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ORIGINAL PAPER

Contribution to the aroma of white wines by controlled *Torulaspora delbrueckii* cultures in association with *Saccharomyces cerevisiae*

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Abstract Although the positive role of non-*Saccharomyces* yeasts on the overall quality of wine is encouraging research into their oenological potential, current knowledge on the topic is still far from satisfactory. This work analyzes the contribution of starter cultures of *Torulaspora delbrueckii*, inoculated sequentially with *Saccharomyces cerevisiae* (multi-starter fermentation), on the fermentation and aromas of two different white style wines, i.e., dry and sweet wines. Chemical analysis of Soave and Chardonnay wines (dry wines) showed that multi-starter fermentation greatly affected the content of several important volatile compounds, including 2-phenylethanol, isoamyl acetate, fatty acid esters, C_4–C_{10} fatty acids and vinylphenols. Moreover, strain-specific contributions have been shown by testing two different *T. delbrueckii* strains. Evidence of the positive impact of *T. delbrueckii* activity on wine quality was also demonstrated in Vino Santo, a sweet wine. Due to its low production of acetic acid, this non-*Saccharomyces* yeast is recommended for the fermentation of high sugar grapes. *T. delbrueckii* also influenced the content of different variety of chemical groups, including lactones. From a sensory perspective, all wines produced by multi-starter fermentation have greater aromatic intensity and complexity than wines resulting from a mono-culture fermentation. These results emphasize the potential of employing *T. delbrueckii,* in association with *S. cerevisiae*, for the production of white wines of different styles with improved and enhanced flavour.

Keywords *Torulaspora delbrueckii* · Multi-starter fermentation · Wine aroma · White wine · Sweet wine

Introduction

In recent years, utilization of non-*Saccharomyces* yeasts has been considered for industrial wine production as several have shown high oenological potential (Ciani and Maccarelli 1998; Romano et al. 2003; Renault et al. 2009; Suarez-Lepe and Morata 2012). Several studies have reported that some species, such as *Torulaspora delbrueckii*, *Lachancea* (*Kluyveromyces) thermotolerans*, *Metschnikowia pulcherrima, Pichia kluyveri*, *Hanseniaspora uvarum, Hansenula anomala*, *Rhodotorula mucilaginosa* and *Candida* spp., when used as a pure culture or in association with *Saccharomyces cerevisiae*, were able to enhance the flavour properties in different types of wine (Anfang et al. 2009; Izquierdo Cañas et al. 2011; Calabretti et al. 2012; Sadoudi et al. 2012; Andorrà et al. 2012; Cordero-Bueso et al. 2013; Gobbi et al. 2013). In particular, the importance of the contribution of non-*Saccharomyces* yeasts to the aromatic complexity of the wine, when used to drive multi-starter fermentations, has been shown (Calabretti et al. 2012; Azzolini et al. 2012; Sadoudi et al. 2012).

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The success of non-*Saccharomyces* yeasts has led to the industrial production of selected cultures for winemaking. Currently, *T. delbrueckii* and *Lachancea thermotolerans* are commercially available as active dry yeasts, *P. kluyveri* as frozen form. *T. delbrueckii* was the first non-*Saccharomyces* species produced for that purpose, about half a century after the introduction of the first *S. cerevisiae* commercial wine starter cultures, and different strains of *T. delbrueckii* are now available for winemaking. The impact of some of these strains on the fermentation and aroma of different wines has been documented (Izquierdo Cañas et al. 2011; Azzolini et al. 2012; Takush and Osborne 2012; van Breda et al. 2013).

Despite the increasing number of studies on non-*Saccharomyces* yeasts, the level of current knowledge on their impact on wine quality is still far from satisfactory. Indeed, the effects of pure and mixed cultures on wine aroma have been often evaluated in conditions (e.g., laboratory-scale trials carried out on synthetic media or sterilized grape musts) very different from those normally found in the winery (Andorrà et al. 2012). Moreover, unlike *S. cerevisiae*, knowledge about strain heterogeneity within a single non-*Saccharomyces* species, like *T. delbrueckii*, and about the impact of strain-specificity on wine aromas is still limited. Some studies underline the importance of intra-species heterogeneity within non-*Saccharomyces* yeasts for wine production (Ciani and Maccarelli 1998; Renault et al. 2009; van Breda et al. 2013). Given the interest in these yeasts from winemakers, and the possibility of widespread use encouraged by the availability of commercial active dry yeasts, further investigation is recommended to evaluate their behaviour under different winery conditions.

The objective of this study was to investigate the effects of *T. delbrueckii*, in association with *S. cerevisiae* (multi-starter fermentation) on the aroma of different types of white wine. Experiments in winery conditions were carried out to produce two dry table wines, one international (Chardonnay) and one local (Italian Soave), and one sweet white wine (Vino Santo, an Italian *passito* wine). Bely et al. (2008) reported that a mixed *T. delbrueckii* and *S. cerevisiae* culture was the best combination of yeasts for improving the analytical profile of sweet wine, particularly in terms of volatile acidity. Moreover, in our previous investigation (Azzolini et al. 2012) the use of mixed cultures of *T. delbrueckii* and *S. cerevisiae* had a relevant impact on the fermentation and aroma of Amarone wine, a dry red *passito* wine. The chemical and sensory profiles of wines obtained by multi-starter fermentation were analyzed and compared to those traditionally produced with *S. cerevisiae* cultures alone or by spontaneous fermentation with indigenous microflora.

Materials and methods

Starter strains

Two strains of *T. delbrueckii* and one of *S. cerevisiae* yeasts, all commercially available, were used in this study. The *T. delbrueckii* strains were Zymaflore® Alpha (Laffort, Bordeaux, France) and BIODIVA® (Lallemand, Montreal, Canada); the *S. cerevisiae* strain was Lalvin EC1118® (Lallemand). In this study, the three strains are called Td1, Td2 and Sc, respectively. These strains were used in the form of active dry yeast according to the manufacturer's instructions.

Vinifications

Three vinifications were carried out to produce the Soave (Verona, Italy) and Chardonnay wines, as the dry white wines, and Vino Santo (Trent, Italy), as the sweet white wine.

The Soave and Chardonnay wines were obtained in the winery through traditional winemaking methods using Garganega and Chardonnay grapes, respectively. After crushing the grapes with 40 mg L^{-1} of SO_2, the juices were separated from the pomace and carefully divided among 150 L steel tanks to obtain homogenous trials each containing 80 L of must. The Garganega juice was pH 3.35, reducing sugars 21.4° Brix, titratable acidity 5.95 g L^{-1} as tartaric acid. The Chardonnay was pH 3.20, reducing sugars 22.5° Brix, titratable acidity 6.72 g L^{-1} as tartaric acid. The musts for the multi-starter fermentations (Td1 + Sc and Td2 + Sc) were inoculated sequentially with *T. delbrueckii* and *S. cerevisiae*, added when the ethanol content was about 3–4 % v v^{-1}. Musts inoculated with *S. cerevisiae* (Sc) only were used as control. *T. delbrueckii* and *S. cerevisiae* strains were inoculated at the concentration approximately of 3–4 × 10^6 and 2–3 × 10^6 cfu mL^{-1}, respectively. Alcoholic fermentation (AF) was carried out in a cellar room without temperature controls ranging from 16 to 20 °C.

To produce the Vino Santo wine, at laboratory level, withered Nosiola grapes were crushed and sulphited (SO_2 50 mg L^{-1}), then the resulting juice (pH 3.87, reducing sugars 42.7° Brix, titratable acidity 8.38 g L^{-1} as tartaric acid) was divided into trial lots of 500 mL each. Multi-starter fermentation (Td2 + Sc) was induced by adding the *T. delbrueckii* strain Td2, then *S. cerevisiae* when the weight loss corresponded to theoretical ethanol content of about 3 % v v^{-1}. The other trial batches were inoculated with *S. cerevisiae* only (Sc) or not inoculated at all (Spn), in which fermentation was carried out by spontaneous indigenous microflora. AF was carried out in a cellar room without temperature controls ranging from 14 to 17 °C, and

was stopped in order to have wines with ethanol and residual sugars in compliance with the regulations for Vino Santo wine production.

All three trials were carried out in triplicate. After fermentation, the wines were removed from the containers and allowed to settle for 2 days through natural sedimentation.

Isolation and identification of yeasts

Grape juices and fermenting juice samples were collected to count the yeasts by plate counting on WL agar (Fluka, Seelze, Germany) and lysine agar medium (Oxoid Ltd., London, UK). Colonies were isolated (about 50 for each trial) according to their morphology (texture, surface, margin, elevation and colour) and frequencies. Analysis of cell morphology was performed using a phase-contrast microscopy at a magnification of ×1,000. All isolates were preserved on YPD agar (1 % w v^{-1} yeast extract, 2 % w v^{-1} glucose, 2 % w v^{-1} peptone and 1.5 % w v^{-1} agar), stored at 4 °C and also kept at −80 °C with 25 % w v^{-1} glycerol. Yeast identification was carried out by ITS-RFLP analysis. The 5.8S-ITS region was amplified using ITS1 and ITS4 primers (White et al. 1990) and the PCR conditions were identical to those reported by to Esteve-Zarzoso et al. (1999). Microsatellite multiplex PCR was carried out according to Vaudano and Garcia-Moruno (2008) to discriminate strains among *S. cerevisiae* isolates. PCR product (ITS and microsatellite) and ITS restriction fragments were separated by electrophoresis on 2.0 % w v^{-1} agarose gels with 1 × TAE buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA, pH 8) strained with ethidium bromide.

Analysis of must and wine

Ethanol was analyzed by near-infrared (NIR) spectroscopy using an Alcolyzer Wine Analysis System (Anton Paar GmbH, Graz, Austria). Sugar content and titratable acidity were determined according to standard analysis methods. Acetic acid was quantified using an enzyme kit (La Roche, Basel, Switzerland).

Analysis of the volatile composition of wines was been performed using gas chromatography-mass spectrometry (GC–MS) Solid Phase Extraction (SPE), as described by Tosi et al. (2013).

SPE was performed to quantitatively extract the volatile compounds with an automated Aspec™ XL solid phase extraction apparatus (Gilson Inc., Middleton, WI, U.S.) using Isolute® ENV + 1 g cartridges (IST Ltd., Mid Glamorgan, U.K.). The free volatile compounds were eluted with 9 mL of dichloromethane. The solution was dried with Na_2SO_4 and concentrated to 0.4 mL with a gentle nitrogen flow.

The GC–MS analysis was performed with an Agilent 6890 N Network Gas Chromatography system coupled with a 5978B inert XL EI/CI MS (Agilent Technologies, Milan, Italy), equipped with a HP-WAX Bonded PEG fused silica capillary column (60 m × 320 µm i.d. × 0.25 µm film thickness; Agilent Technologies). The MS conditions included electron impact energy 70 eV, and MS source temperature 230 °C. The temperatures of the transfer line and GC injector temperature were 200 and 250 °C respectively, and helium was used as the carrier (flow: 1.5 mL min^{-1}). The column temperature program was 50 °C for 4 min, increased to 240 °C at 4 °C min^{-1}, then 240 °C for 16 min.

Identification of volatile compounds was achieved by means of pure reference standard (Sigma-Aldrich, St. Louis, MO, U.S.) co-injections; when the authentic standard were not available the identification was based on the comparison with the spectral data of the NIST library.

Quantitative analysis was performed by total ion chromatograph using the response factors calculated for each compound by calibration curves in a hydroalcoholic solution, containing 12 % v v^{-1} ethanol with 5 g L^{-1} tartaric acid, like a model wine. As for compounds for which commercial standards were not available, the response factors of compounds with similar chemical structures were utilized.

The odour activity values (OAV) were calculated as the ratio between the measured quantitative concentration of a substance in the wine and its odour threshold, when available.

Sensory analysis

The Soave and Chardonnay wines were tested by a panel of six judges in one session using a total of six olfactive-gustative descriptors (aroma intensity, complexity, floral, green apple, tropical fruit, and ripe fruit) and six mouthfeel attributes (acidity, bitterness, herbaceous, persistence, freshness and body). A number from 0 to 10 was assigned to each one and the average of the two repetitions was used as final score.

Statistical treatment of data

Student *t* test was used for the wine compounds and sensory scores to evaluate the differences in the samples. Principal component analysis (PCA) was performed on volatile compounds determined by the SPE/GC–MS of all the wines using PAST software package (version 1.89).

Results

Fermentation of dry table wines and starter implantation analysis with *Torulaspora delbrueckii*

The course of fermentation was similar in both vinifications depending on the type of inoculation and *T. delbrueckii* strain

Table 1 Identification of yeasts by colony morphology and ITS-RFLP analysis isolated from grape musts and fermenting wines during Soave (S1–S29), Chardonnay (C1–C26) and Vino Santo (VS1–VS33) vinification

Strain	Colony morphology[1]		ITS[2]	ITS-RFLP			Species[3]
	Color	Topography		*Hae*III	*Hinf*I	*Cfo*I	
S1	Red	Convex, smooth	600	600	290 + 130 + 120 + 50	300 + 210 + 100	Unidentified
S2	Cream with red halo	Convex, smooth	390	280 + 100	200 + 180	200 + 100 + 90	*Metschnikowia pulcherrima*
S3	Dark green	Flat, smooth	760	760	350 + 200 + 180	330 + 310 + 100	*Hanseniaspora uvarum*
S4	White	Convex, smooth	650	430 + 170 + 130	320 + 320	310–290 + 50	Unidentified
S5	Cream to light green	Umbonate, smooth	880	310 + 220 + 180 + 150	350 + 150	385 + 365	*Saccharomyces cerevisiae*
S6–20	Cream to light green	Flat, smooth	800	800	410 + 380	330 + 220 + 150 + 100	*Torulaspora delbrueckii*
S21–29	Cream	Umbonate, smooth	880	310 + 220 + 180 + 150	350 + 150	385 + 365	*Saccharomyces cerevisiae*
C1	Cream with red halo	Convex, smooth	390	280 + 100	200 + 180	200 + 100 + 90	*Metschnikowia pulcherrima*
C2	Dark green	Convex, smooth	480	400 + 80	230 + 130 + 120	200 + 170 + 70 + 50	Unidentified
C3	Dark green	Flat, smooth	760	760	350 + 200 + 180	330 + 310 + 100	*Hanseniaspora uvarum*
C4	Cream to light green	Knoblike, smooth	880	310 + 220 + 180 + 150	350 + 150	385 + 365	*Saccharomyces cerevisiae*
C5–16	Cream to light green	Flat, smooth	800	800	410 + 380	330 + 220 + 150 + 100	*Torulaspora delbrueckii*
C17–26	Cream	Umbonate, smooth	880	310 + 220 + 180 + 150	350 + 150	385 + 365	*Saccharomyces cerevisiae*
VS1	Intense green	Flat, smooth	760	760	350 + 200 + 180	330 + 310 + 100	*Hanseniaspora uvarum*
VS2	Green	Flat, smooth	700	410 + 195 + 90	390 + 300	310 + 200 + 100 + 90	Unidentified
VS3	White to cream	Convex, smooth	790	700 + 80	340 + 230 + 170 + 50	310 + 290 + 100 + 90	*Zygosaccharomyces bailii*
VS4	Cream	Convex, irregular	750	400 + 200 + 100	340 + 250 + 130	270 + 200 + 180 + 100	*Zygosaccharomyces rouxi*
VS5	Cream with red halo	Convex, smooth	390	280 + 100	200 + 180	200 + 100 + 90	*Metschnikowia pulcherrima*
VS6	Cream to green	Convex, smooth	630	390 + 120 + 90	320 + 300	300 + 270 + 60	*Pichia guilliermondii*
VS7	Dark green	Convex, smooth	480	450	240 + 220	480	Unidentified
VS8	Light green	Knoblike, smooth	880	490 + 230 + 140	350 + 150	385 + 365	*Saccharomyces pastorianus*
VS9	Cream to light green	Umbonate, smooth	880	310 + 220 + 180 + 150	350 + 130	385 + 365	*Saccharomyces cerevisiae*
VS10	Light green	Convex, smooth	450	230 + 150 + 50	210 + 200 + 40	nd	Unidentified
VS11–25	Cream to light green	Convex, smooth	800	800	410 + 380	330 + 220 + 150 + 100	*Torulaspora delbrueckii*
VS26	Cream to light green	Umbonate, smooth	880	310 + 220 + 180 + 150	350 + 150	385 + 365	*Saccharomyces cerevisiae*
VS27–32	Cream	Umbonate, smooth	880	310 + 220 + 180 + 150	350 + 150	385 + 365	*Saccharomyces cerevisiae*
VS33	Cream to green	Umbonate, smooth	880	310 + 220 + 180 + 150	350 + 150	385 + 365	*Saccharomyces cerevisiae*

[1] Description of yeast colonies on WL medium

[2] Band size obtained by PCR amplification

[3] Species assignation according to Esteve-Zarzoso et al. (1999)

nd Not determined

used (Supplementary Table S1). Ethanol production was significantly faster in control trials than in the musts inoculated with both yeasts; among the latter, the Td2 + Sc wines produced higher ethanol levels than did the Td1 + Sc wines (Supplementary Figure S1).

Spontaneous microflora in both grape musts was composed by different yeasts such as *H. uvarum*, *S. cerevisiae*, *M. pulcherrima*, and other unidentified species (Table 1). The former two species were the most frequent and the total concentration of all wild yeasts was approximately 3.0×10^4 and 5.8×10^3 cfu mL^{-1} in Soave and Chardonnay must, respectively.

Representative *S. cerevisiae* isolated from both grape juices displayed different microsatellite profiles and were also distinguishable from EC1118 strain profile (Supplementary Figure S2). Identification of isolates collected during the vinifications was carried out to ascertain the population dynamics of indigenous yeasts and the colonization of *T. delbrueckii* and *S. cerevisiae* starter strains during AF. The evolution of cell populations in both types of wine was similar and *T. delbrueckii* was able to dominate rapidly the fermentation. No wild yeasts were further detected after the starter inoculation. The different profile of colonies in WL plate and different cell morphology between *T. delbrueckii* and *S. cerevisiae* allowed to carry out a reliable quantitative analysis of the two starter strain populations. Usually, the former had flat colony or lightly convex, while the latter had clearly convex or umbonate colony elevation (Table 1). At the optical microscopy *T. delbrueckii* cells appeared rounded, small with a large vacuole, while those of *S. cerevisiae* were ellipsoidal and bigger with small vacuoles (data not shown). The populations of the Td1 and Td2 strains, inoculated at the concentration of $2–4 \times 10^6$ cfu mL^{-1}, increased rapidly and after 4 days the concentration was approximately $7–8 \times 10^7$ cfu mL^{-1}. The inoculation of *S. cerevisiae* was carried out at the 5th and 6th day, when ethanol was about 3 % v v^{-1}, in Td2 + Sc and Td1 + Sc, respectively (Supplementary Figure S1). Microsatellite multiplex PCR analysis of representative yeasts isolated after the starter inoculation confirmed that *S. cerevisiae* population was composed mainly by EC1118 strain. Indeed, all isolates identified as *S. cerevisiae* (S21–29 and C17–26, Table 1) displayed a microsatellite profile similar to that of EC1118 (Supplementary Figure S2). *S. cerevisiae* colonized rapidly the fermenting must and its population increased, progressively overwhelming *T. delbrueckii* population. Nevertheless, *T. delbrueckii* cells remained at relatively high concentrations (about $2–3 \times 10^6$ cfu mL^{-1}) up to the end of AF, a cell density similar to that of *S. cerevisiae* population. Figure 1 shows the percentage of *T. delbrueckii* estimated during the AF in all sequentially inoculated trials with respect to total yeast concentration, mainly composed by *S. cerevisiae*.

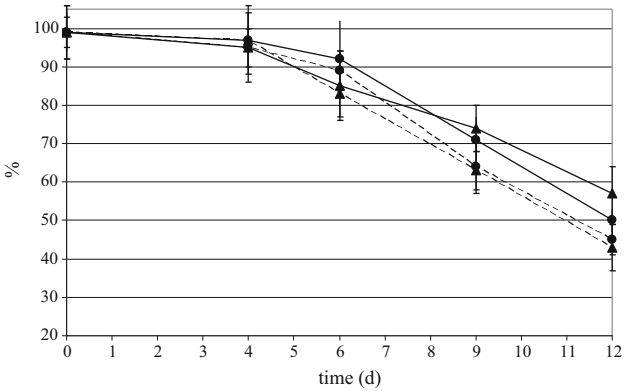

Fig. 1 Percentage of *T. delbrueckii* during the alcoholic fermentation for Soave (*full line*) and Chardonnay (*dashed line*) wine production by the sequential inoculation of *T. delbrueckii* and *S. cerevisiae* (Td1 + Sc and Td2 + Sc, *circle* and *triangle* symbol respectively). *Bars* are standard deviation of three independent trials

Chemical analysis of dry table wines

The volatile composition of wines produced by multi-starters differed from the control wines due to variations in the content of several molecules that greatly impact to wine aroma (Table 2). Interesting differences were also observed between wines inoculated with the Td1 and Td2 strains.

The content of several alcohols changed in one or both type of wines and relevant modifications involved benzyl alcohol, furfuryl alcohol, 1-octen-3-ol, vanillic alcohol, methionol (3-methylthio-1-propanol) and 2-phenylethanol. The increase of methionol (potato note) was particularly important in Chardonnay wine as it was detected above or near the threshold (1 mg L^{-1}; Ferreira et al. 2000). The OAV was 1.5 and 1.0 in the wine fermented with the Td1 and Td2 strains, respectively. High OAV (3–4) were also observed for 2-phenylethanol (rose note) in both wines fermented with *T. delbrueckii*.

Esters and fatty acids were the chemical groups that underwent very consistent modifications related to the presence of *T. delbrueckii* during the fermentation. Ester acetates clearly decreased in wines that were sequentially inoculated with mixed cultures, especially due to the reduction in isoamyl acetate (banana note), detected at levels much higher than its threshold (30 μg L^{-1}; Ferreira et al. 2000). Also, esters of C_4–C_{10} fatty acids, responsible for the fruity scent, strongly decreased. Ethyl octanoate was detected at concentrations fourfold to fivefold lower in the wines inoculated with *T. delbrueckii* than in the control wines, while ethyl decanoate was found below the threshold (200 μg L^{-1}; Ferreira et al. 2000). The OAV was <1.0 in the Td1 + Sc Soave wine and in the Td1 + Sc and Td2 + Sc Chardonnay wines.

Table 2 Composition of Soave and Chardonnay wines produced with multi-starter fermentation using *T. delbrueckii* and *S. cerevisiae* yeasts (Td1 + Sc and Td2 + Sc) and with a monoculture of *S. cerevisiae* (Sc)

	Soave			Chardonnay		
	Sc	Td1 + Sc	Td2 + Sc	Sc	Td1 + Sc	Td2 + Sc
Ethanol (% v v^{-1})	12.1 b[1] ± 0.0	11.9 a ± 0.0	12.1 b ± 0.0	12.9 b ± 0.0	12.7 a ± 0.1	12.9 b ± 0.0
pH	3.6 ± 0.0	3.6 ± 0.0	3.6 ± 0.0	3.2 ± 0.0	3.2 ± 0.0	3.2 ± 0.0
Residual sugars (g L^{-1})	1.4 ± 0.1	1.5 ± 0.4	1.4 ± 0.1	2.0 ± 0.0	2.5 ± 0.5	2.1 ± 0.0
Acetic acid (g L^{-1})	0.27 a ± 0.01	0.34 b ± 0.03	0.35 b ± 0.01	0.42 c ± 0.01	0.36 b ± 0.02	0.25 a ± 0.02
Titratable acidity (g L^{-1})[2]	4.5 ± 0.1	5.3 ± 0.3	4.6 ± 0.1	7.0 b ± 0.0	7.2 b ± 0.1	6.7 a ± 0.0
1-Hexanol[3]	595.6 ± 26.0	613.7 ± 18.7	658.0 ± 30.6	1575.9 a ± 15.7	1857.9 b ± 22.0	2014.2 b ± 81.7
trans-3-Hexen-1-ol	33.8 a ± 0.9	39.7 b ± 1.5	36.8 ab ± 3.2	28.9 ± 1.5	28.6 ± 1.4	27.6 ± 2.1
cis-3-Hexen-1-ol	9.7 ± 1.1	11.2 ± 0.7	10.2 ± 1.0	25.8 a ± 1.2	40.1 c ± 2.2	35.0 b ± 1.6
2-Hexen-1-ol	4.2 a ± 0.3	5.4 b ± 0.3	4.5 ab ± 0.4	3.2 ± 0.2	3.2 ± 0.2	3.5 ± 0.1
Benzyl alcohol	117.9 a ± 11.4	231.7 b ± 16.1	142.9 a ± 23.9	100.0 b ± 2.2	87.4 a ± 3.6	89.7 a ± 3.3
3-Methylthio-1-propanol	1480.4 ± 138.5	1719.3 ± 576.4	1549.2 ± 27.7	878.6 a ± 17.3	1527.7 b ± 7.3	989.8 ab ± 328.5
Furfuryl alcohol	10.8 b ± 0.6	7.1 a ± 1.5	7.5 a ± 0.7	10.4 a ± 0.3	17.5 b ± 1.2	14.0 ab ± 5.7
Homovanillic alcohol	127.6 ± 4.9	111.0 ± 16.4	131.2 ± 21.8	47.9 b ± 1.8	39.5 a ± 1.1	51.5 b ± 2.0
1-Octen-3-ol	1.7 a ± 0.1	3.7 c ± 0.4	2.6 b ± 0.1	1.7 a ± 0.5	3.3 b ± 0.3	2.9 b ± 0.3
Vanillic alcohol	6.7 ± 0.7	7.2 ± 1.7	10.4 ± 2.2	5.1 a ± 0.1	6.4 b ± 0.5	9.7 c ± 0.8
2-Phenylethyl alcohol	37197.0 a ± 989.8	60309.5 c ± 3999.8	45604.6 b ± 360460	36443.2 a ± 884.8	57035.1 b ± 2342.8	41586.8 b ± 1644.0
Alcohols[4]	39585.4 a ± 960.4	63059.5 c ± 4592.6	48157.3 b ± 3663.9	39120.6 a ± 876.1	60646.6 c ± 2353.0	44824.9 b ± 403.2
Hexyl acetate	141.0 a ± 5.8	25.5 b ± 19.3	39.8 b ± 14.8	247.6 b ± 19.2	33.1 a ± 3.3	72.8 a ± 38.5
Isoamyl acetate	1869.8 b ± 92.4	988.4 a ± 232.0	639.6 a ± 200.7	1338.7 b ± 83.0	748.2 a ± 72.0	624.2 a ± 175.7
Phenylethyl acetate	357.5 ± 27.8	487.8 ± 112.2	339.5 ± 105.9	265.9 b ± 4.0	207.7 a ± 4.4	212.2 ab ± 52.9
Ethylphenyl acetate	2.7 b ± 0.2	2.9 b ± 0.3	1.7 a ± 0.1	3.1 b ± 0.2	2.6 a ± 0.1	2.2 a ± 0.3
Ethyl butyrate	126.1 b ± 14.8	83.1 a ± 17.0	87.1 a ± 8.4	110.2 b ± 8.6	80.8 a ± 1.7	81.6 a ± 12.5
Ethyl hexanoate	589.1 c ± 43.5	139.6 a ± 49.7	268.7 b ± 35.0	547.6 b ± 36.0	195.2 a ± 4.8	258.8 a ± 58.4
Ethyl octanoate	1048.7 c ± 103.0	233.2 a ± 17.0	317.9 b ± 28.3	836.5 b ± 37.9	147.2 a ± 3.2	189.4 a ± 46.3
Ethyl decanoate	269.1 a ± 21.2	79.0 b ± 4.6	280.7 a ± 23.9	264.3 c ± 3.4	77.6 a ± 7.8	167.7 b ± 28.6
Ethyl 9-decenoate	24.4 a ± 1.1	28.1 b ± 0.8	44.1 c ± 0.9	21.9 a ± 1.8	19.4 a ± 1.0	30.8 b ± 2.1
Ethyl 3-hydroxybutyrate	314.1 ± 17.3	288.3 ± 32.6	229.2 ± 76.5	289.7 b ± 5.3	131.1 a ± 2.1	224.1 ab ± 65.1
Ethyl 4- hydroxybutyrate	3849.9 b ± 128.6	2216.5 a ± 457.9	1845.1 a ± 398.2	2752.5 b ± 37.8	1523.2 a ± 48.1	1180.8 a ± 224.1
Ethyl 2-hydroxyisovalerate	1.5 a ± 0.2	36.0 c ± 1.3	6.0 b ± 0.4	2.4 a ± 0.2	7.3 b ± 0.4	7.3 b ± 0.8
Ethyl 2-hydroxy-4-methylpentanoate	19.6 a ± 1.6	52.3 b ± 3.3	15.0 a ± 2.5	27.5 b ± 0.9	23.3 b ± 2.1	17.6 a ± 1.4
Diethyl succinate	369.0 a ± 11.5	4649.6 b ± 1606.8	846.2 a ± 300.9	545.7 a ± 19.4	852.2 b ± 17.4	579.6 a ± 37.5

Table 2 continued

	Soave			Chardonnay		
	Sc	Td1 + Sc	Td2 + Sc	Sc	Td1 + Sc	Td2 + Sc
Ethyl lactate	6598.5 ± 391.5	15816.7 ± 8535.2	8004.2 ± 784.3	3914.9 b ± 53.6	4513.2 c ± 225.4	3193.4 a ± 68.3
Isoamyl lactate	35.3 a ± 1.9	177.4 c ± 23.9	84.8 b ± 6.7	14.7 a ± 0.8	40.3 b ± 1.1	16.1 a ± 0.8
Ethyl-isoamyl succinate	5.9 a ± 0.6	70.2 c ± 18.0	21.1 b ± 5.9	10.7 a ± 1.8	19.0 b ± 1.3	7.3 a ± 1.4
Diethyl malate	280.9 c ± 35.9	29.5 a ± 3.5	40.3 b ± 0.5	1053.3 b ± 27.1	903.1 a ± 17.4	606.2 ab ± 189.3
Diethyl 2-hydroxyglutarate	33.9 ab ± 1.0	31.9 a ± 9.0	54.6 b ± 11.2	79.7 b ± 2.7	68.2 a ± 1.5	77.9 ab ± 12.0
Ethyl vanillate	1.7 ± 0.2	1.4 ± 0.1	1.7 ± 0.2	1.2 ± 0.1	1.0 ± 0.2	1.3 ± 0.2
Methyl vanillate	3.6 b ± 0.3	2.7 a ± 0.3	2.8 a ± 0.4	5.6 ± 0.1	5.8 ± 0.3	5.9 ± 0.5
Ethyl cinnamate	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
Ethyl salicylate	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
Methyl salicylate	3.2 ± 0.3	3.4 ± 0.2	4.1 ± 1.2	13.5 b ± 0.5	8.6 a ± 0.9	10.1 a ± 0.3
Esters	15945.5 ± 458.2	25577.0 ± 9157.1	13418.3 ± 1566.7	12347.4 b ± 119.6	9608.2 a ± 84.4	7933.6 a ± 1065.7
Butyric acid	336.6 ab ± 42.0	361.2 b ± 20.3	297.2 a ± 12.7	319.8 b ± 1.8	268.6 a ± 8.8	255.6 a ± 19.0
Isovalerianic acid	237.5 a ± 17.2	337.8 a ± 45.2	178.1 b ± 19.6	212.2 ± 4.1	231.6 ± 57.6	159.3 ± 27.2
Hexanoic acid	1958.4 b ± 209.1	779.6 a ± 46.3	789.0 a ± 107.3	1792.6 b ± 56.9	651.9 a ± 15.5	648.9 a ± 18.2
Octanoic acid	4328.0 b ± 366.0	1089.6 a ± 83.9	1221.8 a ± 181.2	3707.5 b ± 58.0	500.1 a ± 23.9	503.6 a ± 210.3
Decanoic acid	1200.9 b ± 188.0	342.6 a ± 42.3	1151.5 b ± 140.4	1183.6 c ± 27.5	182.3 a ± 17.4	596.9 b ± 33.5
Fatty acids	8061.4 b ± 814.5	2910.8 a ± 147.0	3637.5 a ± 433.5	7215.7 b ± 65.7	1834.5 a ± 38.9	2164.1 a ± 152.5
Linalool	4.4 ab ± 0.4	4.0 b ± 0.1	3.6 a ± 0.2	9.5 ± 0.6	10.8 ± 0.1	10.5 ± 0.4
α-Terpineol	1.9 ± 0.4	2.5 ± 0.2	2.1 ± 0.1	5.4 a ± 0.2	7.2 b ± 0.1	5.5 a ± 0.5
Citronellol	3.1 ± 0.3	3.2 ± 0.6	2.7 ± 0.4	2.1 a ± 0.5	4.5 b ± 0.5	1.6 a ± 0.2
Nerol	1.5 ± 0.8	2.9 ± 1.0	1.4 ± 0.2	1.9 b ± 0.1	1.4 a ± 0.1	2.4 ab ± 1.0
Geraniol	3.9 ± 0.6	5.6 ± 0.8	4.4 ± 0.6	3.6 ± 0.1	3.8 ± 0.8	4.6 ± 0.8
trans-Furanic linalool oxide A	<1.0	<1.0	<1.0	4.1 ± 0.1	4.3 ± 0.3	4.4 ± 0.4
cis-Furanic linalool oxide B	<1.0	<1.0	<1.0	2.5 ± 0.1	2.3 ± 0.1	2.6 ± 0.2
trans-Pyranic linalool oxide C	2.5 ± 0.4	2.7 ± 0.4	2.5 ± 0.1	1.9 b ± 0.1	<1.0 a	1.3 ab ± 0.2
cis-Pyranic linalool oxide D	<1.0	1.3 ± 0.5	<1.0	1.0 ± 0.0	<1.0	1.0 ± 0.1
ho-Diendiol 1	12.4 ± 1.5	11.5 ± 0.4	12.6 ± 0.6	6.0 ± 0.2	6.3 ± 0.1	5.1 ± 0.3
ho-Diendiolo 2	<1.0	1.1 ± 0.3	<1.0	2.1 b ± 0.3	<1.0 a	<1.0 a
Endiol	1.8 b ± 0.3	<1.0 a	<1.0 a	15.0 ab ± 0.7	16.7 b ± 1.1	13.6 a ± 0.5
trans-8-dihydroxylinalool	7.6 b ± 1.6	<1.0 a	2.2 a ± 1.5	<1.0	<1.0	<1.0
cis-8-dihydroxylinalool	8.0 b ± 1.6	2.0 a ± 1.2	3.1 a ± 0.4	6.2 ab ± 3.2	8.9 b ± 0.1	<1.0 a
4-Terpineol	1.0 ± 0.2	1.0 ± 0.0	<1.0	<1.0	<1.0	<1.0

Table 2 continued

	Soave			Chardonnay		
	Sc	Td1 + Sc	Td2 + Sc	Sc	Td1 + Sc	Td2 + Sc
Monoterpenes	51.3 ± 7.2	40.7 ± 0.4	39.4 ± 2.8	63.0 ab ± 4.8	70.2 b ± 2.5	55.9 a ± 1.8
β-Damascenone	6.6 ± 0.1	4.7 ± 1.2	6.9 ± 0.9	6.1 ± 0.2	6.0 ± 0.3	5.9 ± 0.4
Actinidol 1	1.5 b ± 0.2	1.1 a ± 0.2	<1.0 a	2.3 a ± 0.2	2.9 b ± 0.2	2.1 a ± 0.2
Actinidol 2	3.8 b ± 0.5	2.6 ab ± 0.1	2.4 a ± 0.1	6.0 ± 0.0	6.2 ± 0.2	5.2 ± 0.6
3-Oxo-α-ionol	33.6 ± 2.4	35.5 ± 3.1	42.8 ± 6.4	36.7 a ± 1.6	44.1 b ± 1.1	51.2 c ± 2.5
Norisprenoids	45.4 ± 3.1	43.8 ± 4.3	52.8 ± 7.2	51.1 a ± 1.6	59.2 b ± 1.0	64.4 c ± 1.4
Phenylacetaldehyde	2.7 ± 0.2	4.4 ± 1.3	5.6 ± 1.6	2.6 a ± 0.3	5.5 b ± 0.2	4.8 ab ± 2.4
Benzaldehyde	12.7 a ± 2.9	25.1 b ± 0.9	14.5 a ± 0.6	10.3 ± 1.4	7.8 ± 0.3	6.5 ± 1.9
Furfurol	3.2 ± 0.2	4.0 ± 1.4	4.5 ± 0.8	9.9 b ± 0.3	5.7 a ± 0.7	4.1 a ± 0.1
Syringaldehyde	1.4 ab ± 0.3	1.2 b ± 0.1	<1.0 a	1.8 ± 0.5	1.0 ± 0.2	1.2 ± 0.5
5-Methylfurfural	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
Furaneol	5.6 ± 0.5	4.6 ± 0.9	6.3 ± 0.9	4.3 ± 0.5	4.7 ± 0.8	3.7 ± 1.9
Homo-furaneol	5.1 a ± 0.7	8.8 b ± 0.4	18.3 c ± 2.2	2.7 ± 0.7	3.3 ± 0.5	3.0 ± 0.8
Nor-furaneol	96.2 a ± 11.4	103.6 a ± 11.5	137.3 b ± 15.6	27.5 ± 9.4	31.0 ± 3.3	29.7 ± 20.2
Carbonyl compounds	126.9 a ± 10.4	151.7 ab ± 12.8	187.4 b ± 16.5	59.2 ± 7.3	59.0 ± 3.7	53.0 ± 23.2
4-Ethylphenol	<1.0	<1.0	2.8 ± 0.9	<1.0	<1.0	<1.0
4-Ethylguaiacol	<1.0 a	2.7 b ± 0.1	3.2 ab ± 1.4	<1.0	<1.0	<1.0
4-Vinylguaiacol	136.4 b ± 2.0	49.3 a ± 6.5	97.6 ab ± 31.1	85.4 b ± 3.8	20.5 a ± 0.8	13.4 a ± 8.4
4-Vinylphenol	89.3 b ± 5.7	43.1 a ± 11.9	92.6 ab ± 28.0	25.3 b ± 2.6	9.9 a ± 1.0	7.3 a ± 4.0
Eugenol	<1.0	1.1 ± 0.1	<1.0	2.3 ± 0.1	2.4 ± 0.1	1.7 ± 0.5
o-Cresol	1.1 b ± 0.1	1.4 b ± 0.1	<1.0 a	<1.0	<1.0	<1.0
p-Cresol	1.9 ± 1.3	4.0 ± 1.3	1.9 ± 0.2	2.0 ab ± 0.4	1.9 b ± 0.1	1.3 a ± 0.2
Vanillin	3.7 b ± 1.0	2.7 b ± 0.5	1.3 a ± 0.3	3.0 c ± 0.1	1.5 a ± 0.2	2.0 b ± 0.2
Acetovanillone	116.2 b ± 7.5	99.2 a ± 8.3	102.3 ab ± 14.9	92.2 ± 2.5	92.5 ± 3.0	92.4 ± 3.8
Phenol	3.4 b ± 0.2	3.9 b ± 0.3	2.5 a ± 0.3	2.4 ± 0.1	2.1 ± 0.2	3.8 ± 2.2
Homovanillic acid	2.8 ± 0.4	2.3 ± 0.4	2.5 ± 0.4	1.5 b ± 0.2	<1.0 a	1.3 ab ± 0.4
Volatile phenols	357.4 b ± 4.4	210.3 a ± 25.4	337.3 ab ± 87.7	216.5 b ± 7.9	134.0 a ± 2.6	125.5 a ± 11.5
Ethyl pyroglutamate	296.1 b ± 26.0	146.9 a ± 15.3	162.4 a ± 12.6	663.0 c ± 6.6	588.0 b ± 3.3	455.7 c ± 17.6
γ-Nonalactone	6.1 ± 0.3	5.6 ± 0.2	6.7 ± 1.0	3.8 ± 0.2	3.9 ± 0.3	3.5 ± 1.0
γ-Butyrolactone	874.4 b ± 49.1	1188.1 c ± 77.4	629.0 a ± 61.9	728.5 c ± 7.4	560.8 b ± 11.1	486.9 a ± 4.9
4-Carboxyethoxy-*γ*-butyrolactone	235.4 b ± 12.2	116.4 a ± 21.2	153.1 a ± 6.3	408.7 ± 13.9	383.1 ± 5.3	435.7 ± 81.7
Lactones	1411.9 b ± 84.5	1457.1 b ± 82.1	951.2 a ± 68.6	1804.0 b ± 26.0	1535.7 a ± 8.3	1381.8 a ± 95.6

Table 2 continued

	Soave			Chardonnay		
	Sc	Td1 + Sc	Td2 + Sc	Sc	Td1 + Sc	Td2 + Sc
N-(3-methylbutyl)-acetamide	836.1 b ± 48.6	225.0 a ± 158.9	64.7 a ± 2.4	128.5 b ± 1.9	36.6 a ± 5.2	26.8 a ± 0.3

Values (±standard deviation) are the mean of three independent trials

[1] Values with different letter are significantly different for $p < 0.05$

[2] As tartaric acid

[3] Expressed as μg L^{-1}

[4] Obtained by summing up all molecules of same chemical group (alcohols, esters, fatty acids, monoterpenes, norisoprenoids, carbonylic compounds, volatile phenols and lactones)

Other noteworthy variations involved esters of hydroxy-acids, lactic acid, succinic acid and malic acid, compounds that have high sensory thresholds (20–200 mg L^{-1}; Campo et al. 2006).

The decrease in fatty acids (cheese, butter and rancid notes) was particularly marked in both types of wine inoculated with *T. delbrueckii*, as the total amount was two to four folds lower than in the control wines. Greater differences were observed for hexanoic and octanoic acid, the most abundant fatty acids. Decanoic acid was the only one that decreased under its threshold (1 mg L^{-1}; Ferreira et al. 2000). The OAV was <1.0 in wines inoculated with the Td1 and in Td2 + Sc Chardonnay wine.

The carbonylic compounds involved in consistent modifications were phenylacetaldehyde, benzaldehyde, furfural, homo- and nor-furaneol, most of which occurred in the Soave wines.

T. delbrueckii also affects the content of some volatile phenols, like 4-vinylguaiacol and 4-vinylphenol, which decreased in the Td1 + Sc Soave wines and in the Chardonnay wines inoculated with both non-*Saccharomyces* strains. In the latter wines, 4-vinylguaiacol (pharmaceutical note) was detected below its threshold (40 μg L^{-1}; Guth 1997). The OAV was <1.0, while in the control wine the OAV was 2.1.

PCA was applied to volatile compound data (Table 2) to discriminate the wines. The wines of both vinifications were clearly distinct from each other, including those inoculated with the two different *T. delbrueckii* strains (Fig. 2). About 50 % of compounds used to PCA analysis had high PC1 and PC2 loading values (<−8.0 and >8.0) in both types of wines (Supplementary Table S2). In general, compounds that described the wines inoculated with *T. delbrueckii* were different between Soave and Chardonnay wines, while both Sc wines were well described mainly by C_4–C_{10} fatty acids and their esters.

The sensory properties of the Soave and Chardonnay wines produced by *T. delbrueckii* and *S. cerevisiae* were distinct from the related controls (Supplementary Figure S3). In both wines those inoculated with *T. delbrueckii* were significantly distinct ($p < 0.05$) for the descriptors of freshness and acidity, which scored lower than in Sc wines, as well as for flavour intensity, complexity and persistence, which were higher than in Sc. According to the judges' comments, floral and tropical fruits contributed most to the complexity of the wines inoculated with non-*Saccharomyces* yeast, despite these two descriptors did not differ significantly among the wines. In both types of wine, no significant differences resulted in the wines produced with the two different non-*Saccharomyces* strains.

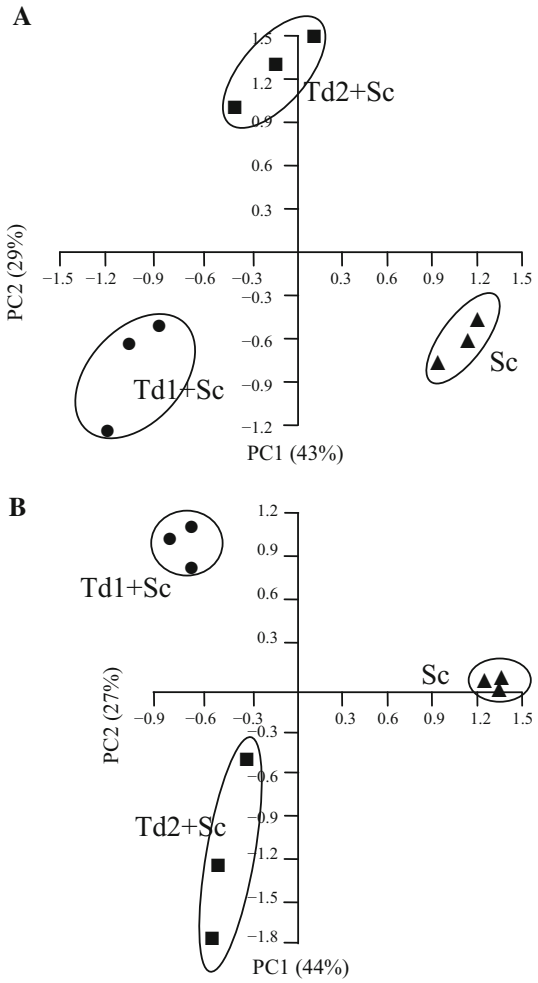

Fig. 2 **a** Principal component analysis of aroma composition of Soave wines obtained by the sequential inoculation of *T. delbrueckii* and *S. cerevisiae* (Td1 + Sc and Td2 + Sc) and the inoculation *S. cerevisiae* as control (Sc). **b** Principal component analysis of aroma composition of Chardonnay wines obtained by the sequential inoculation of *T. delbrueckii* and *S. cerevisiae* (Td1 + Sc and Td2 + Sc) and the inoculation *S. cerevisiae* as control (Sc)

Fermentation of dessert wine and microbiological analysis

The kinetics of AF differed among the trial wines and those inoculated with *S. cerevisiae* only (the control wines), with trial wines displaying the highest rate of CO_2 production, while the trial wines fermented by multi-starters and spontaneous microflora were quite similar, especially in the second half of AF (Supplementary Figure S4).

Several spontaneous yeasts were isolated on grape must and some of them were identified as *H. uvarum*, *Zygosaccharomyces bailii*, *Z. rouxi*, *M. pulcherrima*, *Pichia guilliermondii*, *S. cerevisiae* and *S. pastorianus* (Table 1). The total concentration of these wild yeasts was about 8.2×10^4 cfu mL^{-1} (Fig. 3a). Molecular analysis by microsatellite multiplex PCR of representative isolates identified as *S. cerevisiae* (VS9, VS26 and VS33, Table 1) displayed three profiles different from that of EC1118 starter strain (Supplementary, Figure S2).

The evolution of yeast cell concentration in all trials was monitored and, at the beginning of AF, cell density on Td2 + Sc and Sc wines increased rapidly just after the inoculation of *T. delbrueckii* strain Td2 and *S. cerevisiae* strain, respectively (Fig. 3a, b). Conversely, yeast density in the non-inoculated trial (Spn wine) was more slowly than the inoculated trials. In this last vinification non-*Saccharomyces* species (*H. uvarum*, *P. gulliermondii* and *Zygosaccharomyces* spp. were the most frequent) colonized the early phase of AF but progressively they were replaced by indigenous *Saccharomyces* spp. (Figure 3c). In multi-starter trial (Td2 + Sc wine) the population of *T. delbrueckii*, inoculated in the must at the concentration of 3.7×10^6 cfu mL^{-1}, remained high (10^7 cfu mL^{-1}) throughout the fermentation and started to decline after the inoculation of *S. cerevisiae* strains, carried out at the 11th day (Fig. 3b). Subsequently, *S. cerevisiae* dominated up to the end of fermentation. *S. cerevisiae* strain inoculated at the concentration of 1.5×10^6 cfu mL^{-1} in grape must of Sc trial dominated rapidly the fermenting wine until the end of AF (data not shown). Both in Td2 + Sc and Sc wine, only *S. cerevisiae* yeasts (VS27–32, Table 1), that displayed the same microsatellite multiplex PCR profile of EC1118, were found after starter inoculation (Supplementary Figure S2).

Chemical analysis of dessert wines

Multi-starter fermentation produced lower amounts of acetic acid during the course of AF than fermentations carried out by *S. cerevisiae* only and by indigenous yeasts (Fig. 4). At devatting, the multi-starter wines contained 0.29–0.37 g L^{-1} of acetic acid less than the other wines.

T. delbrueckii significantly affected the volatile composition of the Vino Santo wine, and the trend to variations of several molecules agreed with the data of the Soave and Chardonnay wines (Table 3). However, the particularity of Vino Santo wine provided interesting information on the effects of this non-*Saccharomyces* yeast on the aroma of sweet style wines.

The major effects of *T. delbrueckii* on alcohols were observed on the content of benzyl alcohol, 3-methylthio-1-propanol and 2-phenylethanol. The latter was twice as high in the Td2 + Sc wine (OAV = 2.9) than in the other two wines (OAV = 1.6).

Among esters, the decrease of wine isoamyl acetate was observed in the Td2 + Sc wine, down to half compared to the other two wines. The decrease in C_6–C_{10} fatty acid esters was similar to the dry table wines described above. Moreover, the content of ethyl 4-hydroxybutyrate, ethyl

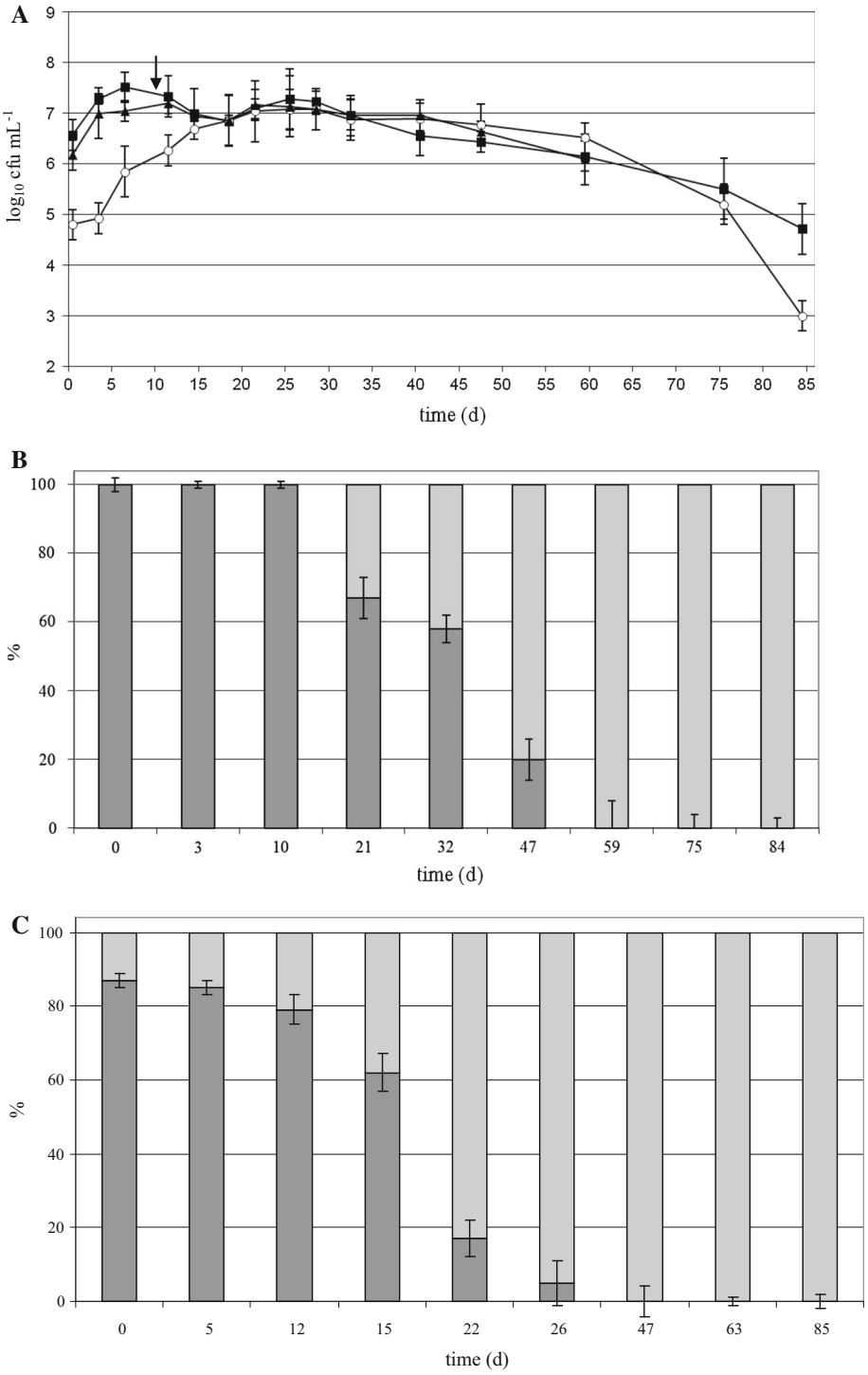

Fig. 3 **a** Total yeast cell concentration ($\log_{10}$ cfu mL^{-1}) measured during the alcoholic fermentation carried out spontaneously (Spn, *white circle*) or induced by the sequential inoculation of *T. delbrueckii* and *S. cerevisiae* (Td2 + Sc, *black square*) and the inoculation of *S. cerevisiae* (Sc, *black triangle*) to produce Vino Santo wine. *Bars* are standard deviation of three independent trials and *arrow* indicates the inoculation of *S. cerevisiae* in Td2 + Sc wine. **b** Percentage of *T. delbrueckii* (*dark grey bar*) and other yeasts, mainly *S. cerevisiae* (*light grey bar*) on Td2 + Sc wine during the alcoholic fermentation. *Bars* are standard deviation of three independent trials. **c** Percentage of non-*Saccharomyces* (*dark grey bar*) and *Saccharomyces* (*light grey bar*) during spontaneous alcoholic fermentation (Spn wine). *Bars* are standard deviation of three independent trials

2-hydroxy-4-methylpentanoate, diethyl 2-hydroxyglutarate and ethyl cinnamate decreased, confirming the strong impact of *T. delbrueckii* on volatile ester composition. It is interesting to note the high content of ethyl cinnamate that determined high OAV in all wines (OAV = 18.4, 11.5 and 12.3 in the Sc, Spn and Td2 + Sc wines, respectively) due to its low sensory threshold (1.1 μg L^{-1}; Ferreira et al. 2000). In the Spn wine, the behaviour of these esters was similar to that observed in the Td2 + Sc wine.

A significant decrease of content of C_6–C_{10} fatty acids was found in the Td2 + Sc wine compared to the Sc wine. This result agreed with the dry table wine data, although the decrease was not as drastic. Isovalerianic acid and hexanoic acid were detected above their thresholds, and in

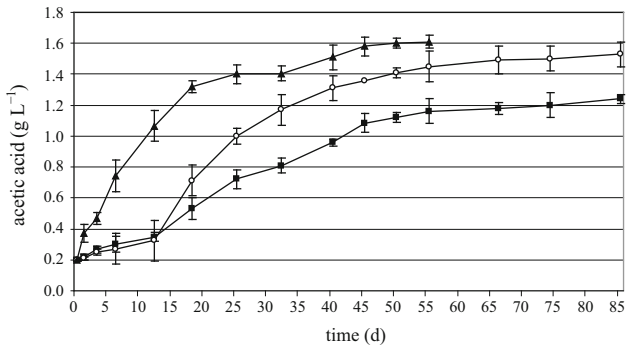

Fig. 4 Acetic acid (g L^{-1}) production during alcoholic fermentation carried out spontaneously (Spn, *white circle*) or induced by the sequential inoculation of *T. delbrueckii* and *S. cerevisiae* (Td2 + Sc, *black square*) and the inoculation of *S. cerevisiae* (Sc, *black triangle*) to produce Vino Santo wine. *Bars* are standard deviation of three independent trials

the wine inoculated with *T. delbrueckii* the isovalerianic acid was about half (OAV = 5.1) of that in the wine inoculated with *S. cerevisiae* (OAV = 9.2). The decrease in hexanoic acid was less marked (OAV = 1.4 vs 1.7). In contrast, in all the wines, butyric acid was above 173 1.1 μg L^{-1}, its threshold (Ferreira et al. 2000), and consistently increased in the Td2 + Sc wine (OAV = 2.0). Similar modifications for isovalerianic acid, hexanoic acid and octanoic acid were observed in the Td2 + Sc and Spn wines.

The varietal aromas (monoterpenes and norisoprenoids) were found at particularly high concentrations compared to dry table wines, and the variations among the wines were generally slight (<30 %), with the most relevant observed for citronellol, endiol and 3-oxo-α-ionol.

More important were the changes in carbonylic compounds, in particular benzaldehyde, furfural, syringaldehyde and homo- and nor-furaneol, although their concentrations remained under their respective thresholds: 0.2, 14.1, 50.0, 0.04 and 2.0 mg L^{-1}, respectively (Ferreira et al. 2000; Peinado et al. 2004; Campo et al. 2006; Sarrazin et al. 2007).

The effect of *T. delbrueckii* on vinylphenols in the Vino Santo wines supported the data of the previously produced Chardonnay wine, as both molecules decreased (especially 4-vinylphenol) in the Td2 + Sc wine. Moreover, it was observed to have consistent impacts on vanillin and homovanillic acid content in the wines.

Among the lactones, which were present in very high amounts, it is worth noting the increase of the two sherry lactones and the decrease in ethyl pyroglutamate (detected at high concentrations in all three wines), γ-butyrolactone and 4-carboetoxy-γ-butyrolactone related to the presence of *T. delbrueckii* during the fermentation with respect to the Sc wine. Unlike the other two wines, the wine that fermented spontaneously (Spn wine) had a γ-nonalactone (sweet, caramel note) level lower than its threshold of 30 μg L^{-1} (Ferreira et al. 2000). The OAV were <1.0.

Similar to the dry table wines, PCA clearly distinguished the wines according to their volatile composition (Supplementary Figure S5). More than 50 % of compounds to PCA analysis had high PC1 and PC2 loading values (<−8.0 and >8.0; Supplementary Table S2). Td2 + Sc wines were mainly described by 2-phenylethyl alcohol, 3-methylthio-1-propanol, ethyl lactate, and butyric acid, Sc wines by ethyl hexanoate, isovalerianic acid, 4-terpineol, 4-vinylphenol, while Spn wines by furfural alcohol and *cis*-3 hexenol.

The three different fermentation strategies used to produce the Vino Santo wines generated substantial differences regarding to the wine's olfactive and gustative properties. In particular, the Sc wines were unanimously judged negatively, as their bouquet and mouthfeel were characterized by a pronounced acetic acid note detrimental to the wine quality. This note was also perceptible in wine obtained through spontaneous fermentation (Spn wine), but only in the mouth and to a lesser extent than the Sc wines, although that did not prejudice the overall quality like Sc wine. Due to this sensory defect of Sc and Spn wines the comparative descriptive sensory analysis of the three wines was not carried out. The Vino Santo wine produced by multi-starter fermentation (Td2 + Sc wine) was appreciated for its balance both in flavour and mouthfeel, and, at the same time, was characterized by aroma complexity, similar to the Spn wines, giving the wines a more harmonious flavour.

Discussion

The results of the fermentation kinetics agreed with the previous studies (Cabrera et al. 1988; Bely et al. 2008; Comitini et al. 2011; Azzolini et al. 2012; Cordero-Bueso et al. 2013), which reported a slower rate of ethanol production in the trials where the musts were inoculated with *T. delbrueckii* yeast. However, the use of *T. delbrueckii* cultures together with *S. cerevisiae* ensured outstanding fermentative performances for both styles of wines (dry and sweet). Moreover, the case of the Vino Santo wine highlighted the advantage of using *T. delbrueckii*, which resulted in a considerable decrease in acetic acid production, as reported by Bely et al. (2008). The consequence of such decrease can be extremely important, as the results of sensory analysis of the Vino Santo wines demonstrated. Indeed, the results of this study suggest that the use of non-*Saccharomyces* yeast can be recommended for the production of this type of wine, while inoculation with *S. cerevisiae* only could even be risky as volatile acidity can increase to unacceptable levels.

Table 3 Composition of Vino Santo wines produced with spontaneous fermentation (Spn), a monoculture of *S. cerevisiae* (Sc) and multi-starter fermentation using *T. delbrueckii* and *S. cerevisiae* yeasts (Td2 + Sc)

	Spn	Sc	Td2 + Sc
Ethanol (% v v^{-1})	12.6 b[1] ± 0.2	14.0 c ± 0.1	12.0 a ± 0.1
pH	4.2 b ± 0.0	4.1 a ± 0.0	4.2 b ± 0.0
Residual sugars (g L^{-1})	196.4 b ± 6.0	171.0 a ± 2.6	208.6 b ± 11.2
Acetic acid (g L^{-1})	1.53 b ± 0.08	1.61 b ± 0.04	1.24 a ± 0.03
Titratable acidity (g L^{-1})[2]	7.7 b ± 0.1	7.7 b ± 0.1	7.3 a ± 0.0
1-Hexanol[3]	1277.0 b ± 20.4	1300.8 b ± 6.3	1019.8 a ± 7.8
trans-3-Hexen-1-ol	37.7 b ± 0.5	35.0 a ± 1.1	36.2 ab ± 1.4
cis-3-Hexen-1-ol	19.3 b ± 0.8	16.9 a ± 0.9	17.2 a ± 0.9
2-Hexen-1-ol	3.1 ± 0.4	3.6 ± 0.4	3.6 ± 0.5
Benzyl alcohol	170.1 ab ± 36.5	179.1 b ± 10.4	101.1 a ± 7.5
3-Methylthio-1-propanol	112.1 b ± 7.9	83.8 a ± 5.9	375.7 c ± 67.9
Furfuryl alcohol	26.8 b ± 2.5	20.7 a ± 2.2	20.6 a ± 2.3
Homovanillic alcohol	624.0 a ± 18.1	597.0 a ± 15.3	723.3 b ± 1.8
1-Octen-3-ol	6.9 a ± 0.8	8.2 ab ± 0.3	9.0 b ± 0.8
Vanillic alcohol	19.6 a ± 1.0	18.3 a ± 0.2	22.0 b ± 0.9
2-Phenylethyl alcohol	22502.2 a ± 356.2	21829.2 a ± 329.7	41521.9 b ± 425.5
Alcohols[4]	24798.8 b ± 312.6	24092.6 a ± 307.3	43850.3 c ± 337.4
Hexyl acetate	3.5 ± 0.8	3.8 ± 0.2	2.7 ± 1.0
Isoamyl acetate	226.9 c ± 9.3	203.0 b ± 4.0	104.9 a ± 6.2
Phenylethyl acetate	30.1 ± 3.0	27.7 ± 1.9	36.4 ± 4.2
Ethylphenyl acetate	34.0 a ± 0.6	36.1 b ± 0.7	37.6 b ± 1.0
Ethyl butyrate	113.4 ± 5.5	120.9 ± 10.9	147.2 ± 28.0
Ethyl hexanoate	178.5 a ± 2.9	279.3 b ± 1.2	182.3 a ± 2.8
Ethyl octanoate	86.2 b ± 2.3	108.8 c ± 1.4	71.5 a ± 2.6
Ethyl decanoate	25.0 c ± 0.5	21.7 b ± 1.1	8.9 a ± 0.1
Ethyl 9-decenoate	<1.0	<1.0	<1.0
Ethyl 3-hydroxybutyrate	132.8 b ± 0.2	135.8 c ± 0.7	94.3 a ± 4.1
Ethyl 4- hydroxybutyrate	4652.7 b ± 154.1	10891.9 c ± 567.9	3824.3 a ± 92.5
Ethyl 2-hydroxyisovalerate	5.4 ± 0.4	5.6 ± 0.1	5.5 ± 0.2
Ethyl 2-hydroxy-4-methylpentanoate	19.5 b ± 0.7	23.6 c ± 0.4	13.5 a ± 1.2
Diethyl succinate	6294.9 ± 74.3	6188.8 ± 181.2	6538.3 ± 272.0
Ethyl lactate	5059.0 ab ± 246.0	4997.9 a ± 10.2	6288.0 b ± 260.7
Isoamyl lactate	15.6 ± 1.2	17.8 ± 2.9	15.5 ± 0.6
Ethyl-isoamyl succinate	24.0 a ± 1.0	23.4 a ± 0.8	27.9 b ± 0.4
Diethyl malate	4332.1 ab ± 239.0	4832.9 b ± 42.8	4135.0 a ± 125.8
Diethyl 2-hydroxyglutarate	63.7 b ± 5.1	61.5 b ± 0.6	42.4 a ± 1.3
Ethyl vanillate	65.0 a ± 2.1	63.0 a ± 0.3	73.1 b ± 1.4
Methyl vanillate	7.5 ab ± 1.0	9.0 b ± 0.7	6.0 a ± 0.3
Ethyl cinnamate	12.7 a ± 0.5	20.3 b ± 0.6	13.5 a ± 0.1
Ethyl salicylate	1.7 ± 0.1	1.8 ± 0.1	1.8 ± 0.1
Methyl salicylate	1.0 ± 0.1	1.2 ± 0.0	<1.0
Esters	21385.1 a ± 217.7	28075.7 b ± 720.8	21670.5 a ± 239.3
Butyric acid	253.6 a ± 2.2	249.8 a ± 0.7	348.1 b ± 0.6
Isovalerianic acid	185.6 a ± 1.4	305.6 b ± 14.3	169.7 a ± 7.6
Hexanoic acid	455.2 a ± 15.3	716.6 c ± 15.0	601.6 b ± 24.7
Octanoic acid	204.5 ab ± 31.3	242.3 b ± 5.4	208.7 a ± 10.4
Decanoic acid	54.9 b ± 10.9	41.5 b ± 1.6	16.9 a ± 1.8

Table 3 continued

	Spn	Sc	Td2 + Sc
Fatty acids	1153.8 a ± 53.9	1555.6 c ± 23.1	1345.0 b ± 40.4
Linalool	150.6 ab ± 2.1	148.2 a ± 0.3	152.8 b ± 1.7
α-Terpineol	65.5 ab ± 2.0	61.2 a ± 0.7	70.3 b ± 0.3
Ho-Trienol	72.0 ± 6.0	72.4 ± 2.0	73.8 ± 6.9
Citronellol	23.5 b ± 0.8	27.3 c ± 0.2	17.5 a ± 0.2
Nerol	17.3 ab ± 1.2	14.8 a ± 0.2	18.8 b ± 0.3
Geraniol	17.3 a ± 0.6	16.6 a ± 0.8	18.9 b ± 0.2
trans-Furanic linalool oxide A	24.4 ± 0.9	23.5 ± 0.4	23.4 ± 0.2
cis-Furanic linalool oxide B	15.2 b ± 0.1	14.6 a ± 0.0	15.4 b ± 0.2
trans-Pyranic linalolo oxide C	85.6 ± 1.3	88.2 ± 2.1	85.6 ± 1.6
cis-Pyranic linalolo oxide D	15.6 ± 1.0	15.5 ± 0.5	15.8 ± 0.6
ho-Diendiol 1	917.8 ± 37.8	968.1 ± 10.1	942.6 ± 31.4
ho-Diendiol 2	118.7 ± 6.6	115.0 ± 1.5	116.0 ± 4.2
Endiol	32.1 ab ± 15.5	34.1 a ± 0.3	44.0 b ± 2.2
trans-8-dihydroxylinalool	39.6 ± 1.2	46.4 ± 0.2	44.3 ± 0.9
cis-8-dihydroxylinalool	78.8 ± 6.8	74.3 ± 2.0	85.7 ± 2.5
4-Terpineol	182.4 ± 2.8	197.8 ± 4.1	181.1 ± 1.3
p-Cymene	<1.0	1.3 ± 0.4	1.0 ± 0.1
Monoterpenes	1856.4 ± 73.3	1918.8 ± 18.2	1906.7 ± 46.4
β-Damascenone	9.9 ± 0.1	10.4 ± 0.4	8.8 ± 0.2
Actinidol 1	2.8 ± 0.4	2.4 ± 0.2	2.5 ± 0.1
Actinidol 2	6.0 b ± 0.2	5.6 a ± 0.1	5.5 ab ± 0.3
3-Oxo-α-ionol	160.9 b ± 5.1	143.8 a ± 4.4	168.3 b ± 3.3
Norisprenoids	179.6 b ± 5.7	162.1 a ± 4.3	185.1 b ± 3.6
Phenylacetaldehyde	9.8 ± 0.4	10.6 ± 1.1	14.7 ± 4.5
Benzaldehyde	313.2 b ± 26.6	212.1 a ± 3.2	343.4 b ± 27.5
Furfurol	346.0 b ± 5.9	263.7 a ± 12.1	353.7 b ± 35.3
Syringaldehyde	18.5 b ± 1.5	9.0 a ± 0.4	19.7 b ± 0.7
5-Methylfurfural	5.2 b ± 0.1	4.2 a ± 0.1	5.0 b ± 0.3
Furaneol	4.2 ± 0.9	10.7 ± 2.9	4.4 ± 0.4
Homo-furaneol	26.1 b ± 3.7	84.0 c ± 3.3	7.9 a ± 2.9
Nor-furaneol	44.1 a ± 1.8	101.6 b ± 9.4	50.7 a ± 18.7
Carbonylic compounds	767.2 ± 33.1	695.9 ± 6.5	799.6 ± 51.4
4-Ethylphenol	<1.0	<1.0	<1.0
4-Ethylguaiacol	<1.0	<1.0	<1.0
4-Vinylguaiacol	151.1 b ± 12.1	151.6 ab ± 22.7	121.3 a ± 6.0
4-Vinylphenol	37.9 b ± 2.0	77.8 c ± 6.6	28.7 a ± 1.8
Eugenol	2.5 ± 0.2	2.6 ± 0.1	2.6 ± 0.0
Guaiacol	1.9 ± 0.2	1.8 ± 0.1	1.6 ± 0.1
o-Cresol	1.7 ± 0.1	1.2 ± 0.2	1.7 ± 0.1
p-Cresol	3.1 b ± 0.1	2.8 b ± 0.1	2.1 a ± 0.0
Vanillin	24.0 b ± 1.8	12.2 a ± 0.4	27.3 b ± 2.7
Acetovanillone	86.3 ± 4.7	81.3 ± 1.9	81.2 ± 2.9
Phenol	5.6 ab ± 0.5	4.7 a ± 0.1	5.3 b ± 0.2
Homovanillic acid	31.7 b ± 0.7	35.8 c ± 0.8	21.7 a ± 0.8
Volatile phenols	345.9 b ± 19.9	371.6 b ± 31.4	293.6 a ± 2.8
Ethyl pyroglutamate	2696.7 b ± 136.8	3335.8 c ± 52.7	2333.0 a ± 7.1
γ-Nonalactone	25.3 a ± 0.2	30.1 b ± 1.5	32.7 a ± 1.1

Table 3 continued

	Spn	Sc	Td2 + Sc
γ-Butyrolactone	2826.5 a ± 48.3	3390.3 c ± 78.5	3069.1 b ± 32.5
4-Carboxyethoxy-γ-butyrolactone	186.3 b ± 11.1	178.1 b ± 2.0	117.5 a ± 2.1
Sherry lactone 1	3769.7 b ± 102.9	3391.8 a ± 69.7	4428.7 c ± 211.8
Sherry lactone 2	4952.5 b ± 594.3	3498.4 a ± 65.4	6227.4 b ± 183.1
Lactones	14457.2 a ± 597.0	13824.4 a ± 262.6	16208.4 b ± 359.3
N-(3-methylbutyl)-acetamide	161.5 a ± 8.7	244.5 b ± 12.4	270.6 c ± 8.2

Values (±standard deviation) are the mean of three independent trials

[1] Values with different letter are significantly different for $p < 0.05$

[2] As tartaric acid

[3] Expressed as μg L^{-1}

[4] Obtained by summing up all molecules of same chemical group (alcohols, esters, fatty acids, monoterpenes, norisoprenoids, carbonylic compounds, volatile phenols and lactones)

The impact of *T. delbrueckii* on aroma compounds affected different chemical groups, and particularly informative were the compounds that behaved similarly in all three trials.

The increase in 2-phenylethanol was a clear effect of the co-presence of *T. delbrueckii* and *S. cerevisiae* yeasts on the alcohol composition of the three wines, as previously reported (Azzolini et al. 2012; Sadoudi et al. 2012; Cordero-Bueso et al. 2013). The differences in the 2-phenylethanol content between the wines fermented by Td1 and Td2 could be strain-related, although Renault et al. (2009) did not find significant differences among several *T. delbrueckii* strains on the production of this alcohol. Other alcohols, like methionol, a potent volatile sulphur flavour compound, seems to be affected by *T. delbrueckii* activity, but its synthesis by this yeast has not yet been investigated.

The impact of *T. delbrueckii* on important volatile esters appears to be pronounced, as demonstrated by the strong decrease in isoamyl acetate and C_6–C_{10} fatty acid esters in wines obtained by multi-starter fermentation. Similar behaviours were previously documented, comparing wines fermented by *S. cerevisiae* and co-cultures of *T. delbrueckii* and *S. cerevisiae* (Viana et al. 2008; Azzolini et al. 2012; Sadoudi et al. 2012; Cordero-Bueso et al. 2013). In this study, the contribution of *T. delbrueckii* on their general decrease appears to be even more evident. This is more significantly reflected at sensory level, especially in table wines, such as the Soave and Chardonnay, than in sweet wines, like the Vino Santo, due to the higher odour potency these esters generally have in young wines (Sumby et al. 2010). The difference in the scores for green apple and freshness between Td1 and Td2 + Sc and Sc wines could be related to their different fatty acid ethyl ester content. Similar observations were reported in Madeira wines submitted to oxidative aging (Câmara et al. 2006). Nevertheless, it is important to note that the perception of fruity aroma is the result of a complex interaction between various compounds of different chemical groups, including powerful mercaptans and pyrazines not detected in this study (Mateo-Vivaracho et al. 2010). Thus, further chemical analysis is necessary for a better understanding of the differences in the fruity attributes among these wines.

As fatty acid esters can also have high odour activity in aged dessert wines (Bailly et al. 2009; Bowen and Reynolds 2012), the role of *T. delbrueckii* on the modulation of the sensory properties of Vino Santo, due to these aromatic esters, could be relevant. Certainly, ethyl cinnamate can be considered a good candidate for be odour-potent compounds in Vino Santo wine, whose content can vary significantly depending on the yeast metabolism, as this study demonstrated.

The contribution of *T. delbrueckii* activity on wine aroma was also clearly evident at the level of fatty acids. Their strong decrease can be considered to be positive as they are generally responsible for negative effects on overall wine aroma, especially in young wines. Therefore, the use of co-cultures of *T. delbrueckii* and *Saccharomyces* yeasts could be strategic in order to modulate the production of ethyl esters and fatty acids, formed enzymatically during fermentation, especially in table wines such as Soave and Chardonnay.

In sweet wines, like Vino Santo, *T. delbrueckii* may affect the wine's aroma profile by acting on carbonylic compounds, although most of them were detected below their threshold. Interestingly, the behaviour of these compounds was similar among the wines produced by multi-starter and spontaneous fermentations compared to those inoculated with *S. cerevisiae* only. The presence of various indigenous non-*Saccharomyces* yeasts in the natural fermentation of dessert wines provided great metabolic diversity, including those involved in the metabolism of aromatic aldehydes and ketones (Krings and Berger 1998),

that contribute to the particular complexity in the aroma of these wines. Because the production of these molecules by *T. delbrueckii* is still unknown, this study provided some insights for its investigation.

The results for vinylphenols suggest that multi-starter fermentation may consistently decrease their content, as observed for the Chardonnay wines inoculated with *T. delbrueckii* where 4-vinylguiacol decreased to under its threshold. Moreover, this occurrence could reflect positively on the sensory properties considering the unpleasant flavours of these phenols, which are also precursors for ethylphenols, which have lower thresholds. Further assay of hydroxycinnamate decarboxylase activity in *T. delbrueckii* would be necessary to ascertain the possibility of minimizing ethylphenol as shown previous investigations (Benito et al. 2011).

The effects of *T. delbrueckii* on lactones were pronounced in the Vino Santo wine, and the consistent variations in sherry lactones agreed with our previous study carried out on Amarone wine, a raisin wine like Vino Santo (Azzolini et al. 2012). The same tendency for these lactones, observed in wines produced by spontaneous and multi-starter fermentation, suggests the importance non-*Saccharomyces* yeasts have on sherry-like notes related to these molecules, precisely these found in sherry wines (Muller et al. 1973). Also, the sensory effects of the decrease of ethyl pyroglutamate linked to *T. delbrueckii* do not appear to be negligible, as this lactone is responsible for burnt, caramel and honey notes that are the key odour descriptors for Vino Santo wine, as well as for sweet fortified wines (Schneider et al. 1998; Campo et al. 2006).

In conclusion, this study of the utilization of co-cultures of *T. delbrueckii* and *S. cerevisiae* yeasts, sequentially inoculated into must to produce two different styles of white wines, demonstrates significant differences distinguishing the chemical and sensory properties, compared to the same wines obtained by traditional (monoculture or spontaneous) fermentation. Furthermore, the relevance of strain-specificity within *T. delbrueckii* to these differences was shown, providing insights for further investigations on the effects of strain diversity on wine quality for non-*Saccharomyces* yeasts. Naturally, the ability of the *T. delbrueckii* and *S. cerevisiae* co-cultures to specifically impact on volatile wine compounds should be further studied to broaden the understanding of the winemaking potential of these multi-starter fermentations.

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