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Using *Torulaspora delbrueckii* killer yeasts in the elaboration of base wine and traditional sparkling wine

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ABSTRACT

For still wines, killer strains of *Torulaspora delbrueckii* can be used instead of non-killer strains to improve this species' domination during must fermentation, with an ensured, reliable impact on the final wine quality. The present work analysed the usefulness of these killer yeasts for sparkling-wine making. After the first fermentation, the foaming capacity of *T. delbrueckii* base wines was very low compared to *Saccharomyces cerevisiae* base wines. Significant positive correlations of foaming parameters were found with the amounts of C_4–C_{16} ethyl esters and proteins, and negative with some anti-foaming alcohols produced by each yeast species. There were, however, no evident positive effects of polysaccharides on those parameters. The organoleptic quality of the *T. delbrueckii* base wines was judged inappropriate for sparkling-wine making, so that the following second-fermentation experiments only used a single *assemblage* of *S. cerevisiae* base-wines. While second fermentation was completed with inoculation of *S. cerevisiae* (both alone and mixed with *T. delbrueckii*) to yield dry sparkling wines with high CO_2 pressure, single inoculation with *T. delbrueckii* did not complete this fermentation, leaving sweet wines with poor CO_2 pressure. Yeast death due to CO_2 pressure was much greater in *T. delbrueckii* than in *S. cerevisiae*, making any killer effect of *S. cerevisiae* over *T. delbrueckii* irrelevant because no autolysed cells were found during the first days of mixed-inoculated second fermentation. Nonetheless, the organoleptic quality of the mixed-inoculated sparkling wines was better than that of wines single-inoculated with *S. cerevisiae*, and showed no deterioration in foam quality. This seemed mainly to be because *T. delbrueckii* increased the amounts of ethyl propanoate and some acids (e.g., isobutyric and butanoic), alcohols (e.g., 3-ethoxy-1-propanol), and phenols (e.g., 4-vinylguaiacol). For these sparkling wines, no significant correlations between foaming parameters and aroma compounds were found, probably because the differences in foaming parameter values among these wines were fairly small. This is unlike the case for the base wines for which there were large differences in these parameters, which facilitated the analysis of the influence of aroma compounds on base-wine foamability.

1. Introduction

Torulaspora delbrueckii is probably the non-*Saccharomyces* yeast most frequently used for wine fermentation. It has been suggested that this yeast was first domesticated for winemaking by Romans about 1900 years ago, and for other food fermentations during the Neolithic era about 4000 years ago (Albertin et al., 2014). Besides winemaking, several *T. delbrueckii* strains have also been considered for olive and bread fermentations (Ohshima et al., 1987; Psani and Kotzekidou, 2006). This yeast can improve wine complexity, decrease volatile acidity and acetaldehyde content, and increase dried-fruit and pastry aromas (Azzolini et al., 2012, 2015; Bely et al., 2008; Jolly et al., 2006; Ramírez et al., 2016; Renault et al., 2009; Velázquez et al., 2015). Also, it has recently been found that sequential inoculation of *T. delbrueckii* and *Saccharomyces cerevisiae* increases glycerol concentration, reduces volatile acidity, and exerts a positive effect on the foaming properties of base wine for sparkling-wine making (González-Royo et al., 2014).

Mixed inoculation of the must with *T. delbrueckii* and *S. cerevisiae* seems to be preferred over single *T. delbrueckii* inoculation (Azzolini et al., 2012; Bely et al., 2008; Ciani et al., 2006; Comitini et al., 2011; Herraiz et al., 1990; Renault et al., 2015, 2016, 2009; Sadoudi et al., 2012; Zhang et al., 2018). This is mainly because the lower fermentation vigour and slower growth rate of *T. delbrueckii* with respect to *S. cerevisiae* under wine fermentation conditions (González-Royo et al., 2014; Mauricio et al., 1998). Therefore, *T. delbrueckii* usually does not complete wine fermentation by itself, or has difficulty in dominating

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Table 1
Yeast strains used.

Strain	Genotype [relevant phenotype]	Origin
Sc EX85	*MAT a/α HO/HO cyh2^{s}/cyh2^{s}* LA M2 [K2^{+}]	Wine (Ramírez et al., 1998; Regodón et al., 1997)
Sc EX229	*MAT a/α HO/HO cyh2^{s}/cyh2^{s}* LA Mlus [Klus^{+}]	Wine (Rodríguez-Cousiño et al., 2011)
Sc EX85R	*MAT a/α HO/HO CYH2^{R}/cyh2^{s}* [cyh^{R}, K2^{0}]	*Sc* EX85 (Pérez et al., 2000)
Sc EX229-R1	*MAT a/α HO/HO CYH2^{R}/cyh2^{s}* LA [cyh^{R}, Klus^{0}]	*Sc* EX229 (Velázquez et al., 2016)
Sc E7AR1	*MAT a/α HO/HO CYH2^{R}/CYH2^{R}* LA M2 [cyh^{R}, K2^{+}]	*Sc* 7AR (Ramírez et al., 1998)
Sc Rod23-1B	*MAT a/α HO/HO PDR5/PDR5* LA M2 [RHOD^{PC}, K2N^{+}]	*Sc* EX88 (Ambrona et al., 2006)
Td EX1180	wt LA M-barr-1 [Kbarr-1^{+}]	Wine (Ramírez et al., 2015)
Td EX1180-11C4	cyh^{R} LAbarr Mbarr-1 [cyh^{R} Kbarr-1^{+}]	*Td* EX1180 (Ramírez et al., 2015)
Td EX1257	wt LAbarr Mbarr-2 [Kbarr-2^{+}]	Wine (Ramírez et al., 2015)
Td EX1257-CYH5	cyh^{R} LAbarr Mbarr [cyh^{R} Kbarr-2^{+}]	*Td* EX1257 (Ramírez et al., 2015)
Td EX1180-2K^{-}	cyh^{R} LAbarr Mbarr-0 [cyh^{R} Kbarr^{0}]	*Td* EX1180 (Ramírez et al., 2015)

Sc, *Saccharomyces cerevisiae*. Td, *Torulaspora delbrueckii*.

this process in the presence of a relevant population of wild or contaminating *S. cerevisiae* yeasts. Full domination and completion of wine fermentation has been found in only a few studies for some *T. delbrueckii* killer strains (Ramírez et al., 2016; Velázquez et al., 2015). For this reason, these yeasts are usually not recommended as single inoculum for fermentation under very stringent conditions such as those of the second fermentation in traditional sparkling-wine making under high pressure conditions or in the elaboration of wines with high alcohol content.

Some effects frequently found for mixed *T. delbrueckii* + *S. cerevisiae* inoculation on wine quality are: the reduction of undesirable compounds such as acetic acid, acetaldehyde, and acetoin (Bely et al., 2008; Ciani et al., 2006; Herraiz et al., 1990), and the increase of interesting compounds such as 2-phenylethanol, terpenols, and lactones (Azzolini et al., 2012; Comitini et al., 2011; Herraiz et al., 1990; Sadoudi et al., 2012). Despite this, while an increase of total ester concentration has been found for mixed *T. delbrueckii* + *S. cerevisiae* inoculation relative to single inoculation with *T. delbrueckii* or *S. cerevisiae* (Herraiz et al., 1990), the contrary effect has also been reported (Azzolini et al., 2012; Comitini et al., 2011; Sadoudi et al., 2012). These contradictory results may be because the production and degradation of esters by each yeast species during must fermentation, and the eventual occurrence of malolactic fermentation (Ramírez et al., 2016). Moreover, the production of esters by *T. delbrueckii* can be strain dependent, and the aromas produced by this yeast may vary when it interacts with *S. cerevisiae* in mixed cultures (Renault et al., 2015, 2016, 2009).

Although several yeast companies market different strains of *T. delbrueckii* for winemaking (e.g., http://www.enartis.com/eu/products/yeast, http://www.lallemandwine.com/es/spain/products/, https://www.chr-hansen.com/en/food-cultures-and-enzymes/wine/cards/product-cards/prelude, http://www.agrovin.com/agrv/index.php/web/enologia/no_saccharomyces/es, and https://www.laffort.com/es/productos/470-zymaflore-alpha), this yeast is still not generally used in the wine industry. This is mostly because of the lack of reliable knowledge about the biotechnological advantages claimed for this yeast, particularly when compared to knowledge about *S. cerevisiae* which is considered to be the most reliable yeast for winemaking. It is of general belief that there is a need to implement knowledge of *T. delbrueckii*'s fermentation behaviour and aroma production in a practical approach to its use in winemaking. In this sense, there has been a recent analysis of the influence of sequential inoculation of this yeast with *S. cerevisiae* on the composition and quality of base wine for sparkling wine production (González-Royo et al., 2014). The study showed that sequential inoculation of these yeasts may be an interesting tool with which to obtain base wines with different characteristics, although no actual sparkling wine was made with *T. delbrueckii*. Beside this, a recent publication (Canonico et al., 2018) showed that two single-inoculated *T. delbrueckii* strains completed the secondary fermentation of sparkling wine even more efficiently than single-inoculated *S. cerevisiae*; which is an impressive result for non-*Saccharomyces* yeasts. However, unfortunately, these authors did not determine the proportion of inoculated yeast during second fermentation. Therefore, the presence of contaminating *S. cerevisiae* yeasts cannot be definitively ruled out, and further research should be done to confirm this result.

With the aim of complementing these interesting results, we decided to analyse the utility of new killer strains of *T. delbrueckii* (Kbarr) – strains which kill *S. cerevisiae* yeasts (Ramírez et al., 2015) and can easily dominate must fermentation (Ramírez et al., 2016; Velázquez et al., 2015) – for base-wine and sparkling-wine elaboration. Also, since the killer effect can improve yeast autolysis and sparkling wine quality (Velázquez et al., 2016), we also tested *T. delbrueckii* killer-sensitive strains. We addressed the following issues: (i) the capacity of Kbarr strains to dominate and complete single-inoculated base-wine fermentation; (ii) the ability of Kbarr strains to perform sparkling-wine second fermentation; and (iii) the analysis of the aroma profile and foaming properties of base wine and sparkling wine made with *T. delbrueckii* as compared with those wines made with *S. cerevisiae*. The usefulness of killer *T. delbrueckii* strains for sparkling-wine making will be discussed.

2. Materials and methods

2.1. Yeast strains and culture media

The yeast strains used in this work are summarized in Table 1. *Sc* EX85 is prototrophic, K2-killer, and homothallic *S. cerevisiae* wine yeasts previously isolated and selected for winemaking (Ramírez et al., 1998; Regodón et al., 1997). *Sc* E7AR1 is a K2-killer and cycloheximide-resistant (cyh^{R}) wine yeast. The *S. cerevisiae* K2-killer strains kill other killer-sensitive *S. cerevisiae* strains, but we confirmed that they do not kill *Torulaspora delbrueckii* yeasts, as previously found (Ramírez et al., 2015). *S. cerevisiae Sc* EX229 is a Klus-killer strain that kills other *S. cerevisiae* and *T. delbrueckii* yeasts (Rodríguez-Cousiño et al., 2011); *Sc* EX85R (originally named JP85R; Pérez et al., 2000) and *Sc* EX229-R1 are virus-free killer-sensitive cycloheximide-resistant (cyh^{R}) strains from *Sc* EX85 (originally named JP85; Pérez et al., 2000) and *Sc* EX229, respectively; *Sc* Rod23-1B is K2N-killer, cyh^{R}, and rhodamine pink colony (RHOD^{PC}) (originally named RhodM2H3-1B; Ambrona et al., 2006), derived from the K2 *Sc* EX88 strain. *Sc* Rod23-1B shows a new weak killer activity against other K2-killer strains. The *T. delbrueckii* Kbarr wine yeasts, *Td* EX1180 and *Td* EX1257, are prototrophic strains that kill all *S. cerevisiae* killer and non-killer strains, and the non-killer *T. delbrueckii* strains. *Td* EX1180-11C4 and *Td* EX1257-CYH5 are cyh^{R} spontaneous mutants from *Td* EX1180 and *Td* EX1257, respectively. *Td* EX1180-2K^{-} is a cyh^{R} non-killer spontaneous mutant from *Td* EX1180. These cyh^{R} spontaneous mutants were obtained as previously described (Ramírez et al., 2015; Pérez et al., 2000). The industrial use of *Td* EX1180 and *Td* EX1257 is under patent application (Ramírez et al., 2015).

YEPD + cyh is YEPD-agar supplemented with cycloheximide (cyh) to a final concentration of 2 μg/mL (Pérez et al., 2000). YEPD + rhod is

YEPD-agar supplemented with rhodamine 6G (rhod) to a final concentration of 5 μg/mL (Ambrona et al., 2006).

2.2. Analytical methods

Alcohol content, pH, total acidity, volatile acidity, glucose + fructose, and density were determined according to EC recommended methods (E.C., 1999). The sparkling-wine pressure was measured at room temperature using an aphrometer. The values were then corrected to 20 °C by using the Henry's law constant. The wine protein content was determined using the Bradford method (Bradford, 1976), with some modifications to avoid interferences from ethanol and phenolic compounds. In particular, the wine samples were ultrafiltered (Marchal et al., 1997) and protein content was determined as previously described (Cilindre et al., 2010). The value of the ultrafiltrate (containing interfering compounds) was subtracted from that of the wine sample to determine the wine protein content. Bovine serum albumin was used as standard. The results were the mean of four independent measurements.

Wine mannan (mannoproteins) content was determined as previously by isolation of polysaccharides, followed by acid hydrolysis of soluble polysaccharides, and separation of monosaccharides by HPLC/ion chromatography (Quiros et al., 2012; Velázquez et al., 2016). Monosaccharide concentrations were calculated by external calibration. The amount of mannose was considered proportional to the amount of mannan in the sample. Each value was the mean of two independent measurements.

Aliquots of the same samples of purified polysaccharides before the acid hydrolysis were used to determine total polysaccharide concentrations by the phenol-sulfuric acid method (Dubois et al., 1956). Each value was the mean of four independent measurements.

Foaming parameters were determined with the Mosalux system (Maujean et al., 1990) as previously described (Martínez-Lapuente et al., 2015; Velázquez et al., 2016). HM (the maximum height reached by foam after CO_2 injection through the glass frit) represents the wine's ability to foam. HS (the foam stability height during CO_2 injection) represents the wine's ability to produce stable foam. TS (the foam stability time in seconds) is the time until all bubbles collapsed when CO_2 injection was interrupted, and represents the foam stability time once effervescence has decreased. These parameters were determined four times at 20 °C for each base wine and for each bottle of sparkling wine.

The wine aroma compounds were isolated and pre-concentrated following a solid-phase extraction (García-Carpintero et al., 2011), and then assayed by gas chromatography–mass spectrometry (GC–MS) as previously described (Velázquez et al., 2015).

2.3. Determination of killer activity

Killer activity was tested on low-pH (pH 3.2 or 4.0) methylene blue plates (3.2 MB or 4.0 MB) (Kaiser et al., 1994) seeded with 100 μL of a 48-h grown culture of the sensitive strain (Ramírez et al., 2004). The strains being tested for killer activity were either loaded as 4 μL aliquots of 48-hour cultures, patched from solid cultures, or replica plated with sterile velvets onto the seeded MB plates. The plates were incubated during 4–8 days at 12 or 20 °C.

2.4. Grape must fermentation for base winemaking

Three fermentation sets were prepared independently with the same yeast strains but at different dates. Each set consisted of four vinifications – two inoculated with *S. cerevisiae* (*Sc* E7AR1 or *Sc* EX85R) and two with *T. delbrueckii* (*Td* EX1180-11C4 or *Td* EX1180-2K$^-$) – so that there were 12 vinifications in total (three for each yeast). Must fermentations were performed in 100-L stainless steel tanks with 80 L of cold-settled Macabeo grape must (17–19 °Brix, pH 2.92–3.25, 88–98 NTU, 50 mg/L SO_2, malic acid 1.35–1.46 g/L, lactic acid 0.06–0.12 g/L, and 0.3 g/L Actimax nutrients from Productos Agrovin S.A.). Yeast cells were cultured in YEPD broth for 2 days at 30 °C, washed twice (by centrifugation) with sterile water, and inoculated in the must to a final concentration of 2–4 × 10^6 cells/mL for *S. cerevisiae* and 2–4 × 10^7 cells/mL for *T. delbrueckii*. The fermentations were done at 16–18 °C and the density, °Brix, and yeast growth (total and viable yeast cells) were monitored. When reducing sugars reached around 1%, the tanks were hermetically closed to avoid wine oxidation. The settled solids were discarded at the end of fermentation, and a sample of each wine was centrifuged for assay. The wines were stored at 12 °C. The settled solids were again discarded at 30 days from the beginning of fermentation. Settled solids were discarded once again at 60 days, and assays for organoleptic quality, aroma compound, and foaming parameter (HM, HS, and TS) were performed. The organoleptic characteristics (flavour and colour) of each wine were tested once using normalized glasses (ISO 3591.1977) as previously described (Ramírez et al., 2016).

2.5. Base wine fermentation for sparkling-wine making

All second fermentations were done with an *assemblage* of some *S. cerevisiae* (*Sc* E7AR1 or *Sc* EX85R) single-inoculated base wines (pH 2.95, 6.1 g/L total acidity, 1.1 g/L reducing sugars, and 10.2% alcohol v/v). For stabilization, base wine was cold-settled at 10 °C for 5 months at which time the settled solids were discarded. Sparkling wines were made by the traditional method of elaborating "cava" (closed-bottle-fermented sparkling wine, a VECPRD according to the European Union CEE 1993/1999) (Boletín Oficial del Estado, 1991). Before base wine inoculation, yeasts were adapted to growth in this base wine as follows. Yeast pellets (2–4 × 10^9 CFU/mL) from a 48-hour YEPD cultures were resuspended in sterile water supplemented with 2.4% sucrose and 0.2% diammonium phosphate ($(NH_4)_2$ PO_4), and incubated at room temperature (18–22 °C) for about 2 h. The cultures were then diluted with one volume of a mixture 1:1 of water:base-wine (pH 2.95, 6.1 g/L total acidity, 1.1 g/L reducing sugars, 10.2% alcohol) containing 2.4% sucrose and incubated at 18–22 °C for about 5 h. Thereafter, the cultures were then diluted again with one volume of base wine supplemented with 2.4% sucrose and incubated overnight at room temperature. Finally, the cultures were diluted again with 1.5 volumes of base wine with 2.4% sucrose and incubated for about 5 h at 18–22 °C. This culture contained a final yeast concentration of 2–6 × 10^8 CFU/mL.

For experiments under low-pressure conditions (LPC), base wine was supplemented with 2.4% sucrose and 0.02% diammonium phosphate, and single inoculated with each *S. cerevisiae* (*Sc* EX85, *Sc* EX85R, *Sc* EX229, or *Sc* EX229-R1) or *T. delbrueckii* strain (*Td* EX1180, *Td* EX1257, *Td* EX1180-11C4, or *Td* EX1180-2K$^-$) (killer or non-killer) or mixed inoculated with *S. cerevisiae* and *T. delbrueckii* (*Sc* EX85R + *Td* EX1180, *Sc* Rod23-1B + *Td* EX1180, *Sc* EX229 + *Td* EX1180-2K$^-$, or *Sc* EX85 + *Td* EX1180-2K$^-$) (killer and sensitive) in 15-mL capped Falcon conical polypropylene centrifuge tubes from Fisher Scientific (where the pressure cannot exceed 1.5 atm because the CO_2 leaks through the cap-tube joining). The total number of second fermentations was twelve. The occurrence of killer activity was expected in mixed-inoculated wines, while no killer activity was expected in single-inoculated wines. The intended amount of yeast inoculum was about 2–4 × 10^7 viable cells/mL for *S. cerevisiae* and *T. delbrueckii* in both single- and mixed-inoculated wines.

For sparkling-wine making under high pressure conditions (HPC), base wine was supplemented with 2.4% sucrose and 0.02% diammonium phosphate. Thereafter, the base wine was single- or mixed-inoculated in 0.75 L capped bottles where high pressure above 6 atm could be reached after the second fermentation. Four independent sets of second fermentations were done for each condition. Each replicate was made with the same base wine, but different adapted yeast culture. The replicates were done at different times. A total of 80 bottles were

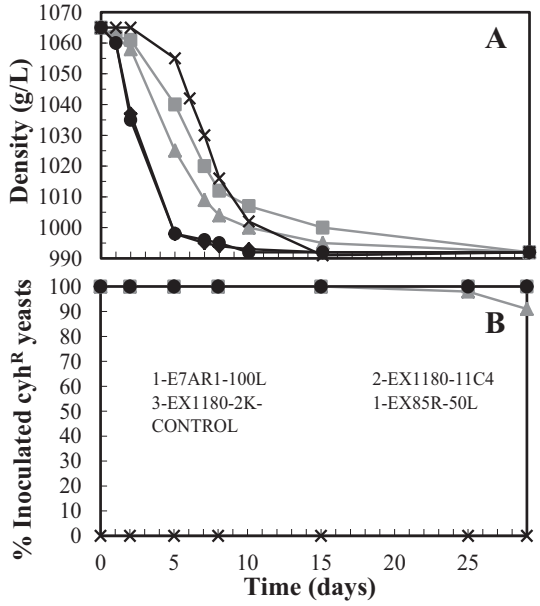

Fig. 1. Must fermentation kinetics and yeast population dynamics of one set of vinifications done with Macabeo grape must. Each yeast was (single) inoculated in the fresh must at a cell concentration of 2–4 × 10^6 CFU/mL for the *S. cerevisiae* strains *Sc* E7AR1 (K2, cyh^R) or *Sc* EX85R (non-killer, cyh^R), and 2–4 × 10^7 CFU/mL for the *T. delbrueckii* strains *Td* EX1180-11C4 (Kbarr-1, cyh^R) or *Td* EX1180-$2K^-$ (non-killer, cyh^R). A: Evolution of must/wine density. B: Evolution of the percentage of each inoculated yeast (cyh^R) during the must fermentation. Symbols: non-inoculated control, (−×−), *Sc* E7AR1 (−♦−), *Sc* EX85R (−●−), *Td* EX1180-11C4 (—■—), and *Td* EX1180-$2K^-$ (−▲−).

done for each condition. For each set, base wine was single inoculated with *S. cerevisiae* (*Sc* EX229), *T. delbrueckii* (*Td* EX1180-$2K^-$), or mixed inoculated with *S. cerevisiae* + *T. delbrueckii* (*Sc* EX229 + *Td* EX1180-$2K^-$) strains. The total number of sparkling wines was thus twelve. The intended amount of yeast inoculum was about 2–4 × 10^6 viable cells/mL for *S. cerevisiae*, and 2–4 × 10^7 viable cells/mL for *T. delbrueckii*, in both single- and mixed-inoculated wines. As in LPC, to avoid any putative interference with foam-influencing compounds released by yeasts, no bentonite was added.

Since 20 °C is the maximum temperature allowed by the law regulating cava sparkling-wine making, the second fermentation (in LPC and HPC) was done at 18–19 °C for the first 15 days to increase the killer effect, and thereafter at 12–14 °C. Samples for microbiological and chemical assay were taken in duplicate at time 0, and then at around 7, 15, 30, 60, 90, 180, and 270 days of fermentation/aging. Finally, after 270 days of aging, the HPC sparkling wines were riddled for 15–60 days to move the lees to the bottle neck. Finally, after disgorging, the organoleptic and foam assays were done.

The descriptive organoleptic analyses were done by an expert panel of 12 judges as previously described (Velázquez et al., 2016). The olfactory intensity, full body (mouthfeel), aged flavour descriptors (such as toasty and yeasty), and flavour persistence of wines were evaluated. The experts scored the wines on a six-point scale (0 = very poor, 1 = deficient, 2 = acceptable, 3 = good, 4 = very good, and 5 = excellent) and the maximum score possible (60 points) was considered 100% preference. Additionally, to evaluate the effect on sparkling-wine quality of single or mixed inoculation with *T. delbrueckii*, the wines were tasted again by ten different expert consumers. In this case, two sensory triangle tests (Jackson, 2002) were conducted to compare *S. cerevisiae* single-inoculated with both *T. delbrueckii* single-inoculated and *S. cerevisiae* + *T. delbrueckii* mixed-inoculated wines. The objective of this additional trial was to determine whether tasters were able to differentiate wines according to the yeast inocula that had been used.

2.6. Determination of inoculated yeast proportions during first and second fermentation

The percentage of genetically marked cyh^R and $RHOD^{PC}$ yeasts was determined by the replica-plating method (Pérez et al., 2000) as described previously (Velázquez et al., 2016). The percentage of wild yeasts was determined by mtDNA restriction pattern analysis (Maqueda et al., 2010). Eventually, this same procedure was used to validate the results obtained by the replica-plating analysis (Velázquez et al., 2016).

2.7. Determination of the amount of damaged/dead and destroyed/autolysed yeast cells

Cell death was determined by staining with methylene blue followed by observation under a microscope with a Nomarski 60× objective (Velázquez et al., 2016). Since the morphological changes in the yeast cells during the second fermentation of sparkling wine are very variable, the total percentage of damaged/dead cells was calculated as the sum of blue, empty, and destroyed/autolysed cells. Blue cells include dead yeasts and near-death yeast (with sublethal damages) that can still grow in fresh YPD medium.

2.8. Statistical analysis

The data were analysed for statistical significance by a one-way analysis of variance ($p < 0.05$), and bivariate correlations between the variables analysed were determined by Pearson's correlation coefficient. Duncan's test was used for multiple comparisons of means. SPSS version 20.0 (Chicago, IL) was used for the calculations.

3. Results

3.1. Base-wine making with T. delbrueckii and S. cerevisiae yeasts

Killer and non-killer strains of *T. delbrueckii* and *S. cerevisiae* were single inoculated in carefully clarified must (< 100 NTU turbidity). Therefore, the amount of wild yeasts present originally in the must was fairly low (2.15–7.5 × 10^3 CFU/mL). Three fermentation sets were done with three similar musts from Macabeo grapes. A non-inoculated spontaneous fermentation was performed in each set of fermentations. The fermentations inoculated with *S. cerevisiae* strains were always the fastest ones. The *T. delbrueckii* fermentations started as quick as those of *S. cerevisiae*, but they slowed down progressively. Non-inoculated fermentations were the slowest to start, but finished earlier than vinifications inoculated with *T. delbrueckii* (see Fig. 1A for a set of fermentations as example). Wild *Saccharomyces* yeasts, which were monitored by mtDNA-RFLP analysis (Maqueda et al., 2010), were only detected at the end of the fermentations inoculated with *T. delbrueckii* (Supplementary Fig. S1), and were found in lower proportions in fermentations inoculated with killer yeasts than with non-killer yeasts (see the example vinifications in Fig. 1B). The inoculated *S. cerevisiae* strains (killer or non-killer) fully dominated (100% ratio) from the beginning to the end of fermentation. The mean *T. delbrueckii* dominance ratio decreased to 93.8 ± 4.5% at tumultuous fermentation and to 76.4 ± 17% at the end of fermentation (Table 2). Nevertheless, the inoculated *T. delbrueckii* strain can still be considered as the dominant yeast in the process. Similar fermentation kinetics and yeast population dynamics were found in the three sets of independent vinification trials. There was no malolactic fermentation in any wine, even in the wines inoculated with *T. delbrueckii* strains that had > 5 g/L reducing sugars (Table 2), which favours the growth of lactic acid bacteria (Ramírez et al., 2016; Velázquez et al., 2015).

According to the wine parameters values, both base wine types were non-defective good-quality products. Significant differences were found for the amount of polysaccharides, proteins, Σ ethyl esters, and Σ acids; and also for the fermentation kinetics (T15 and T100) and foaming

Table 2
Macabeo must fermentation parameters and results of the corresponding base wine analyses to study the differences between inoculations with *S. cerevisiae* (*Sc*) or *T. delbrueckii* (*Td*) yeasts.

Parameter	Yeast species		p^a
	Sc	*Td*	
T15 (days)	1.58 ± 0.05	3.81 ± 0.3	0.000
T100 (days)	5.80 ± 0.5	18.2 ± 2.2	0.001
Preference (%)	59.0 ± 3.6	51.4 ± 4.4	0.174
Proportion at TF (%)	100 ± 0.0	93.8 ± 4.5	0.209
Proportion at EF (%)	100 ± 0.0	76.4 ± 17	0.205
Alcohol (% v/v)	10.5 ± 0.3	9.78 ± 0.4	0.206
pH	2.98 ± 0.07	3.12 ± 0.05	0.189
Total acidity (g/L)	6.28 ± 0.07	6.10 ± 0.1	0.197
Volatile acidity (g/L)	0.23 ± 0.03	0.27 ± 0.02	0.258
Density (g/L)	991 ± 0.0	995 ± 0.0	0.143
Reducing sugars (g/L)	1.14 ± 0.1	6.46 ± 3.9	0.211
Malic acid (g/L)	1.34 ± 0.1	1.31 ± 0.1	0.898
Lactic acid (g/L)	0.06 ± 0.0	0.10 ± 0.0	0.084
Glycerol (g/L)	6.1 ± 0.2	5.65 ± 0.3	0.315
Polysaccharides (mg/L)	150 ± 5	241 ± 32	0.000
Proteins (mg/L)	9.3 ± 0.4	6.2 ± 0.2	0.000
Σ ethyl esters (mg/L)	19 ± 2.3	11 ± 1.8	0.027
Σ acetate esters (mg/L)	167 ± 16	152 ± 18	0.542
Σ acids (mg/L)	23 ± 1.2	7.3 ± 1.2	0.000
Σ alcohols (mg/L)	153 ± 12	162 ± 16	0.652
Σ monoterpenes (mg/L)	3.25 ± 0.04	3.25 ± 0.02	0.919
Σ furans + phenols (mg/L)	0.20 ± 0.07	0.09 ± 0.03	0.183
Σ lactones (mg/L)	0.24 ± 0.04	4.3 ± 4.23	0.363
Σ norisoprenoids (mg/L)	0.1 ± 0.1	0.3 ± 0.08	0.131
Σ carbonyl compounds (mg/L)	0.01 ± 0.0	0.01 ± 0.0	0.952
Σ other compounds (mg/L)	0.16 ± 0.02	0.22 ± 0.05	0.325
HM (mm)	174 ± 15	33 ± 3.7	0.000
HS (mm)	137 ± 8.7	19 ± 3.3	0.000
TS (s)	111 ± 22	161 ± 33	0.248

The data are the mean ± standard error of 6 independent experiments done with *S. cerevisiae* (*Sc*) and 6 with *T. delbrueckii* (*Td*).
TF, tumultuous fermentation; EF, end of fermentation; T15, time needed to ferment 15% of the total sugars present in the must; T100, time needed to ferment 100% of the total sugars or to get to a non-fluctuating, stabilized level; HM, foam maximum height; HS, foam stability height; TS, foam stability time.
[a] *p*-Values from the ANOVA performed for the wines made with the two yeast species.

parameters HM and HS. Noticeable but statistically non-significant differences were found for the organoleptic quality score, pH, total acidity, density, and amount of reducing sugars (Table 2).

The organoleptic quality was compared only for wines whose fermentation was clearly dominated by the inoculated yeast (*S. cerevisiae* or *T. delbrueckii*). Wines inoculated with a non-killer *T. delbrueckii* yeast that became replaced by wild *S. cerevisiae* yeasts, falling to proportions of < 95% at TF and 70% at EF, were not considered for this analysis (actually, this led to only one fermentation inoculated with non-killer EX1180-2K$^-$ strain being discarded).

In the sensory triangle test, all ten expert consumers were able to distinguish *S. cerevisiae* from *T. delbrueckii* wines ($p < 0.001$), mainly because *T. delbrueckii* wines were aged, with few aromas of fresh fruit, and rare compared to the usual base wines for cava making. In the descriptive organoleptic analysis, *S. cerevisiae* wines had medium-intensity fresh fruity aromas, while *T. delbrueckii* wines had low-intensity fresh fruity aromas, unusual dried fruit (cooked fruit, pastry, and candy) aromas, and intense aged/evolved taste. Although *S. cerevisiae* wines were preferred over *T. delbrueckii* wines (59.0 ± 3.6% vs 51.4 ± 4.4% preference, respectively), no statistically significant differences were found in the organoleptic quality scores. While the total (summatory) amount of ethyl esters, acetate esters, organic acids, and furans + volatile phenols was greater in the *S. cerevisiae* than in the *T. delbrueckii* wines, and the contrary was the case for the total amount of alcohols, lactones, and norisoprenoid compounds, only the ethyl ester and organic acid differences were statistically significant. No differences were found for the amounts of monoterpenes and carbonyl compounds (Table 2).

The *S. cerevisiae* wines had significantly greater values of the amount of proteins, foam maximum height (HM), and foam stability (HS) than the *T. delbrueckii* wines, but the contrary was the case for the amount of polysaccharides and stability time (TS), although this last difference was not statistically significant (Table 2). Generally, with all the wines considered together, the results found for the values of HM and HS were opposite to those for TS: low HM and HS coincided with high TS, and vice versa. There were significant positive correlations of HM and HS with the amounts of proteins and 31 volatile compounds (mainly C_4–C_{16} ethyl esters), and of TS with isoamyl lactate, isobutyric acid, and several alcohols (isobutanol, 3-exen-1-ol, and benzyl alcohol). On the contrary, there were negative correlations of HM and HS with the amount of polysaccharides, ethyl propanoate, ethyl isobutyrate, 3-ethoxy-propan-1-ol, and isobutyric acid, and of TS with various esters (ethyl lactate, 2-phenyl-ethyl-acetate, ethyl malate, and ethyl succinate) and alcohols (1-butanol, 1-pentanol, 1-hexanol, linalool, 1-octanol, and terpineol). The correlation of TS with polysaccharides was also negative, although not statistically significant (Fig. 2).

Only *S. cerevisiae* single-inoculated base wines were used to get the stabilized base wine for sparkling-wine second fermentation because the organoleptic quality of the *T. delbrueckii* single-inoculated base wines was considered to be atypical for this purpose, although it may be adequate for the elaboration of other wine styles.

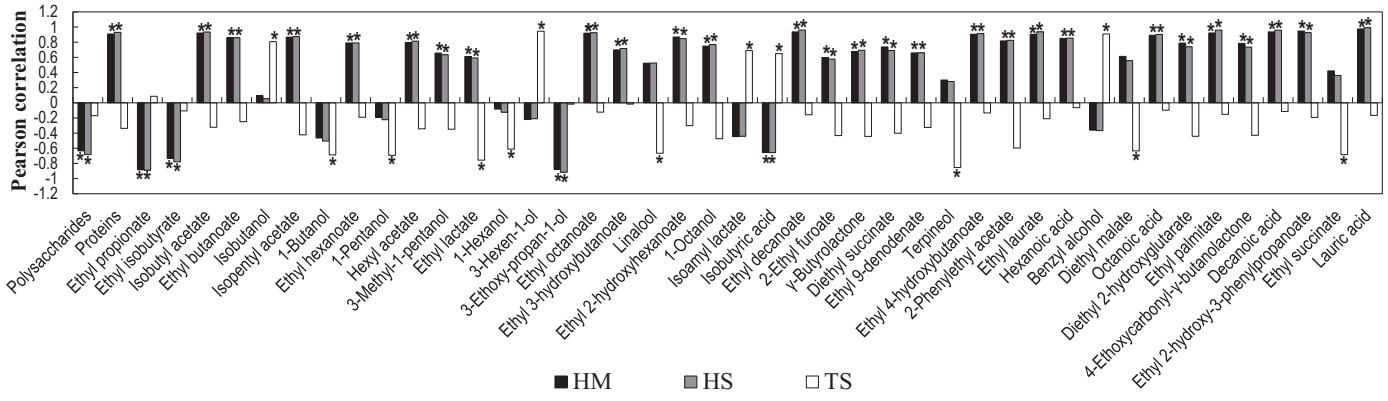

Fig. 2. Pearson correlation coefficients between each of the foaming parameters (HM, HS, and TS) and the concentrations of polysaccharides, proteins, and 42 aroma compounds of the twelve base wines (from independent experiments, six with *S. cerevisiae* and six with *T. delbrueckii*). *Compounds for which the correlation (two-tailed) was statistically significant at the $p < 0.05$ level. Only those compounds for which at least one of the three foaming parameter correlations was significant are shown.

3.2. Sparkling-wine making with T. delbrueckii and S. cerevisiae yeasts

Before the elaboration of sparkling wines under the usual industrial conditions (in particular, in glass bottles), the ability of *T. delbrueckii* to perform second fermentation of the stabilized base wine supplemented with sucrose was tested in tightly-capped plastic tubes (Falcon). Under these conditions, CO_2 pressure can increase to up to 1.5 atm (low-pressure conditions - LPC). The stabilized base wine was an *assemblage* of several wines made with Macabeo must that was single inoculated with *S. cerevisiae*. Single inoculation with *S. cerevisiae* and mixed inoculation with *S. cerevisiae* + *T. delbrueckii* yeasts completed the second fermentation in about 10 days. However, fermentation following single inoculation with *T. delbrueckii* stopped after 40 days, still leaving 2.4–11.1 g/L reducing sugars. The viable yeast population increased during the first ten days of fermentation in all vinifications, and, interestingly, in most cases there were still $> 10^5$ CFU/mL viable yeasts remaining after 9 months. In mixed-inoculated fermentations, the proportion of *T. delbrueckii* decreased while that of *S. cerevisiae* increased to become the dominant yeast in the process, independently of whether one of these yeasts was killer or non-killer (Supplementary Fig. S2). The yeasts *S. cerevisiae* EX229 and *T. delbrueckii* EX1180-2K$^-$ were selected for the next experiment: HPC sparkling-wine making in glass bottles. EX1180-2K$^-$ was selected because it had the best fermentation kinetics of all the *T. delbrueckii* strains tested, and EX229 because it had good fermentation kinetics and killed *T. delbrueckii* in the low pH condition (< 3.3) that is required for sparkling-wine making (Supplementary Fig. S3).

In the HPC experiments, four sets of three in-bottle second fermentations were done with the same stabilized base wine. Single (*S. cerevisiae*) as well as mixed (*S. cerevisiae* + *T. delbrueckii*) inoculated yeasts completed second fermentation to leave < 4 g/L glucose + fructose after 30 days, and fermentation was fully complete after 60 days. However, single-inoculated *T. delbrueckii* showed slow fermentation kinetics that stopped after 60 days, leaving 7.4 g/L glucose + fructose in the wine (see Fig. 3A for a set of fermentations as example). The result was that *S. cerevisiae* inoculated wines achieved high pressures of about 6 atm after 60 days of fermentation, while *T. delbrueckii* single-inoculated wine only reached about 3 atm, with this value remaining after nine months (Fig. 3B). The amount of viable cells in the (single- or mixed- inoculated) *T. delbrueckii* wines increased during the first ten fermentation days and decreased progressively thereafter, while in the *S. cerevisiae* single-inoculated wines it increased during the first twenty days, was maintained until day 60, and then progressively decreased. The fastest decrease occurred in the *T. delbrueckii* single-inoculated wine, and the slowest in the *S. cerevisiae* single-inoculated wine. Contrary to the LPC results (Supplementary Fig. S2), no viable *T. delbrueckii* yeast was found after 90 days of fermentation/aging, and no viable *S. cerevisiae* yeast after 270 days of aging (Fig. 3C).

The proportion of damaged/dead cells increased during the first thirty days more rapidly in the *T. delbrueckii* than in the *S. cerevisiae* fermentations (Fig. 3E), similar to what was found in LPC (Supplementary Fig. S2). The proportion of viable killer *S. cerevisiae* yeasts increased while that of killer-sensitive *T. delbrueckii* yeasts decreased in the mixed-inoculated fermentations (Fig. 3D). This was in agreement with the findings that *Sc* EX229 (killer Klus) killed *Td* EX1180-2K$^-$ (killer-sensitive) in the killer plate assay (Fig. S3). However, no difference in the proportion of damaged/dead cells was found between *T. delbrueckii* mixed-inoculated and single-inoculated fermentations during the first 30 days (Fig. 3E). This result was unexpected because the proportion of damaged/dead cells should have increased more quickly in mixed-inoculated fermentations than in single-inoculated, if there really was killer activity. Neither were any destroyed/autolysed cells detected during the first 15 days of second fermentation (data not shown), contrary to previous findings in sparkling wine mixed-inoculated with killer and sensitive *S. cerevisiae* strains (Velázquez et al., 2016). Moreover, after 30 days, the proportion of damaged/dead cells increased faster in *T. delbrueckii* single-inoculated than in mixed-inoculated fermentations, reflecting the presence of viable *S. cerevisiae* cells in the latter.

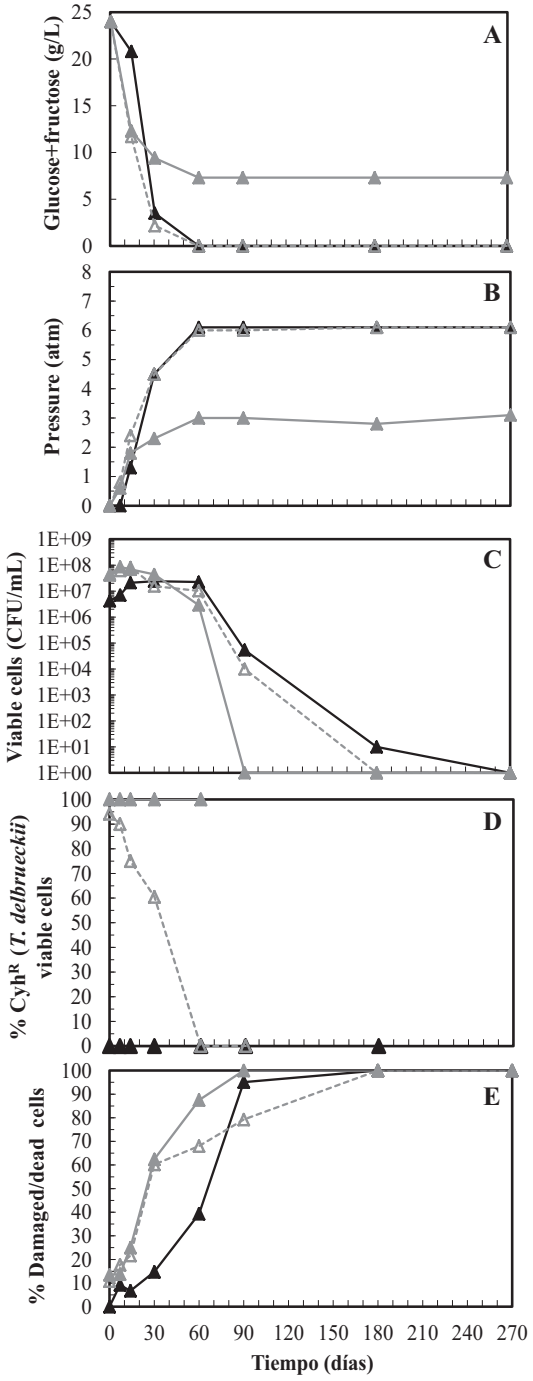

Fig. 3. Fermentation kinetics and yeast-population dynamics during one set of sparkling-wine second fermentations. Base wine was single inoculated with *S. cerevisiae* (*Sc* EX229) or *T. delbrueckii* (*Td* EX1180-2K$^-$) strains, or mixed inoculated with *S. cerevisiae* + *T. delbrueckii* (*Sc* EX229 + *Td* EX1180-2K$^-$) strains under high-pressure conditions (HPC). A: Evolution of sugar uptake (glucose + fructose). B: Pressure inside the bottle. C: Viable yeasts. D: Percentage of cyh^R yeast cells in each fermentation. E: Percentage of damaged/dead cells. Symbols: *Sc* EX229 (—▲—), *Td* EX1180-2K$^-$ (—△—), *Sc* EX229 + *Td* EX1180-2K$^-$† (--△--). The symbol (†) indicates the *Torulaspora* strain that was replaced by *Saccharomyces* in mixed-inoculated fermentations. Note that the cyh^R strain was not the dominant yeast in the mixed-inoculated wine.

Table 3
Some relevant parameters and the organoleptic quality (taster preferences) of sparkling wines (cavas) made by single or mixed inoculating base wine with strains of *S. cerevisiae* and *T. delbrueckii* under HPC.

Parameter	Yeast species			p^a
	Sc	*Td*	*Sc* + *Td*	
Alcohol (%, v/v)	11.4 ± 0.01a	10.6 ± 0.15b	11.3 ± 0.32a	0.050
pH	3.16 ± 0.01a	3.57 ± 0.04c	3.28 ± 0.07b	0.010
Total acidity (g/L)	5.82 ± 0.05a	5.15 ± 0.05b	5.35 ± 0.05b	0.010
Volatile acidity (g/L)	0.27 ± 0.02a	0.47 ± 0.01b	0.44 ± 0.01b	0.010
Glucose + fructose (g/L)	0.06 ± 0.0a	7.4 ± 0.1b	0.07 ± 0.01a	0.000
Density (g/L)	989 ± 0.0a	998 ± 0.0b	992 ± 0.0a	0.007
Pressure (atm)	6.1 ± 0.05a	3.2 ± 0.90b	6.05 ± 0.05a	0.000
Preference (%)	65 ± 0.00a	47 ± 1.50b	78 ± 2.50c	0.000

The data are the mean ± standard error of four independent experiments done with each of *S. cerevisiae* (*Sc*), *T. delbrueckii* (*Td*), and *Sc* + *Td*.
Different lower case letters (a, b, and c) in a given row mean significantly different homogeneous groups found with the Duncan test at $p < 0.05$.
[a] *p*-Values from the ANOVA performed for the wines made with the three types of inoculum.

3.3. Physicochemical and organoleptic analyses of sparkling wines

The wine parameter values indicated that all the *S. cerevisiae* sparkling wines, single- or mixed-inoculated, were non-defective and good-quality products. However, the single-inoculated *T. delbrueckii* sparkling wines presented lower pressure, alcohol, and total acidity than those inoculated with *S. cerevisiae*, while the contrary was the case for volatile acidity, amount of reducing sugars, and pH. These differences were statistically significant in all cases. Those differences related to pressure, volatile acidity, reducing sugars, and pH may already explain the lowest organoleptic quality score (preference) of the *T. delbrueckii* sparkling wines (Table 3).

In general, the sparkling-wine foaming parameters (HM, HS, and TS) were much lower than those of base wine fresh made with *S. cerevisiae* and of stabilized base wine (especially HS and TS in this latter case). Sparkling wines inoculated with *S. cerevisiae* (single or mixed) had the best HM, and those inoculated with *T. delbrueckii* (single or mixed) the best TS (Fig. 4A). These last wines also contained the greatest amounts of total polysaccharides and mannan. No difference among the sparkling wines was found for the amount of proteins, although this increased to > 10 mg/L from the original 3.3 mg/L found in stabilized base wine (Fig. 4B). Neither were there any increases in the amounts of polysaccharides, mannan, or proteins in mixed-inoculated wines (possible killer effect) with respect to *T. delbrueckii* single-inoculated wines (no killer effect). No significant correlations between foaming parameters and aroma compounds were found in the sparkling wines, where the level of the foaming parameters was much lower than in *S. cerevisiae* base wine, and the difference among sparkling wines was much lower than that which had been observed between *S. cerevisiae* and *T. delbrueckii* base wines (Table 2).

All ten expert consumers were able to distinguish *T. delbrueckii* single-inoculated from both *S. cerevisiae* single-inoculated and *S. cerevisiae* + *T. delbrueckii* mixed-inoculated sparkling wines in the sensory triangle test ($p < 0.001$). This was mainly due to the fact that all tasters detected low CO_2 pressure and almost no fresh fruit aroma in *T. delbrueckii* wines. Only one taster failed to distinguish *S. cerevisiae* single-inoculated from *S. cerevisiae* + *T. delbrueckii* mixed-inoculated wines. Eight of the ten tasters preferred the mixed-inoculated wines over the rest ($p < 0.01$), mainly because their good complexity that combined good aromas of fresh fruit with some dry fruit aromas. In the descriptive organoleptic analysis, again, *S. cerevisiae* wines (single- or mixed-inoculated) were preferred over *T. delbrueckii* single-inoculated wines because of their better fruity aroma. Among them, wines mixed-inoculated with *S. cerevisiae* + *T. delbrueckii* were the more appreciated (78 ± 2.5% preference) because of their complexity, better mouth-feel, additional dried-fruit aromas, and some pleasant aged character. On the contrary, *T. delbrueckii* single-inoculated wines got poor (disqualifying) scores (47 ± 1.5% preference) because they reached neither the good maturity and aroma complexity of mixed-inoculated wines nor the legally required pressure (> 3.5 atm), lacked the fresh-fruit aroma of *S. cerevisiae* inoculated wines, and had an unpleasantly strong aged character (see Table 3).

No significant or relevant differences were found between the three types of sparkling wines for the total amounts of ethyl esters, acetate esters, organic acids, alcohols, monoterpenes, lactones, carbonyl compounds, furans + phenols, or norisoprenoids. Significant differences were found, however, for 15 of the 75 volatile compounds analysed independently. The amounts of ethyl propanoate (odour descriptor: banana, apple), isoamyl alcohol (alcohol, nail polish), 3-ethoxy-1-propanol (fruity), isobutyric acid (cheese, sour, butter), butanoic acid (cheese, acidic sour, butter, fruit), 4-vinylguaiacol (smoky, spices), and ethyl succinate (caramel, coffee) were significantly greater in *T. delbrueckii* than in *S. cerevisiae* wines. On the contrary, the amounts of the major ethyl esters responsible for fruity aromas – ethyl hexanoate, ethyl octanoate, ethyl decanoate, and ethyl 9-decenoate – and of γ-butyrolactone (cooked peach, coconut, caramel, toasty), terpineol (lilac), furfural (caramel), and β-damascenone (sweet, fruity) were more abundant in *S. cerevisiae* wines (single or mixed inoculated) than in those made with *T. delbrueckii* (Fig. 4C). With regard to the detection thresholds of these 15 compounds, the greatest odour activity values (OAVs) corresponded to three compounds with fresh fruit odour descriptors: two which were more abundant in *S. cerevisiae* than in *T. delbrueckii* wines (ethyl hexanoate [banana, green apple] and ethyl octanoate [banana, pineapple, pear, floral]), and one which was more abundant in *T. delbrueckii* wines (ethyl propanoate [banana, apple]). The odour impact of ethyl hexanoate and ethyl octanoate seemed to be the most relevant, and was greater in *S. cerevisiae* than in *T. delbrueckii* wines (Fig. 4D). Furthermore, the amount of β-damascenone found in *S. cerevisiae* (but not in *T. delbrueckii*) wines may have indirectly enhanced the fruity aroma of *S. cerevisiae* wines (Fig. 4D) because this compound has a very low detection threshold (Pineau et al., 2007).

4. Discussion

4.1. Influence of T. delbrueckii on must fermentation and quality of base wine

Fermentations of *T. delbrueckii* took longer to complete than those of *S. cerevisiae* or non-inoculated fermentations. This favoured the growth of wild *Saccharomyces* yeasts by the end of fermentation, but was reduced or abolished by using killer strains of *T. delbrueckii* (Fig. 1A and B). The mean dominance ratio of *T. delbrueckii* was high, frequently above 90% at TF and 70% at EF. There frequently remained, however, > 5 g/L of reducing sugars in the final wines (Table 2). These results are coherent with previous findings for the same yeast strains in table wines (Ramírez et al., 2016; Velázquez et al., 2015). There was no

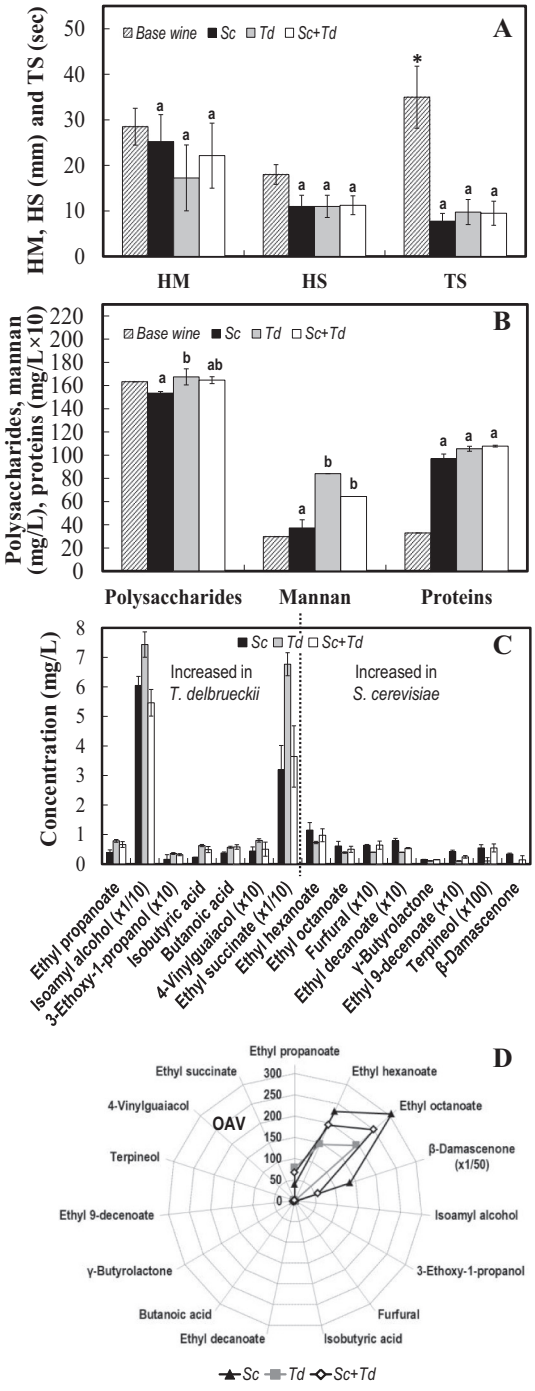

Fig. 4. Analysis of sparkling wines from the second fermentation of stabilized base wine single- or mixed-inoculated with *S. cerevisiae* and *T. delbrueckii*. Parameter values correspond to sampling after nine months of fermentation/aging. A: Foaming parameters – HM, maximum height; HS, foam stability height; TS, foam stability time. *Sc*, *S. cerevisiae* EX229; *Td*, *T. delbrueckii* EX1180-2K$^-$; *Sc* + *Td*, *S. cerevisiae* EX229 + *T. delbrueckii* EX1180-2K$^-$. *TS value of base wine divided by ten. B: Mean polysaccharide, mannan (measured as mannose), and protein concentrations. Different lower case letters (a, b, and c) mean significantly different groups found with the Duncan test at $p < 0.05$. C: Aroma compounds for which statistically significant differences were found between *Sc*, *Sc* + *Td*, and *Td* sparkling wines. D: Mean odour activity values (OAV) for the same aroma compounds. The four highest OAV compounds are shown together in the figure. The data are the mean ± standard error of 12 (four *Sc*, four *Td*, and four *Sc* + *Td*) independent vinifications.

malolactic fermentation in any wine. This, together with the high dominance ratio achieved for the *T. delbrueckii* inoculated strains, provides a good opportunity to compare the parameters of *T. delbrueckii* vs *S. cerevisiae* wines, avoiding the potential influence of other microorganisms such as wild yeasts or lactic acid bacteria.

The two base-wine types were markedly different. Those of *S. cerevisiae* were preferred over those of *T. delbrueckii* because of their better fresh fruity aromas. The unusual dried fruit aroma and aged taste noted in the *T. delbrueckii* wines were similar to previous findings in white table wines made with the same *T. delbrueckii* strains (Velázquez et al., 2015). Now, however, these characters were unacceptable for the tasters, mainly because a standard organoleptic quality is required to elaborate cava-certified sparkling wines. This difference in organoleptic quality can principally be understood as a reflection of the greater amount of ethyl and acetate esters in *S. cerevisiae* than *T. delbrueckii* wines, as had previously been described in white table wines (Velázquez et al., 2015).

The *S. cerevisiae* wines presented greater amounts of proteins and values of the HM and HS foaming parameters than the *T. delbrueckii* wines. A priori, this indicates that proteins may improve the ability of wine to form stable foam, as is also suggested by the significant positive correlations found for HM and HS with the amount of proteins when considering all the wines together. A similar correlation had previously been found in base wine (González-Royo et al., 2014), although in that work the protein content, HM, and HS were greater in *T. delbrueckii* than in *S. cerevisiae* wines. However, we found the amount of polysaccharides to be greater in *T. delbrueckii* than in *S. cerevisiae* wines, which, while coherent with findings reported for synthetic base wine, is in disagreement with the findings of that same work (González-Royo et al., 2014) for white base wine. Other studies have also reported a notable increase of polysaccharides in table wines made with *T. delbrueckii* (Belda et al., 2015; Domizio et al., 2014). There may be a variety of factors behind these conflicting results, such as the strain of *T. delbrueckii* used, its relative abundance with respect to *S. cerevisiae* during each must fermentation stage, the specific composition of the starting must, etc. In any case, no statistically significant positive correlation was found between the amount of polysaccharides and foaming parameters (Fig. 2). The significant positive or negative correlations of the foaming parameters with 31 volatile compounds – especially the positive correlation with C_4–C_{16} ethyl esters as had been described elsewhere for sparkling wine (Gallart et al., 2002; Velázquez et al., 2016) – indicate that other wine compounds may be involved in the wine's foaming quality. If a study compared just a few wine types, any positive correlations it found between foaming parameters and the amounts of both polysaccharides and proteins might just be coincidence or a result of covariance, and hence lead to wrong conclusions being drawn about some relevant positive effect of polysaccharides and proteins on wine foamability. When more wines from independent experiments are compared however, the covariance disappears, and there may even be found a negative correlation. Indeed, in our case, when considering all the wines together, the correlation between polysaccharides and TS was negative (although non-significant) instead of positive. Nevertheless, it should be taken into account that, although *T. delbrueckii*'s wines contained the greatest amount of polysaccharides, their HM and HS may have decreased with respect to *S. cerevisiae*'s wines because the former also contained the greatest amount of some anti-foaming compounds such as the alcohols 1-butanol, 1-pentanol, 1-hexanol, linalool, 1-octanol, and terpineol (Fig. 2).

4.2. Influence of *T. delbrueckii* on the second fermentation of sparkling wines

The preliminary experiment made in LPC (Supplementary Fig. S2) brought out some relevant aspects of the capability of our yeasts to perform base wine fermentation – mainly that single-inoculated *S. cerevisiae* and mixed-inoculated *S. cerevisiae* + *T. delbrueckii* yeasts can

complete second fermentation, single-inoculated *T. delbrueckii* is unable to complete second fermentation, and *T. delbrueckii* dies so quickly that the killer effect of *S. cerevisiae* on *T. delbrueckii* is not clearly detectable in mixed-inoculation fermentations. Furthermore, the results of this experiment allowed us to select *S. cerevisiae* EX229 and *T. delbrueckii* EX1180-2K$^-$ as appropriate yeasts for sparkling-wine making in glass bottles (HPC).

The HPC second-fermentation results confirmed what was to be expected given the LPC results: the selected *S. cerevisiae* strain (single- as well as mixed-inoculated) completed second fermentation to brut nature (< 3 g/L sugar) sparkling wines with great pressure (6 atm), while single-inoculated *T. delbrueckii* showed slow and incomplete fermentation kinetics, leaving about 7.4 g/L glucose + fructose in the wine and a poor pressure (about 3 atm). This confirmation indicated that the preliminary LPC experiment using plastic tubes was a good approach to the evaluation of second-fermentation performance so as to select yeast strains for sparkling-wine making.

Our results are in disagreement with those recently found for two *T. delbrueckii* strains (DiSVA 130 and DiSVA 313). These strains, single-inoculated in a base wine with high ethanol concentration (11.65 vol %), completed the secondary fermentation of sparkling wine very efficiently (Canonico et al., 2018). These contradictory results could be due to the fact that the strains of *T. delbrueckii* used in this research were different from ours. However, as mentioned in the Introduction section, the dominance of DiSVA 130 and DiSVA 313 during the second fermentation was not confirmed. Therefore, there is a possibility that some contaminant yeasts actually were responsible for the high efficiency of the second fermentation. Moreover, to the best of our knowledge, no previous study has been done on the resistance of these DiSVA strains at high ethanol concentration.

Although viable cells decreased faster in the *T. delbrueckii* than in the *S. cerevisiae* populations for HPC as well as for LPC, this decrease was much faster in both yeasts for HPC than for LPC. As a consequence, no viable *T. delbrueckii* yeast remained alive after 90 days, and no viable *S. cerevisiae* yeast after 270 days. Similar results have been found for other *S. cerevisiae* strains under similar high-pressure working conditions (Feuillat and Charpentier, 1982; Martinez-Rodriguez et al., 2002; Núñez et al., 2005; Velázquez et al., 2016). This increased yeast death rate in HPC relative to LPC is clearly due to the sensitivity of yeasts to CO_2 pressure, which seems to be much greater with *T. delbrueckii* than with *S. cerevisiae*. Consequently, *T. delbrueckii* cell death due to a killer effect in mixed inoculation, if it actually occurred, would have been very low compared to cell death due to the unfavourable environmental conditions for the growth of this yeast (mainly high ethanol concentration and rising CO_2 pressure). In particular, no killer effect of killer *S. cerevisiae* over sensitive *T. delbrueckii* yeasts was detected because no autolysed cells were found during the first days of second fermentation. This result is contrary to what had been found previously in second fermentations mixed-inoculated with killer and sensitive *S. cerevisiae* strains in which this strategy was used to improve yeast autolysis and the quality of traditional sparkling wine (Lombardi et al., 2015; Velázquez et al., 2016). Nonetheless, there still exists the possibility that there was a killer effect, but that any destroyed *T. delbrueckii* cells were harder to detect than those of *S. cerevisiae*.

4.3. Influence of T. delbrueckii on the organoleptic quality of sparkling wines

While *S. cerevisiae* sparkling wines (single or mixed-inoculated) were good-quality products, single-inoculated *T. delbrueckii* sparkling wines were poor quality and obligatorily disqualified products. This was mainly because of their low pressure, high amounts of reducing sugars, and relatively high pH (Table 3). The only possible improvement found in single-inoculated *T. delbrueckii* sparkling wines was greater TS than the *S. cerevisiae* sparkling wines, which again coincided with the greater amount of polysaccharides. Despite this, *S. cerevisiae* sparkling wines showed the best overall foaming properties (HM and HS). Additionally, no difference among the sparkling wines was found for the amount of proteins (Fig. 4B). Taking these results together, it again seems that the amounts of proteins and polysaccharides are less relevant than previously described for the foaming capability of wines (Andrés-Lacueva et al., 1996; Cilindre et al., 2010; Coelho et al., 2011a, 2011b; Girbau-Sola et al., 2002a, 2002b; López-Barajas et al., 2001; Moreno-Arribas et al., 2000; Núñez et al., 2006; Vanrell et al., 2007), at least under our working conditions. Also, we found no significant correlations between foaming parameters and aroma compounds when comparing the sparkling wines, probably because the differences in foaming parameters among these wines were fairly small since they came from the same *S. cerevisiae* single-inoculated base wine. Therefore, greater differences in foaming parameters would seem to be required to be able to find significant correlations reflecting any influence of aroma compounds on foaming parameters such as we found comparing base wine single-inoculated with *S. cerevisiae* or *T. delbrueckii*.

Although *T. delbrueckii* single-inoculated wines were disqualified, *S. cerevisiae* + *T. delbrueckii* mixed-inoculated sparkling wines were actually even more appreciated than *S. cerevisiae* single-inoculated wines. Moreover, most tasters were able to distinguish *S. cerevisiae* + *T. delbrueckii* mixed-inoculated wines from *S. cerevisiae* single-inoculated. In particular therefore, *T. delbrueckii* can be used to improve sparkling-wine quality, but only when mixed-inoculated with *S. cerevisiae*, as had been shown elsewhere for the sequential inoculation of base wine (González-Royo et al., 2014). The increased fruity aroma of *S. cerevisiae* wines (single or mixed inoculated) with respect to *T. delbrueckii* single-inoculated wines was due to the former's greater amounts of ethyl hexanoate, ethyl octanoate, and β-damascenone. These compounds had a major impact on fruity aroma perception (Fig. 4D), similar to what had previously been observed for table wines (Ramírez et al., 2016; Velázquez et al., 2015). The significant increase in the amounts of ethyl propanoate, isobutyric acid, and butanoic acid could explain the complexity, additional dried-fruit aromas, and some pleasant aged character of the mixed-inoculated *S. cerevisiae* + *T. delbrueckii* wines as compared with the *S. cerevisiae* single-inoculated wines (Fig. 4C). Of these three compounds, only the ethyl propanoate content was above the perception threshold. While this may appear to indicate that it by itself may have impacted the aroma perception, it is hard to believe that the differences in wine quality reported by the tasters were due just to this single compound. Instead, it is likely that there was a cooperative effect of other compounds, which may have had no sensory implications individually because they were below the perception threshold, but that modulated or enhanced the effect of ethyl propanoate. Apart from the aforementioned isobutyric and butanoic acids, other possibilities are some alcohols such as 3-ethoxy-1-propanol and some phenols such as 4-vinylguaiacol since these which have also been found at increased levels in table wines and base wines made with *T. delbrueckii* compared to *S. cerevisiae* wines (González-Royo et al., 2014; Ramírez et al., 2016; Velázquez et al., 2015). Although previous work has found that improvement of sparkling-wine quality does not correlate with the amount of polysaccharides or mannoproteins (Velázquez et al., 2016), the better mouth-feel of mixed-inoculated sparkling wines may be due in part to their greater content in these compounds than single-inoculated *S. cerevisiae* wines (Fig. 4B), as has been reported previously (Belda et al., 2015). Against this idea, it could be argued that the polysaccharide content of wine is so low (< 0.2 g/L) that its effect on mouth-feel would have been masked by the influence of residual sugars. The amount of sugars in our *S. cerevisiae* wines was, however, very low (< 0.08 g/L in all cases). As we have suggested elsewhere (Velázquez et al., 2016), these discordant results indicate that the mouthfeel and foamability of sparkling wine could be very complex properties which depend on the interactions of a variety of wine compounds which may change from one winemaking protocol to another which is only slightly different.

5. Conclusions

The killer phenotype allowed our *T. delbrueckii* strains to reduce or abolish the presence of wild yeasts during base-wine fermentation. The foaming capability of *T. delbrueckii* base wines was much lower than that of *S. cerevisiae* wines, and the former's unusual dried fruit aromas and aged tastes made them unacceptable for the elaboration of a typical sparkling wine. Moreover, single *T. delbrueckii* inoculation was unable to complete sparkling-wine second fermentation, which discourages the use of these strains for this purpose. Given that other *T. delbrueckii* strains have been found to complete second fermentation, further research is required to find out whether our results can be generalized for most strains of this yeast species. Despite this, mixed *S. cerevisiae* + *T. delbrueckii* inoculation completed second fermentation to get dry sparkling wines with high pressure. Therefore, this mixed-inoculation showed to be a good option to improve the organoleptic quality of sparkling wines with respect to *S. cerevisiae* single-inoculation, mainly because *T. delbrueckii* increased the amounts of some interesting aromatic compounds.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.09.010.

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