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Use of *Schizosaccharomyces pombe* and *Torulaspora delbrueckii* strains in mixed and sequential fermentations to improve red wine sensory quality

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Abstract

One of the main opportunities in the use of non-*Saccharomyces* yeasts is its great intraspecific variability in relation to the synthesis of secondary products of fermentation. Thus, mixed or sequential fermentation with non-*Saccharomyces* can increase the synthesis of certain metabolites that are important for colour stability, such as acetaldehyde and pyruvic acid (vitisin precursors) or vinylphenols (vinylphenolic pyranoanthocyanin precursors). Furthermore, the selection and use of non-*Saccharomyces* yeast strains with good yields in the production of certain volatile compounds (ethyl lactate, 2,3-butanediol, 2-phenylethyl acetate), with limited formation of higher alcohols, is a way to improve the aromatic profile of red wine. The main aim of this work was to evaluate the influence of sequential and mixed fermentations with *Schizosaccharomyces pombe* and *Torulaspora delbrueckii* strains on red wine's sensory quality. Anthocyanins and aromatic profiles, as well as glycerol and organic acid content, were analysed in the red wines obtained. Results show that, in general, mixed fermentations can promote an increment in polyols synthesis, while sequential fermentations can enhance the herbaceous aroma. Moreover, the use of *Torulaspora delbrueckii* in mixed fermentations allowed an increase to the fruity character of red wine. The use of *Schizosaccharomyces pombe* in sequential fermentations increased the stability of the colouring matter by favouring vitisins and vinylphenolic pyranoanthocyanins formation.

Keywords: *Schizosaccharomyces pombe*, *Torulaspora delbrueckii*, mixed/sequential fermentations, red wine, sensory quality

1. Introduction

Recently, non-*Saccharomyces* yeasts have been gaining importance for winemaking due to their high intraspecific variability (Romano *et al*, 1997). This diversity in the yielding of secondary products of the fermentation allows the selection of those strains with interesting metabolic features in order to improve the sensory quality of the wine.

Over recent years, the use of non-*Saccharomyces* yeasts has been studied for multiple and interesting oenological applications including reductions to alcohol content (Contreras *et al*, 2014), sur lie aging (Palomero *et al*, 2009) and improving wine's sensory profile by performing mixed and/or sequential fermentations (Gobbi *et al*, *2013*), among others.

Mixed or sequential fermentations with non-*Saccharomyces* yeasts can potentiate the synthesis of certain important metabolites for colour stability, such as acetaldehyde and pyruvic acid, both involving vitisins synthesis (Clemente-Jiménez *et al*, 2005; Benito *et al*, 2012; Gobbi *et al*, 2013). Similarly, the use of non-*Saccharomyces* yeast with high hydroxycinnamate decarboxylase (HCDC) activity may increase the formation of vinylphenolic pyranoanthocyanins (Benito *et al*, 2011).

With regards to the aromatic profile, mixed and sequential fermentations allow increasing concentrations of some interesting compounds in red wine such as ethyl lactate, 2,3-butanediol, 2-phenylethanol and 2-phenylethyl acetate (Clemente-Jiménez *et al*, 2005; Viana *et al*, 2009; Gobbi *et al*, 2013). Achieving slight reductions to the content of higher alcohol is also interesting (Viana *et al*, 2009), especially the avoidance of exceeding 350 mg/l, the level at which the sensory quality of the wine can be negatively affected (Rapp & Mandery, 1986). Moreover, in wines with lower levels of alcohol content, a fruity character can be more easily appreciated (Viana *et al*, 2009).

The *T. delbrueckii* species is characterized by its high purity fermentation, and as such it has a low production of glycerol, acetaldehyde, acetic acid and ethyl acetate (Renault *et al*, 2009). When used in sequential or mixed fermentations with *S. cerevisiae* it allows for the correcting of certain defects in wines such as volatile acidity (Bely *et al*, 2008).

On the other hand, the *S. pombe* species is highly appreciated in colder regions because of its ability to completely transform the malic acid of the must into ethanol, thanks to its particular metabolism of maloalcoholic fermentation (Suárez-Lepe *et al*, 2012). Moreover, its great ability to synthesize pyruvic acid (a vitisin A precursor) and glycerol was recently reported by Benito *et al*, 2012 & 2013. Therefore, it is an interesting species to consider in order to improve some of the sensory parameters of the wine, especially those related to wine colour stability, despite its main drawback, which is the medium-high yield of acetic acid (Benito *et al*, 2012).

The main aim of this work was to evaluate the potential influence of *S. pombe* and *T. delbrueckii* species on the sensory quality of red wine when used in sequential and mixed fermentations with *S. cerevisiae*, paying particular attention to changes in wine colour and aroma.

2. Materials and methods

2.1 Yeast strains and fermentation media

The *Schizosaccharomyces pombe* (*Sp*) and *Torulaspora delbrueckii* (*Td*) yeast strains assessed were 938, V1, 4.2 (CSIC, Madrid, Spain) and 1880, 7013, 10558 (CECT, Valencia, Spain), respectively. The aforementioned non-*Saccharomyces* strains were used in co-inoculated and sequentially inoculated fermentations with *Saccharomyces*

cerevisiae (*Sc*) 7VA (HCDC+) (EnotechUPM, Madrid, Spain). *S. cerevisiae* 7VA together with *Saccharomyces uvarum* (*Su*) S6U (HCDC-) (Lallemand, Montreal, Canada) were also used in controlled fermentations as single inoculants (PF).

The fermentative assay was performed in triplicate at 23ºC using a fresh free-run juice (no skin content) made from Syrah grapes (*Vitis vinifera* L.) with an initial sugar content of 220 g/l (potential alcohol content of 13 % v/v), pH 3.5, and heat treated at 100 °C for 3 minutes. All inocula were standardised in order to obtain homogenous active populations (10^6 cfu/ml), by adding 100 µl of each strain to 5 ml of YEPD medium (Kurtzman & Fell, 1998) and were grown for 24h at 23ºC, twice in succession. In sequential fermentations (SF), 70 ml of must in 100 ml flasks were inoculated with 1 ml of each non-*Saccharomyces* strain, and after 7 days (when a fermentative power of 8% v/v ethanol was reached) the second inoculation was performed with 1 ml of strain *Sc*7VA. On the other hand, mixed fermentations (MF) were co-inoculated with 1 ml of a non-*Saccharomyces* strain and 100 µl of strain *Sc*7VA (non-*Saccharomyces*:*Saccharomyces* ratio 10:1).

Fermentation kinetics and fermentative power were estimated by the daily weighing of the fermentation flasks, thus registering variations that correspond to the loss of CO_2 associated with the fermentative process. All chemical analyses were performed at the end of the fermentations.

2.2 Determination of anthocyanin profile by HPLC-DAD-ESI/MS

Grape anthocyanins and pyranoanthocyanins were determined by high-performance liquid chromatography with diode array and electrospray ionization/mass spectrometry detection according to Morata *et al* (2012). 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 x 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; minutes 29-30, re-equilibration. Detection was performed by scanning within 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-*O*-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 to 23). One hundred microliter samples of previously filtered (0.45 μm membrane) wines were injected into the HPLC column. The detection limit was 0.1 mg/l.

2.3 Determination of volatile profiles by LLE-GC-MS

Aromatic profiles of wine samples were determined by gas chromatography with mass spectrometric detection after performing a liquid-liquid extraction. Firstly, 5 ml of the wine sample was mixed with 5 ml of a 30% sodium chloride solution and 200 μl of ethyl heptanoate as the internal standard (0.42 g/l in ethanol). The mixture was extracted with 5 ml of a pentane: dichloromethane (2:1 v/v) solution, vigorously shaking the glass tube by hand for 2 min; this extraction procedure was repeated three times. The organic phase was collected, dried with anhydrous sodium sulphate, transferred into a smaller conical glass tube and stored at -18 ºC until GC injection. The equipment used was a Shimadzu GC-17A (Shimadzu, Kyoto, Japan) gas chromatograph coupled with a Shimadzu QP-5000 (Shimadzu, Kyoto, Japan) mass spectrometer detector. The injection was performed in splitless mode with a 60 sec splitless time. The injection volume was 1 μl. Temperatures in the injector and detector were held at 250 ºC and 240 ºC, respectively. The carrier gas was helium, at a linear flow rate of 35 cm/s. Compounds were separated on a DB-Wax capillary column (30 m x 0.25 mm i.d., 0.25

µm film thickness), purchased from Alltech (State College, PA, USA). The column temperature was programmed as follows: 40ºC for 1 min, followed by a gradual increase of temperature at a rate of 4 ºC/min up to 240 ºC, with a final holding time of 15 mins. The MS detector was programmed in positive scanning mode (35-400 *m/z*). Volatile compounds were identified by comparing their mass spectra and retention times with those of standard compounds and/or with data reported in the mass spectrum libraries Wiley 6, NIST21 and NIST107. Moreover, linear retention indexes were calculated from the retention times of *n*-alkanes and compared with those available in literature. Semi-quantitative data were expressed in equivalents of internal standards, considering a concentration of ethyl heptanoate in the sample of 16.9 mg/l and a response factor equal to 1.00.

2.4 Determination of glycerol, organic acids and residual sugar levels by HPLC-UV/RI

Glycerol, residual sugars and organic acids such as citric, tartaric, malic, succinic, lactic and acetic acids, were measured by liquid chromatography (PerkinElmer model 250) coupled with two different detectors: a refractive index detector model RID-10A (Shimadzu, Kyoto, Japan) was used for the detection of sugars and glycerol, while a UV−vis detector, model 875-UV (Jasco Co. Ltd.), was set at a wavelength of 210 nm for the detection of organic acids. H_2SO_4, 0.025M was used as a working solvent with a flow rate of 0.7 ml/min, in isocratic mode. Analytes were separated on an Aminex HPX-87H column (30 cm x 7.8 mm i.d.) (Bio-Rad Laboratories Inc., Hercules, CA, USA) filled with sulfonated copolymer of styrene and divinylbenzene (9 µm particle size) and thermostated at 65°C. The injection volume was 20 µl. Samples were diluted with H_2SO_4 0.025M ten times, then treated with polyvinylpolypyrrolidone (PVPP) (0.15 g/ml) and finally filtered through 0.45 µm pore sized cellulose acetate cartridges (Albet-

Hahnemühle, Barcelona, Spain). A quantitative analysis was performed by measuring peak areas of each compound and comparing them with the response of pure standard compounds: diluted standard solutions were prepared in HPLC mobile phase and injected to obtain calibration curves; concentrations were: 10, 20, 50, 100, 150, 200 g/l, for glucose and fructose; 0.5, 1.0, 2.5, 5.0, 7.5 and 10.0 g/l for organic acids and glycerol.

2.5 Determination of alcoholic content by HPLC-RI

Ethanol content was determined using a liquid chromatograph Waters e2695 Alliance (Waters, Massachusetts, USA) coupled with a refractive index detector model 2414. MilliQ water was used as the working solvent with a flow rate of 0.4 ml/min in isocratic mode. Samples were filtered through 0.45 µm pore sized methyl ester cellulose cartridges. Separation of analytes was held on a reverse phase column PhenoSphere XDB C18 (150 x 4.6 mm, 5 µm particle size) (Phenomenex, California, USA) stabilized at 30°C. Quantification was performed using ethanol (99.5 % purity) (Panreac, Spain) as an external standard with four levels of calibration: 5, 10, 15 and 20 % v/v (r^2=0.9998). Injection volume was 2 µl.

2.6 Determination of colour parameters by spectrophotometry

Colour variables of wines were determined by an absorbance measurement using an Agilent 8453 UV-Visible (Santa Clara, USA) spectrophotometer. The chromatic characteristics were determined at 420, 520 and 620 nm (colour intensity and tonality), using a 1 mm path length quartz cell following the Glories method (Glories, 1984a; 1984b).

2.7 Statistical analysis

The means and standard deviations were calculated and the ANOVA and least significant difference (LSD) tests performed using PC Statgraphics v.5 software (Graphics Software Systems, Rockville, MD, USA). Significance was set at $P<0.05$. Moreover, a Principal Component Analysis (PCA) was carried out on the concentrations measured for the volatile compounds detected.

3. Results and discussion

Sequential fermentations took twice as long (22 days) to complete the fermentation process (**Figure 1**). This longer duration for the fermentative process allows better preservation of aromas due to milder temperature conditions as a result of a less intense fermentation process. Fermentation kinetics were correct in both cases, starting and ending smoothly. Also, fermentations with the controls and with the mixed cultures were more vigorous in the early stages than those with the sequential cultures. All fermentations ended with an alcoholic content that ranged from 13-13.4 % v/v with no significant differences between the types of fermentation (**Table 1**).

In general, the higher glycerol contents were produced in MF, except for strain *Sp*938, with which the maximum concentration of glycerol (7.9 ± 0.5 g/l) was obtained in SF (**Table 1**). Glycerol is an interesting metabolite for wines because of its positive contribution to the mouthfeel sensations such as sweetness, softness, silkiness and body (Moreno-Arribas & Polo, 2009). Its average concentration in wine is around 6-10 g/l and its taste perception threshold is set at 5.2 g/l (Nieuwoudt *et al*, 2002).

The analysis of organic acids and sugar content in wine is of great importance, because both the type and the quantity can affect chemical and sensory characteristics such as pH, total and volatile acidity, microbiological stability, and flavour. Both acidity and pH

are two key aspects of the sensory quality of wine, significantly influencing the perception of its structure and balance. Furthermore, acidity brings freshness to wine, decreases the perception of sweetness and, together with ethanol, limits the development of spoilage microorganisms (Jackson, 2008). All fermentations finished with sugar concentrations of less than 6 g/l and pH between 3.5-3.7 (**Table 1**). During wine fermentation and aging, acids are involved in the formation of esters responsible for the fruity character. With regards to organic acids, SF kept more tartaric acid in free form than MF, with values of up to 3.4 g/l (**Table 1**). As expected, the malic acid concentration was lower in fermentations with *S. pombe* strains, especially noticeable in SF (**Table 1**). Once again, strain *Sp*938 showed its ability to completely degrade the malic acid present in the must (Benito *et al*, 2012). Moreover, strains *Sp*938 in MF and *Td*1880 in SF produced the highest concentrations of lactic acid during fermentation (~0.25 g/l) (**Table 1**). High concentrations of this acid are good for the quality of the wine, since it contributes to the softness in the mouth and is a precursor of ethyl lactate. One of the major disadvantages of the use of the *S. pombe* species is a consequence of its greater acetic acid synthesis (0.5-0.7 g/l) (**Table 1**), however, when used in sequential or mixed fermentations, this value generally does not exceed the threshold of perception (0.4-1.1 g/l) and, therefore, does not cause sensory defects. Above 0.8 g/l, acetic acid is considered a demeaning factor to wine quality providing a bitter taste and a smell likened to vinegar (Maicas *et al*, 1999; Moreno-Arribas & Polo, 2009). Only the strain *Td*10558 was noted for its higher acetic acid production when used in SF (1.0 ± 0.1 g/l). As for the content of citric and succinic acids, no significant differences between the species or types of fermentation were observed (data not shown).

MF had higher values of monomeric, acetylated and coumarylated anthocyanins than SF, especially with *T. delbrueckii* strains, which is reflected in the higher total

anthocyanins content (**Figure 2**). This difference in the anthocyanins content may be explained by the longer duration of the sequential fermentations (22 days vs. 11 days), so that the anthocyanins were being combined for longer or their precipitation was favoured due to the decrease in solubility along with the change of polarity in the medium as a consequence of ethanol synthesis (Benito *et al*, 2011). Regarding the contribution to the stability of the colouring matter, *S. pombe* in SF showed greater vitisins synthesis (**Figure 3A**), specially type A, whose precursor is pyruvic acid. These *S. pombe* strains were previously described as good producers of pyruvic acid (Benito *et al*, 2012). In the case of *Schizosaccharomyces* strains, the highest concentrations of vitisins were achieved in sequential fermentations (range 9.2-11.6 mg/l), while the opposite occurred with *Torulaspora* strains, where the maximum concentrations were obtained in mixed fermentations (range 6.4-7.6 mg/l). As for vinylphenolic pyranoanthocyanins, its synthesis was higher in MF (**Figure 3B**). The S6U strain acts as a negative control to the formation of these compounds, since its HCDC activity is negligible. Maximum concentration was reached with strain *Td*1880 (1.6 ± 0.2 mg/l), despite not being significantly different to the others. This same *Torulaspora* strain was also the one that led to the highest concentration in the SF, indicating its potential use in a combination with strain *Sc*7VA to improve wine colour stability. However, no significant increase was observed with respect to *Sc*7VA positive control strain, thus indicating that most vinylphenolic pyranoanthocyanins synthesis in mixed fermentations was probably due to the *Saccharomyces cerevisiae* strain activity. Moreover, strain *Sp*V1 proved not to be suitable for the formation of these stable compounds, not even in mixed culture, achieving, as sequential fermentations, mean values lower than 1 mg/l.

Concerning colour intensity, no significant differences between the two types of fermentation were observed (range 0.5-0.6 AU), although wines obtained by SF showed slightly higher values of tonality (data not shown), thus indicating the further evolution of these wines towards red-orange colours.

The main purpose of the volatile compounds analysis was to assess the contribution of each strain, and the influence of each type of fermentation, on the aromatic quality of the wine. It was possible to identify a total of 77 different compounds in the wine samples, although palmitic and stearic acids cannot be completely considered as volatiles under our analysis conditions, due to its high boiling point (> 350 ºC). In **Table 2** we only show the identification data of the compounds that were found to be interesting to our study. MF with *Torulaspora delbrueckii* strains allowed a potential increase of fruity aromas in the wine by synthesizing larger amounts of esters (isoamyl acetate, hexyl acetate, ethyl hexanoate and ethyl octanoate) (**Table 3**). Such compounds are interesting because of their fresh and fruity aroma. In turn, MF in general, produced significantly higher concentrations of polyols (2,3-butanediol and 1,2-propanediol). According to Liu (2002), polyols contribute to wine mouthfeel and body by increasing the viscosity. Notwithstanding, even being normally present at concentrations well above their threshold of perception, they have little effect on wine aroma. On the other hand, SF enhances herbaceous aromas (1-hexanol; threshold perception, 8 mg/l (Culleré *et al*, 2004)), but decreases the presence of total higher alcohols, especially with *S. pombe* strains. Certain higher alcohols such as 1-butanol, isobutanol, 1-hexanol, benzyl alcohol and 2-phenylethanol possess particular aromas that help improve the aromatic profile of the wine (Gil *et al*, 2006; Vilanova & Martinez, 2007). However, quantitatively, main higher alcohols in wine are 2 and 3-methyl-1-butanol, both characterized by a strong alcohol aroma (Sánchez-Palomo *et al*, 2012). *T. delbrueckii*

strains in SF, especially strain *Td*1880, can produce significant amounts of 3-ethoxy-1-propanol and 2-phenylethanol. Thus, 3-ethoxy-1-propanol seems to be a compound linked to *T. delbrueckii*'s metabolism, as previously reported by Herraiz *et al* (1990). Its interest lies in its low perception threshold, 0.1 mg/l, (Peinado *et al*, 2004b) and in its blackcurrant aroma descriptor (Tao & Zhang, 2010). As mentioned above, 2-phenylethanol is an interesting compound for red wine because of its contribution of a floral aroma (rose petals) at the same time adding a touch of honey (Mendes, Gonçalves & Camara, 2012). In general, except for strain *Sp*V1, SF produced more furaneol (> 0.15 mg/l) than MF (<0.08 mg/l), where the strain *Td*1880 stands out for its high concentration of furaneol (0.29 ± 0.14 mg/l). In addition to providing an aroma of caramelized sugar, furaneol, along with methional and sotolon, contributes notes of chocolate in red wines (Ferreira, 2007). This compound is particularly interesting due to its low perception threshold: 5-37 µg/l (Culleré *et al*, 2004; Selli *et al*, 2004). Regarding the contribution of each yeast assessed, strain *Td*7013 was characterized by a higher synthesis of 1-butanol, ethyl 4-hydroxybutanoate and γ-butyrolactone, strain *Sp*938 by its greater production of acetoin, ethyl lactate and benzaldehyde, strain *Td*1880 by high concentrations of 3-ethoxy-1-propanol and furaneol, and finally, strain *Sp*4.2 by higher amounts of octanoic and decanoic acid. Some of these particular compounds may be negative to the quality of red wine when found over certain concentrations, as is the case with γ-butyrolactone and acetoin. However, when in low concentrations, they may prove interesting, as they may help to enhance the wine's aromatic complexity. For instance, benzaldehyde is characterized by its bitter almond aroma and has a perception threshold of 2-3 mg/l (Delfini *et al*, 1991). Similarly, acetoin contributes to wine aroma by providing notes of dairy such as butter or cream, so it is undesirable in high concentrations. Its perception threshold is quite high (150 mg/l) compared to the mean values in which it is found in red wines (0.6-253 mg/l) (Bartowsky & Pretorius, 2009).

As a general rule, for all of the analysis performed, the biggest differences between yeast strains were found in sequential fermentations. This proves that this type of fermentation is suitable for enhancing the expression of the non-*Saccharomyces* yeasts' metabolic particularities, whereas with mixed fermentations greater uniformity is achieved in the results.

After performing PCA analysis on the volatile compound data, three main groups could be clearly differentiated (**Figure 4**). One of the groups is located on the positive part of the component 2 axis, and represents those wines obtained from SF with *T. delbrueckii* yeast strains. The other two groups are located on the negative part of the axis. One of them represents those wines obtained from SF with *S. pombe* yeast strains (positive side of component 1), and the other includes all the wines in MF and PF (negative side of component 1). Wines from *T. delbrueckii* SF were characterized by 1-butanol, 3-ethoxy-1-propanol and furaneol, while wines from *S. pombe* SF were correlated with 1-hexanol, benzaldehyde and acetoin. The last group proves that the wines from MF were highly influenced by the coexistence of the fermentation with the control yeast *Sc*7VA from the beginning, so the aroma was less influenced by the different yeast strains. These wines were mainly associated with polyols and saturated fatty acid ethyl esters.

4. Conclusions

The use of *Schizosaccharomyces pombe* and *Torulaspora delbrueckii* strains in sequential and mixed fermentations with *Saccharomyces cerevisiae* may potentially improve the sensory profile of red wine by enhancing the aromatic complexity and increasing colour stability. As for the impact of each type of inoculation, mixed fermentations performed better with regards to the aroma and the structure of the wine

by increasing its fruity character and polyols content, while by carrying out sequential fermentations with *S. pombe,* better results were obtained in the formation of stable pigments.

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Figures and Tables

Table 1. Glycerol, tartaric acid, malic acid, lactic acid, acetic acid, ethanol and residual sugars content of the wines obtained from mixed and sequential fermentations with *S. pombe* and *T. delbrueckii*. Mean ± SD (n=3)

	Mixed fermentation						Sequential fermentation						Pure fermentation	
	S. pombe			*T. delbrueckii*			*S. pombe*			*T. delbrueckii*			*S. cerevisiae*	
	938	V1	4.2	1880	7013	10558	938	V1	4.2	1880	7013	10558	7VA	S6U
Glycerol (g/l)	7.0 ± 0.0^{cd}	7.0 ± 0.0^{cd}	7.1 ± 0.2^{cd}	7.2 ± 0.0^{cd}	7.0 ± 0.1^{cd}	7.0 ± 0.1^{cd}	7.9 ± 0.5^{e}	6.3 ± 0.3^{b}	6.5 ± 0.2^{b}	6.1 ± 0.1^{ab}	5.9 ± 0.0^{a}	6.9 ± 0.3^{c}	7.1 ± 0.0^{cd}	7.3 ± 0.6^{d}
Tartaric acid (g/l)	2.9 ± 0.1^{a}	2.8 ± 0.1^{a}	2.9 ± 0.1^{a}	2.8 ± 0.1^{a}	3.0 ± 0.1^{abc}	2.8 ± 0.1^{a}	3.4 ± 0.0^{ef}	3.3 ± 0.1^{def}	3.2 ± 0.0^{cde}	3.2 ± 0.1^{def}	3.1 ± 0.2^{bcd}	3.4 ± 0.0^{f}	2.9 ± 0.1^{ab}	2.8 ± 0.3^{bcd}
Malic acid (g/l)	0.12 ± 0.02^{a}	0.79 ± 0.01^{c}	0.84 ± 0.04^{c}	1.37 ± 0.02^{ef}	1.34 ± 0.07^{ef}	1.19 ± 0.07^{d}	0.30 ± 0.06^{b}	0.17 ± 0.02^{ab}	0.15 ± 0.03^{ab}	1.20 ± 0.02^{d}	1.46 ± 0.00^{fg}	1.12 ± 0.02^{d}	1.58 ± 0.06^{g}	1.11 ± 0.34^{de}
Lactic acid (g/l)	0.25 ± 0.06^{e}	0.20 ± 0.01^{d}	0.19 ± 0.01^{cd}	0.19 ± 0.00^{bcd}	0.17 ± 0.03^{bcd}	0.15 ± 0.03^{ab}	0.17 ± 0.01^{bcd}	0.15 ± 0.01^{ab}	0.15 ± 0.02^{abc}	0.26 ± 0.04^{e}	0.12 ± 0.05^{a}	0.12 ± 0.01^{a}	0.20 ± 0.00^{cd}	0.26 ± 0.04^{e}
Acetic acid (g/l)	0.55 ± 0.10^{b}	0.54 ± 0.03^{b}	0.54 ± 0.05^{b}	0.49 ± 0.00^{ab}	0.50 ± 0.03^{ab}	0.47 ± 0.03^{ab}	0.51 ± 0.08^{ab}	0.70 ± 0.01^{c}	0.72 ± 0.05^{c}	0.40 ± 0.10^{a}	0.43 ± 0.19^{ab}	1.00 ± 0.14^{d}	0.55 ± 0.01^{b}	0.80 ± 0.03^{c}
Ethanol (% v/v)	13.4 ± 0.1^{def}	13.3 ± 0.1^{cdef}	13.4 ± 0.0^{cdef}	13.3 ± 0.0^{abcd}	13.3 ± 0.1^{bcde}	13.2 ± 0.1^{abcd}	13.2 ± 0.1^{abc}	13.5 ± 0.1^{ef}	13.5 ± 0.0^{f}	13.2 ± 0.0^{abc}	13.4 ± 0.1^{ef}	13.3 ± 0.2^{abcd}	13.2 ± 0.2^{ab}	13.1 ± 0.1^{a}
Residual sugars (g/l)	4.6 ± 0.1^{ab}	4.8 ± 0.1^{ab}	4.8 ± 0.2^{ab}	5.2 ± 0.1^{bc}	5.6 ± 0.1^{cd}	5.2 ± 0.1^{bc}	4.6 ± 0.1^{ab}	4.8 ± 0.1^{ab}	4.9 ± 0.1^{ab}	4.5 ± 0.1^{a}	5.9 ± 0.2^{d}	5.6 ± 0.2^{cd}	5.7 ± 0.1^{cd}	7.9 ± 1.4^{e}
pH	3.6 ± 0.0^{c}	3.5 ± 0.0^{a}	3.5 ± 0.0^{a}	3.5 ± 0.0^{a}	3.5 ± 0.0^{a}	3.5 ± 0.0^{a}	3.5 ± 0.0^{a}	3.7 ± 0.0^{d}	3.6 ± 0.0^{d}	3.5 ± 0.0^{ab}	3.5 ± 0.0^{a}	3.6 ± 0.0^{bc}	3.5 ± 0.0^{a}	3.6 ± 0.0^{bc}

Values in the same row with same letter are not significantly different ($p < 0.05$).

Figure 1.

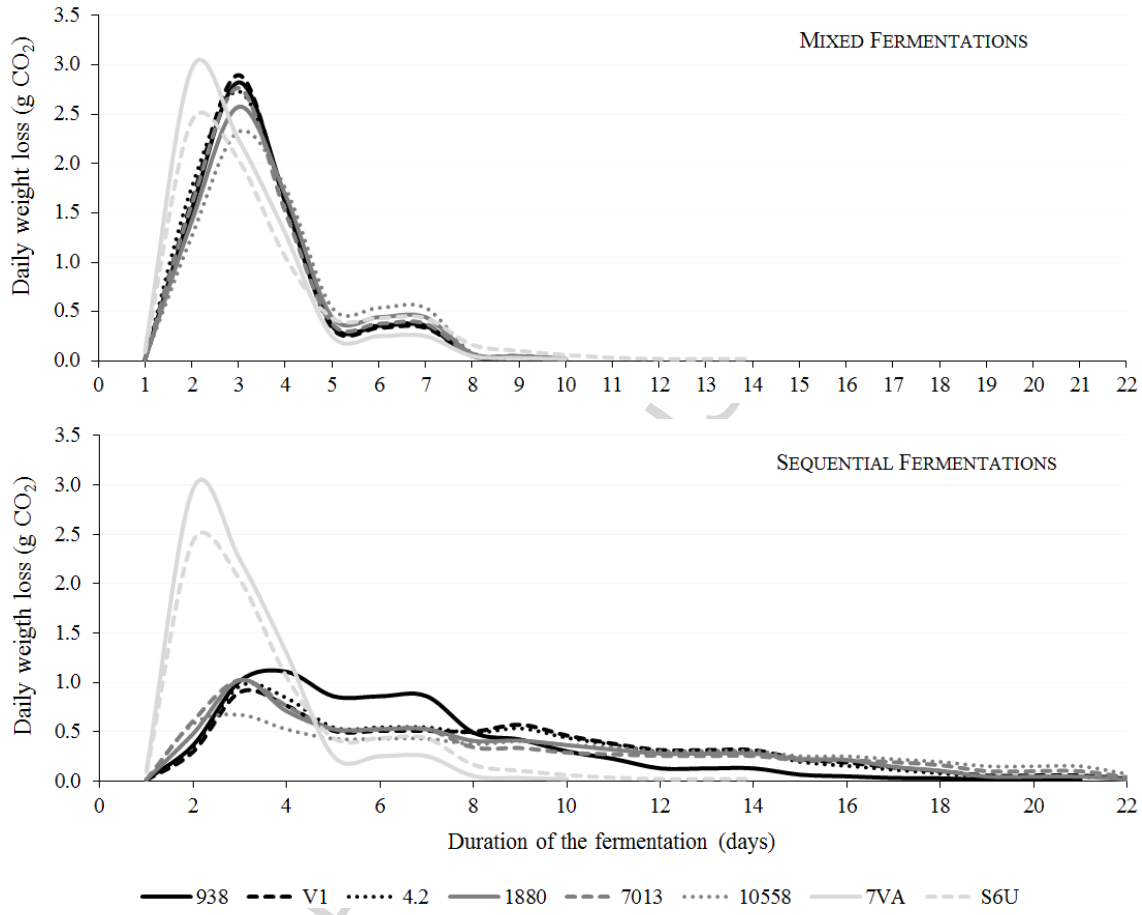

Figure 2.

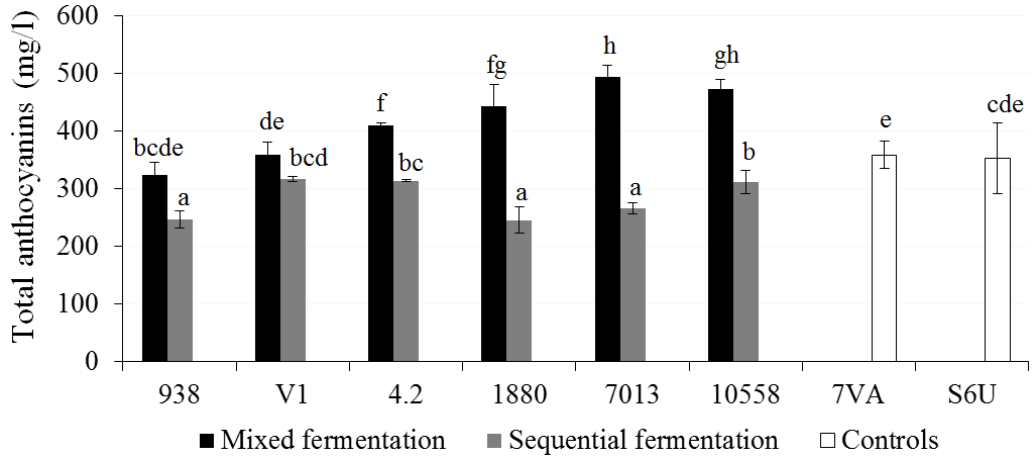

Figure 3.

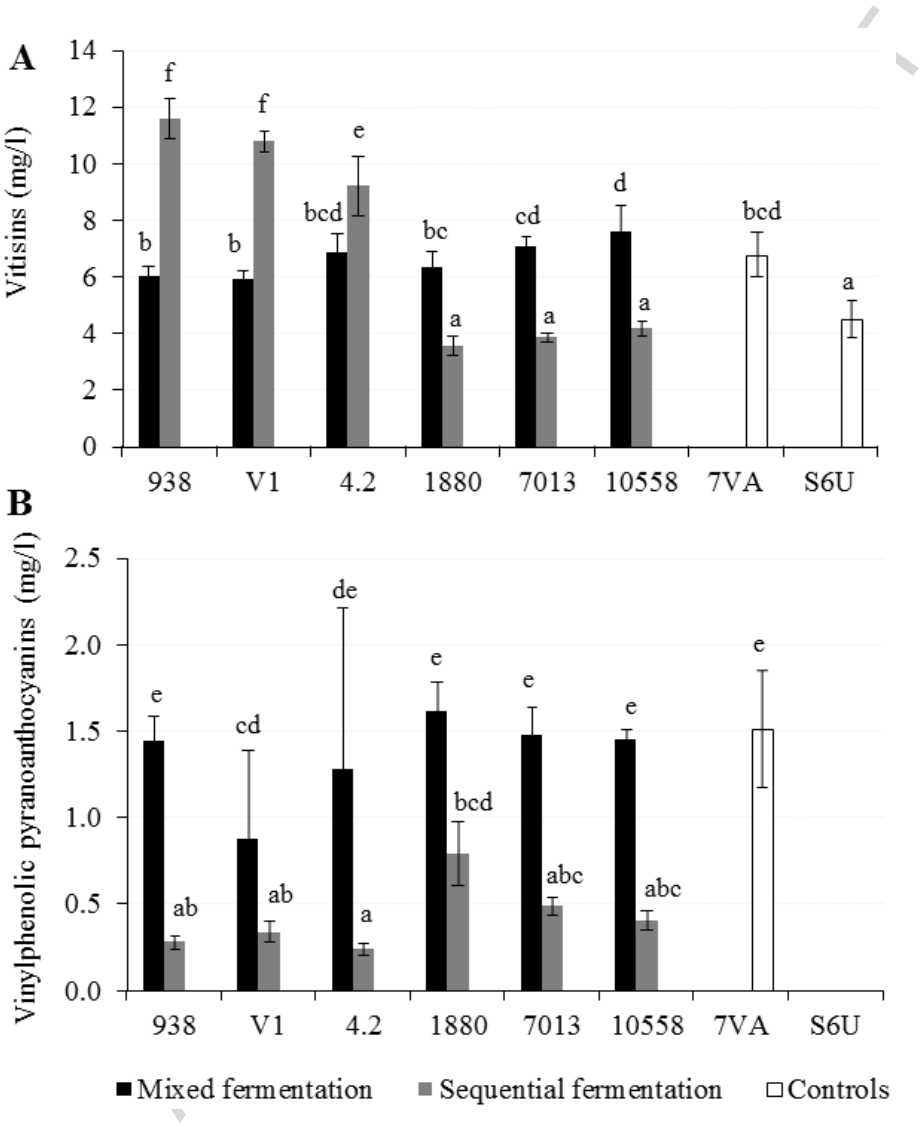

Table 2. Retention index, identification method, aroma descriptors, mean concentrations and perception thresholds of the volatile compounds tentatively identified in the wine samples obtained from mixed and sequential cultures of *S. pombe* and *T. delbrueckii* with *S. cerevisiae*.

Compound	Ri	Ri (lit)	Reference	IM	Aroma descriptors	Mean concentration (mg/l)	Perception threshold (mg/l)
Isoamyl acetate	1124	1123;1125	Sumitani *et al*, 1994; Oliveira *et al*, 2008	MS, IR, S	Fruity: banana, apple[2,3,4,7,9,10]	0.1-3.4[3]	0.03[3,7];0.16[4]
1-butanol	1150	1149	Sumitani *et al*, 1994	MS, IR, S	Alcohol, medicinal[1,2,4,7,16]	0.5-8.5[3,16]	150[3,4,6,16]
Ethyl hexanoate	1235	1234;1238	Oliveira *et al*, 2008; Sumitani *et al*, 1994	MS, IR, S	Fruity: green apple[3,10,17], strawberry[1]	0.03-3.4[3]	0.014[12];0.05[3]; 0.08[17]
Hexyl acetate	1273	1272;1275	Oliveira *et al*, 2008; Sumitani *et al*, 1994	MS, IR	Fruity: pear[1,4] Floral[4,7]	0-4.8[3]	0.67[4];0.7[3];1.5[8]
Acetoin	1281	1286;1291	Sumitani *et al*, 1994; Selli *et al*, 2004	MS, IR	butter, cream[3,4]	0.6-253[3]	150[3,6,13]
Ethyl lactate	1344	1338	Oliveira *et al*, 2008	MS, IR	Fruity: Strawberry[3], Raspberry[1] Dairy[1,4,17]	10-25[11,16]	14[3];150[4];154.6[6]
1-hexanol	1359	1359;1361	López *et al*, 1999; Sumitani *et al*, 1994	MS, IR, S	Herbaceous[1,3,12], Wood[4]	0.3-12[3,11,16]	1.1[4];4[3,16];8[2,8]
3-ethoxy-1-propanol	1379	1369	Oliveira *et al*, 2008	MS, IR	Alcohol[12], organic solvent[14], blackcurrant[14]		0.1[1]
Ethyl octanoate	1434	1434;1435	Oliveira *et al*, 2008; Comuzzo *et al*, 2006	MS, IR, S	Fruity: apple[10], pineapple, pear[1,2,17] Floral[1,2,13,17]	0.05-3.8[3,11]	0.002[2];0.005[1]; 0.02[3];0.58[4,6]
Benzaldehyde	1507	1508	Pino *et al*, 2001	MS, IR	Fruity, sweet[7] Bitter almond[4,16]	0.006-0.5[6]	2[4];3[6]
2,3-butanediol	1545	1542;1545	Lee and Noble, 2003; Sánchez-Palomo *et al*, 2012	MS, IR	Butter, cream[1,2,15] Fruity[7,17]	300-1500[19]	120[12,14];150[7,17]
1,2-propanediol	1593	1594	Wong and Bernhard, 1988	MS, IR			
γ-butyrolactone	1615	1632;1635	Jennings and Shibamoto, 1980; Lee and Noble, 2003	MS, IR, S	caramel, sweet[4,7] cream, milk[1]		0.35[7,8];20[4]; 35[5];50[1,14]
Ethyl 4-hydroxybutanoate	1805	1783;1819	Sánchez-Palomo *et al*, 2012; Selli *et al*, 2004	MS, IR	Fruity[13]		
2-phenylethanol	1906	1908	Oliveira *et al*, 2008	MS, IR, S	Floral, roses[3,9,16] Honey[4,16]	4.0-197[3,11]	10[3,16];14[2];200[4]
Furaneol	2032	2033	Mahajan *et al*, 2004	MS, IR	Caramel[13] Cotton candy[8]		0.005[8];0.037[13]
Octanoic acid	2059	2058;2060	Lee and Noble, 2003; López *et al*, 1999	MS, IR, S	Rancid[1,3,4,12] Cheese[1,7,12,13] Sweat[7]	0.5-2.1[16]	0.5[1,12,13,16];8.8[3]; 10[4,16]
Decanoic acid	2275	2272;2289	Lee and Noble, 2003; Sánchez-Palomo *et al*, 2012	MS, IR, S	Fat[1,2,3,4,12,14] Rancid[4,7,16]	0.09-3[16]	1[1,7,12,14]; 6[3,4,16]; 15[2]

Ri: Retention index on DB-Wax column
Ri (lit): Retention index from literature
MI: Identification method – MS: Comparison with mass spectra in the libraries NIST21, NIST107 y WILEY6; RI: Comparison with retention index from literature; S: Comparison of mass spectra and retention time with those of standard compounds
References for Aroma descriptors, Mean concentration and Perception threshold: [1] Tao and Li, 2009; [2] Jiang and Zhang, 2010; [3] König *et al*, 2009; [4] Peinado *et al*, 2004a; [5] Campo *et al*, 2006; [6] Etiévant, 1991; [7] Sánchez-Palomo *et al*, 2012; [8] Culleré *et al*, 2004; [9] Oliveira *et al*, 2008; [10] Rojas *et al*, 2001; [11] Cabanis *et al*, 2003; [12] Zhang *et al*, 2013; [13] Selli *et al*, 2004; [14] Tao and Zhang, 2010; [15] Lee and Noble, 2003; [16] Bakker and Clarke, 2012; [17] Peinado *et al*, 2004b.

Table 3. Concentration (mg/l) of different volatile compounds tentatively identified in the wines obtained from mixed and sequential fermentations with *S. pombe* and *T. delbrueckii*. Mean ± SD (n=3)

	Mixed fermentation						Sequential fermentation						Pure fermentation	
	S. Pombe			*T. delbrueckii*			*S. pombe*			*T. delbrueckii*			*S. cerevisiae*	
Compound	938	V1	4.2	1880	7013	10558	938	V1	4.2	1880	7013	10558	7VA	S6U
Isoamyl acetate	8.33 ± 0.72^{d}	9.01 ± 0.51^{d}	8.13 ± 1.09^{d}	11.21 ± 2.58^{ef}	11.18 ± 0.57^{ef}	11.79 ± 2.25^{f}	0.00 ± 0.00^{a}	0.08 ± 0.01^{a}	0.10 ± 0.06^{a}	5.99 ± 1.84^{c}	9.59 ± 1.40^{de}	3.63 ± 1.37^{b}	9.96 ± 1.36^{def}	5.91 ± 0.98^{c}
1-butanol	0.52 ± 0.08^{abcd}	0.57 ± 0.09^{abcd}	0.41 ± 0.10^{abc}	0.64 ± 0.02^{abcd}	0.73 ± 0.13^{cd}	0.62 ± 0.09^{abcd}	0.31 ± 0.04^{ab}	0.30 ± 0.03^{a}	0.28 ± 0.06^{a}	1.31 ± 0.60^{e}	3.09 ± 0.02^{f}	0.86 ± 0.39^{d}	0.68 ± 0.02^{bcd}	1.56 ± 0.41^{e}
Ethyl hexanoate	0.56 ± 0.05^{cd}	0.59 ± 0.06^{de}	0.60 ± 0.11^{de}	0.73 ± 0.07^{f}	0.65 ± 0.07^{def}	0.55 ± 0.13^{cd}	0.37 ± 0.03^{ab}	0.47 ± 0.05^{bc}	0.47 ± 0.03^{bc}	0.39 ± 0.09^{ab}	0.59 ± 0.00^{de}	0.35 ± 0.05^{a}	0.76 ± 0.04^{f}	0.69 ± 0.06^{ef}
Hexyl acetate	0.25 ± 0.05^{cd}	0.29 ± 0.04^{d}	0.22 ± 0.03^{bcd}	0.39 ± 0.10^{e}	0.37 ± 0.01^{e}	0.40 ± 0.09^{e}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.18 ± 0.05^{bc}	0.28 ± 0.00^{d}	0.16 ± 0.05^{b}	0.42 ± 0.05^{e}	0.23 ± 0.11^{bcd}
Acetoin	1.17 ± 0.09^{a}	0.82 ± 0.22^{a}	0.70 ± 0.12^{a}	0.85 ± 0.05^{a}	1.07 ± 0.15^{a}	1.85 ± 0.16^{a}	15.05 ± 7.00^{b}	3.37 ± 0.41^{a}	2.76 ± 0.55^{a}	0.41 ± 0.14^{a}	0.88 ± 0.10^{a}	0.83 ± 0.38^{a}	0.89 ± 0.12^{a}	2.68 ± 0.39^{a}
Ethyl lactate	0.83 ± 0.08^{abc}	0.84 ± 0.11^{abc}	0.90 ± 0.04^{bcd}	1.11 ± 0.09^{ef}	1.19 ± 0.11^{ef}	1.02 ± 0.07^{cde}	1.72 ± 0.22^{g}	0.83 ± 0.15^{abc}	0.68 ± 0.03^{a}	1.12 ± 0.08^{ef}	1.07 ± 0.06^{def}	0.81 ± 0.25^{ab}	1.23 ± 0.05^{f}	1.88 ± 0.10^{g}
1-hexanol	1.68 ± 0.05^{bc}	1.44 ± 0.15^{ab}	1.48 ± 0.17^{ab}	1.29 ± 0.18^{a}	1.42 ± 0.15^{ab}	1.32 ± 0.17^{a}	3.17 ± 0.03^{e}	3.56 ± 0.12^{f}	3.32 ± 0.13^{ef}	2.38 ± 0.16^{d}	2.41 ± 0.22^{d}	2.20 ± 0.40^{d}	1.49 ± 0.04^{ab}	1.87 ± 0.24^{c}
3-E-1-P*	0.23 ± 0.02^{a}	0.15 ± 0.03^{a}	0.15 ± 0.02^{a}	0.76 ± 0.03^{a}	0.46 ± 0.11^{a}	1.00 ± 0.07^{a}	0.04 ± 0.03^{a}	0.00 ± 0.00^{a}	0.00 ± 0.01^{a}	11.76 ± 1.77^{d}	4.74 ± 0.27^{b}	6.45 ± 1.36^{c}	0.22 ± 0.03^{a}	0.09 ± 0.01^{a}
Ethyl octanoate	0.60 ± 0.09^{d}	0.59 ± 0.06^{cd}	0.52 ± 0.04^{c}	0.63 ± 0.07^{d}	0.61 ± 0.05^{d}	0.55 ± 0.07^{cd}	0.15 ± 0.01^{a}	0.12 ± 0.01^{a}	0.13 ± 0.02^{a}	0.14 ± 0.03^{a}	0.17 ± 0.00^{a}	0.12 ± 0.03^{a}	0.74 ± 0.08^{e}	0.32 ± 0.08^{b}
Benzaldehyde	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.14 ± 0.05^{b}	0.09 ± 0.12^{b}	0.01 ± 0.02^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
2,3-butanediol	14.75 ± 3.89^{c}	13.52 ± 1.83^{c}	12.27 ± 2.29^{c}	12.99 ± 2.60^{c}	15.46 ± 3.73^{c}	15.38 ± 4.36^{c}	7.07 ± 1.35^{ab}	4.46 ± 0.49^{ab}	3.91 ± 0.51^{a}	6.47 ± 1.52^{ab}	7.64 ± 1.46^{ab}	7.93 ± 0.84^{b}	13.98 ± 1.47^{c}	19.99 ± 3.42^{d}
1,2-propanediol	0.31 ± 0.07^{de}	0.33 ± 0.03^{def}	0.30 ± 0.01^{cde}	0.38 ± 0.01^{ef}	0.40 ± 0.11^{ef}	0.45 ± 0.09^{f}	0.15 ± 0.02^{ab}	0.09 ± 0.01^{a}	0.06 ± 0.05^{a}	0.24 ± 0.04^{bcd}	0.17 ± 0.02^{ab}	0.18 ± 0.05^{abc}	0.45 ± 0.09^{f}	0.59 ± 0.19^{g}
γ-butyrolactone	0.36 ± 0.04^{bc}	0.29 ± 0.03^{ab}	0.37 ± 0.04^{bc}	0.39 ± 0.03^{c}	0.42 ± 0.06^{cd}	0.41 ± 0.07^{c}	0.23 ± 0.04^{a}	0.22 ± 0.01^{a}	0.24 ± 0.06^{a}	0.51 ± 0.05^{de}	0.84 ± 0.03^{f}	0.60 ± 0.07^{e}	0.44 ± 0.04^{cd}	0.60 ± 0.13^{e}
Ethyl 4-HB**	4.76 ± 0.34^{bc}	5.03 ± 0.99^{bc}	5.07 ± 1.46^{bc}	5.11 ± 0.01^{bc}	6.20 ± 0.91^{cd}	5.03 ± 0.87^{bc}	0.43 ± 0.11^{a}	0.58 ± 0.26^{a}	0.66 ± 0.32^{a}	5.11 ± 1.28^{bc}	9.54 ± 0.01^{e}	5.84 ± 1.79^{cd}	6.86 ± 0.75^{d}	4.03 ± 1.51^{b}
2-phenylethanol	19.50 ± 2.52^{def}	22.15 ± 2.34^{fg}	22.70 ± 3.76^{fg}	15.78 ± 1.63^{cde}	21.66 ± 1.68^{fg}	14.28 ± 3.78^{bcd}	6.76 ± 2.83^{a}	8.38 ± 3.64^{a}	8.50 ± 2.11^{ab}	24.62 ± 6.18^{fg}	20.16 ± 1.63^{ef}	20.86 ± 7.44^{efg}	26.18 ± 2.09^{g}	10.35 ± 0.83^{abc}
Furaneol	0.05 ± 0.04^{a}	0.06 ± 0.05^{ab}	0.03 ± 0.02^{a}	0.08 ± 0.02^{abcd}	0.07 ± 0.04^{abc}	0.05 ± 0.03^{a}	0.17 ± 0.05^{d}	0.08 ± 0.04^{abc}	0.15 ± 0.02^{bcd}	0.29 ± 0.14^{e}	0.17 ± 0.06^{d}	0.16 ± 0.05^{cd}	0.07 ± 0.01^{abc}	0.10 ± 0.08^{abcd}
Octanoic acid	5.73 ± 1.42^{def}	5.29 ± 0.73^{cdef}	4.12 ± 1.31^{bcde}	4.44 ± 0.26^{cdef}	4.70 ± 0.70^{cdef}	3.97 ± 0.55^{bcd}	4.05 ± 1.08^{bcde}	3.46 ± 1.37^{abc}	6.12 ± 1.32^{f}	2.35 ± 0.82^{ab}	2.04 ± 0.32^{a}	2.01 ± 0.96^{a}	5.83 ± 0.62^{ef}	5.52 ± 2.20^{def}
Decanoic acid	0.62 ± 0.33^{cd}	0.52 ± 0.10^{bcd}	0.20 ± 0.09^{abc}	0.25 ± 0.12^{abcd}	0.39 ± 0.16^{abcd}	0.31 ± 0.09^{abcd}	0.33 ± 0.18^{abcd}	0.48 ± 0.33^{abcd}	1.27 ± 0.48^{e}	0.07 ± 0.13^{a}	0.15 ± 0.08^{ab}	0.18 ± 0.10^{ab}	0.36 ± 0.14^{abcd}	0.68 ± 0.57^{d}
Higher alcohols	132.19 ± 15.64^{b}	133.34 ± 11.94^{b}	128.42 ± 34.93^{b}	108.69 ± 17.94^{b}	131.25 ± 34.99^{b}	107.15 ± 32.96^{b}	54.42 ± 7.66^{a}	65.73 ± 11.63^{a}	58.33 ± 9.87^{a}	124.21 ± 11.35^{b}	114.83 ± 32.48^{b}	109.73 ± 5.17^{b}	133.39 ± 4.48^{b}	127.25 ± 29.03^{b}

Values in the same row with same letter are not significantly different ($p < 0.05$)

* 3-ethoxy-1-propanol

** Ethyl 4-hydroxybutanoate

Figure 4.

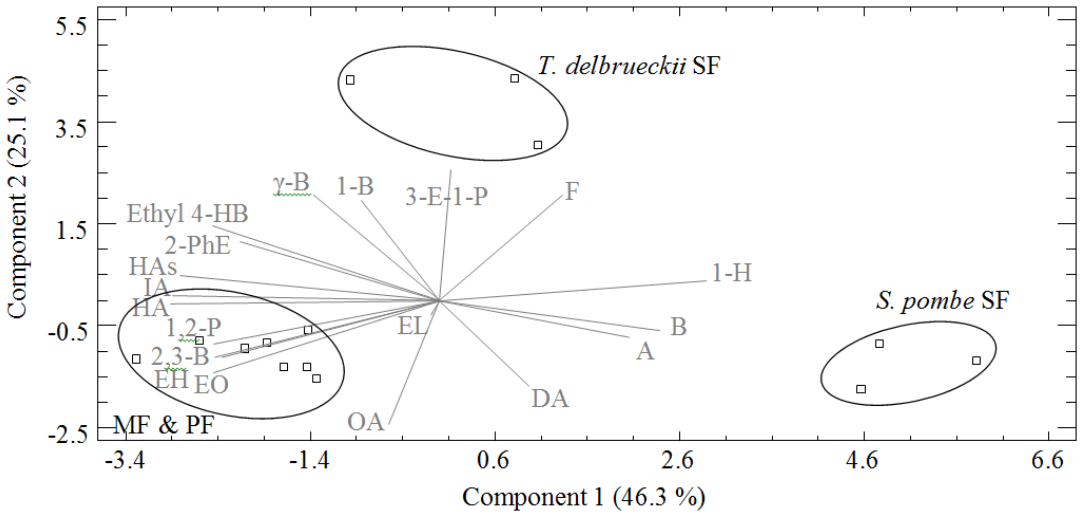

Figure 1. Fermentation kinetics of the mixed and sequential fermentations with *S. pombe* and *T. delbrueckii* strains (measured by the daily loss of CO_2).

Figure 2. Total anthocyanins content (mg/l) in mixed and sequential fermentations with *S. pombe* and *T. delbrueckii* strains. Mean ± SD (n=3). Bars with the same letter are not significantly different ($p<0.05$).

Figure 3. Vitisins (A) and vinylphenolic pyranoanthocyanins (B) content (mg/l) in mixed and sequential fermentations with *S. pombe* and *T. delbrueckii* strains. Mean ± SD (n=3). Bars with the same letter are not significantly different ($p<0.05$).

Figure 4. Principal component analysis (PCA) of volatile compounds in wines by GC/MS. Abbreviations: Ethyl 4-HB: Ethyl 4-hydroxybutanoate; 2-PhE: 2-phenylethanol; HAs: Higher alcohols; IA: Isoamyl acetate; HA: Hexyl acetate; 1,2-P: 1,2-Propanediol; 2,3-B: 2,3-Butanediol; EH: Ethyl hexanoate; EO: Ethyl octanoate; 3-E-1-P: 3-ethoxy-1-propanol; 1-B: 1-butanol; γ-B: γ-butyrolactone; F: furaneol; 1-H: 1-hexanol; B: Benzaldehyde; A: Acetoin; DA: Decanoic acid; OA: Octanoic acid; EL: Ethyl lactate.

Highlights

- Use of *Schizosaccharomyces pombe* in sequential fermentation allows increasing the contents of vitisins, specially A type.
- Use of *Torulaspora delbrueckii* in mixed fermentation allows a potential increase of fruity aromas in the wine.
- Mixed fermentations with non-*Saccharomyces* yeasts may increase polyols content.
- 3-ethoxy-1-propanol was found as a compound linked to *T. delbrueckii*'s metabolism.
- Use of *S. pombe* in mixed or sequential fermentations allows tempering of its characteristic high acetic acid synthesis.