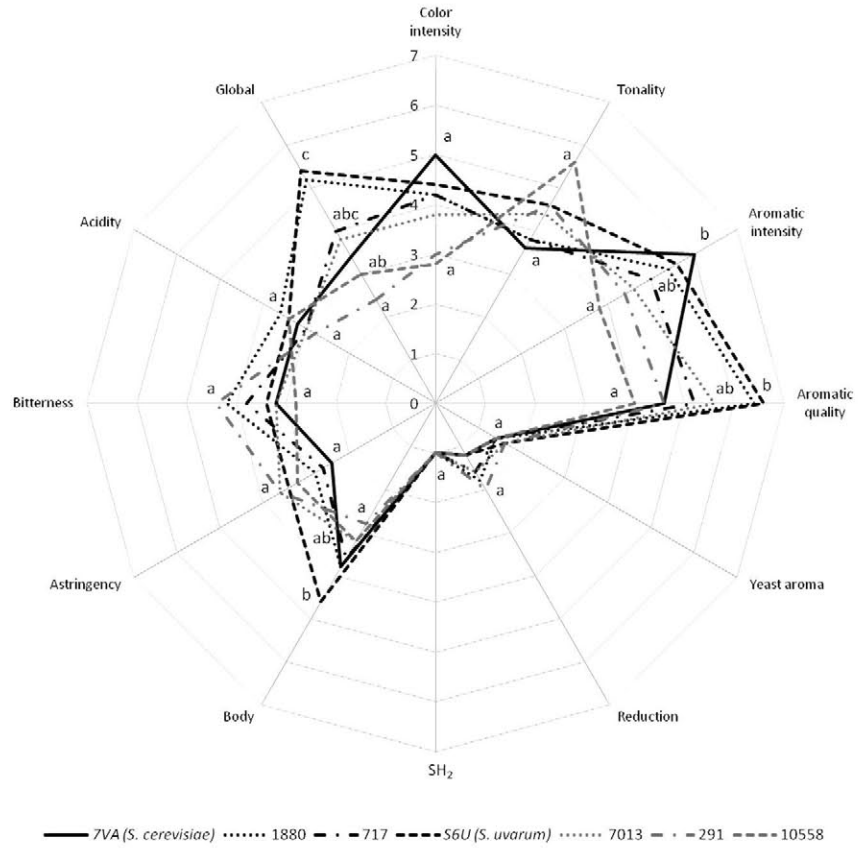

Fig. 4. Spider net graph of taste panel results. Different letters in the same series indicate significant differences between means ($p < 0.05$).

fermentations with *T. delbrueckii* is probably due to the greater production of some volatile compounds (esters, diacetyl and 3-ethoxy propanol) as previously indicated. These findings also support the use of this yeast as a means of improving wine aroma.

The taste panel members did not perceive reductive notes or any sulphuric acid smell in the sequential fermentations or controls. Some strains of *Torulaspora* have been described as medium-strong producers of SH_2. Comitini et al. (2011) analysed SH_2 production by nine strains of *T. delbrueckii* ranked 3–4 on a 0–5 scale, and found them to be strong SH_2 producers. This could be improved via the effect of *S. cerevisiae* at the end of sequential fermentations.

The panellists recorded higher colour intensity and lower tonality for single-culture *S. cerevisiae* fermentations, a reflection of the total anthocyanin concentration. However, the differences were not statistically significant ($p = 0.61$). The sequential use of *S. cerevisiae*, with its positive effect on colour (formation of pyranoanthocyanin pigments), could be a good strategy for overcoming this drawback of *T. delbrueckii*.

The sequential fermentations produced some slight bitterness that might be deemed positive in terms of prolonging sensations in the mouth (Gonzalo-Diago, Dizy, & Fernández-Zurbano, 2014). This might be related to the higher content of succinic acid in these wines (Ciani & Maccarelli, 1998); this compound produces a bitter flavour (Rubico & McDaniel, 1992). Astringency and acidity were moderate in both sequential cultures and controls.

4. Conclusions

This work reports new information on the changing populations of yeasts in sequential fermentations with *T. delbrueckii* and *S. cerevisiae*, and the influence of these fermenters on the formation of aromatic compounds, polyalcohols and pigments. Sequential fermentations with *T. delbrueckii* and *S. cerevisiae* increase the complexity of wine, which could help improve the quality of some wine types. *T. delbrueckii* improves the aromatic complexity of wines by increasing their fruity flavour, while keeping spoilage attributes (volatile acidity, ethyl acetate and acetaldehyde) at suitable levels.

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