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Base Wine and Traditional Sparkling Wine Making Using *Torulaspora delbrueckii* Killer Yeasts †

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Abstract: The killer strains of *Torulaspora delbrueckii* can be used to improve the dominance of this yeast during must fermentation. The present work analyzes its usefulness for traditional sparkling wine making. *T. delbrueckii* killer strain dominated base wine fermentation better than non-killer strains and produced dried wines. The foam ability of *T. delbrueckii* base wines was very low compared to that of *Saccharomyces cerevisiae*. Significant positive correlations of foam parameters were found with some amounts of C_4–C_{16} ethyl esters and proteins, and negative correlations with some antifoam alcohols. The organoleptic quality of *T. delbrueckii* base wines was considered unusual for cava making. While *S. cerevisiae* (single or mixed with *T. delbrueckii*) completed the second fermentation to produce dry sparkling wines with high CO_2 pressure, single *T. delbrueckii* did not complete this fermentation, leaving sweet wines with low CO_2 pressure. Death due to CO_2 pressure was much higher in *T. delbrueckii* than in *S. cerevisiae*, making any killer effect of *S. cerevisiae* on *T. delbrueckii* irrelevant. However, the organoleptic quality of cava inoculated with mixtures of the two yeast species was better than that of wine inoculated exclusively with *S. cerevisiae*, and no deterioration in the quality of the foam was observed.

Keywords: yeast; killer; *Torulaspora delbrueckii*; sparkling wine; aging; autolysis; foam; aroma

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1. Introduction

The use of non-*Saccharomyces* yeasts is being highly recommended for winemaking because it can improve their organoleptic complexity. Specifically, *Torulaspora delbrueckii* is the most non-*Saccharomyces* yeast species used after *Saccharomyces cerevisiae* in the wine industry. It is a torula-shaped yeast that is smaller and grows somewhat slower than *S. cerevisiae*, but it has interesting technological advantages for making still wines. Its usefulness has been confirmed in several published works, it can reduce volatile acidity and acetaldehyde levels in wines and increase some interesting dried fruit and pastry aromas [1]. Furthermore, it has recently been verified that the sequential inoculation of *T. delbrueckii* and *S. cerevisiae* increases the concentration of glycerol, reduces volatile acidity, and exerts a positive effect on the foam properties of base wines to produce sparkling wines [2].

Foam formation and its stability are very important organoleptic characteristics valued by consumers in sparkling wines such as "cava" (closed-bottle-fermented sparkling wine). It has been described that the foam of cava, mainly the foam stability, de-

pends to a great extent on its content of proteins and mannoproteins [3,4]. It has also been reported that the foam maximum height (HM) correlates negatively with C_8, C_{10}, and C_{12} fatty acids, and positively with the ethyl esters of C_6, C_8, and C_{10} fatty acids [5]. These studies have been carried out with cava made entirely with *Saccharomyces* yeasts. Few studies have been carried out with cava made with non-*Saccharomyces* yeasts such as *Torulaspora*.

An alternative to accelerate the yeast autolysis is to use mixtures of killer and sensitive yeasts as inocula in the second cava fermentation. Killer toxins can kill sensitive cells and accelerate their autolysis [6]. This strategy has not been tested at the winery level until very recently. In this work, it was demonstrated that inoculation with mixed cultures of *S. cerevisiae* killer yeast caused cell death and early autolysis of sensitive yeasts during cava-winemaking, without negatively affecting fermentation kinetics or the consequent increase in pressure, improving the cava foam andits organoleptic quality [7]. To complement these results, it is of interest to analyze the utility of killer *T. delbrueckii* yeast strains, which can dominate must fermentation [1,8], in producing base wine and cava. Furthermore, given the killer effect, it can enhance yeast autolysis and cava quality.It is also necessary to analyze the usefulness of *T. delbrueckii* sensitive strains. This work analyzes the capacity of *T. delbrueckii* (killer and sensitive) to dominate and complete the fermentation in basewinemaking, to carry out the second fermentation at high CO_2 pressure, and the aromatic and sparkling wine quality of the base wine and cava made with *T. delbrueckii* with respect to *S. cerevisiae*.

2. Materials and Methods

For base wine making, a cold-settled Macabeo grape must was used, inoculating with two *T. delbrueckii:* EX1180-11C4 (killer Kbarr-1 and resistant to cycloheximide, cyh^R), and EX1180-$2K^-$ (no-killer, cyh^R); and two *S. cerevisiae*: E7AR1 (killer K2, cyh^R) and EX85R (no-killer, cyh^R), yeast strains. For cavawinemaking, a base wine blend of *S. cerevisiae* was used. Before inoculating the wine, the yeasts were adapted to growth in this medium as previously described [9] and 2.4% sucrose and 0.02% diammonium phosphate were added. Subsequently, the base wine, single (with *S. cerevisiae* EX229, killer Klus and sensitive to cycloheximide, cyh^S; or *T. delbrueckii* EX1180-$2K^-$), and mixed (EX229+EX1180-$2K^-$), wasinoculated in 0.75 L cava bottles, inoculating about 1–4×10^6cells/mL for*S. cerevisiae* or2–4×$10^7$for *T. delbrueckii* and incubating at 18–19 °Cfor 15 days, to enhance the killer effect, which is more effective at this temperature, and then at 12–14 °C for up to 9 months. During the first and second fermentation,the yeast population was monitored by analyzing its resistance to cycloheximide (cyh^R) by replica-plating on YEPD (yeast extract peptone dextrose) (1% Bacto yeast extract, 2% Bacto- peptone, 2% glucose, 2% Bacto-agar) plates supplemented with cycloheximide. For the first fermentation, must density was monitored every day; and for the second fermentation, the pressure was measured (expressed in atm at 20°C) using an aphrometer. Cell death was counted by methylene blue staining, mannoprotein, and protein content as previously described [9]. The wine aroma compounds were measured by GC-MS (Gas Chromatography-Mas Spectrometry), and the foaming parameters using a Mosalux system as described previously [9]. The principal analytical parameters were determined according to EC (Amending regulation EEC Nº 2676/90) (Com-recommended methods and the organoleptic analysis was carried out by a wine-tastingexpert as described previously [9]. The statistical analysis of the data was performed with the parametric ANOVA test (p<0.05), Pearson's correlation, and Duncan's test, using SPSS software version 20.0 for Windows (Chicago, IL).

3. Results and Discussion

3.1. Enhance of Killer T. delbrueckii Yeasts on the First Fermentation and Quality of Base Wine

Fermentation kinetics inoculated with *T. delbrueckii* strains were generally slower than those of *S. cerevisiae*. However, base wines inoculated with killer*T. delbrueckii* dominated fermentation more easily than non-killer *T. delbrueckii* and left the wines dried (Figure 1). In the descriptive organoleptic analysis, *T. delbrueckii* wines were clearly different from those of *S. cerevisiae*. Wine tasters appreciated the latter as they were more intense and fruitier, although the differences in valuation were not statistically significant. *S. cerevisiae* wines were foamier, had more protein, and better foamability (HM) and stable foam (HS). *T. delbrueckii* wines were spicier, with more aging notes, more polysaccharides, and better foam stability time (TS). The concentration of ethyl esters, acetate esters, furans, volatile phenols, and organic acids was higher in the *S. cerevisiae* wines, which would explain their greater aromatic intensity and more fruity character. The higher quantity of proteins could also explain its greater foamability, and its higher quantity of glycerol could explain the lower stability of the foam. The higher amount of alcohol in *T. delbrueckii* wines can explain its lower foamability, and its higher amount of polysaccharides can explain that the little foam that is formed is more stable (Table 1).

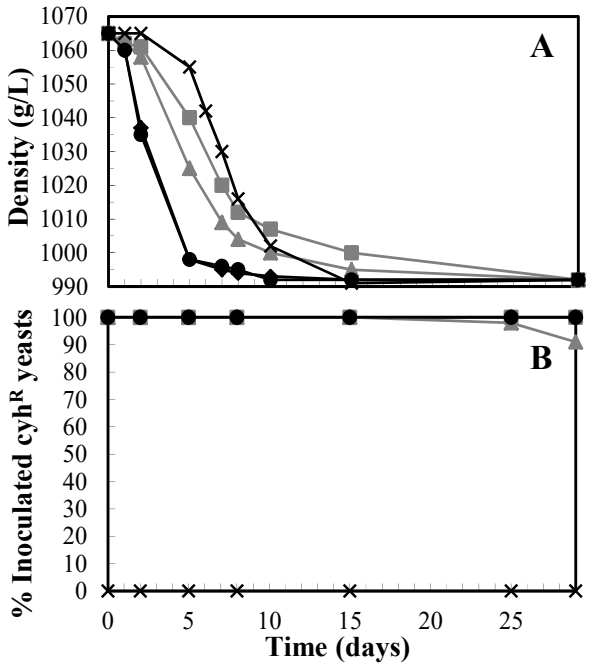

Figure 1. (**A**)Must/wine density. (**B**)Percentage of each inoculated yeast (cyh^R) during the must fermentation. Symbols: non-inoculated control, (—×—), *Sc* E7AR1 (—♦—), *Sc* EX85R (—●—), *Td* EX1180-11C4 (—■—), and *Td* EX1180-2K$^-$ (—▲—). Data taken from [9].

Table 1. White must fermentation parameters and results of the base wines analyses to study the differences between inoculation with *S. cerevisiae*or *T. delbrueckii* yeasts.

Parameter	*S. cerevisiae*	*T. delbrueckii*	*p*[a]
T15 (days)	1.58 ± 0.05	3.81 ± 0.3	0.000
T100 (days)	5.80 ± 0.5	18.2 ± 2.2	0.001
Proportion at EF (%)	100 ± 0.0	76.4 ± 17	0.205
Alcohol (% *v*/*v*)	10.5 ± 0.3	9.78 ± 0.4	0.206
Reducing sugars (g/L)	1.14 ± 0.1	6.46 ± 3.9	0.211
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 Furans + phenols (mg/L)	0.20 ± 0.07	0.09 ± 0.03	0.183
HM (mm)	174 ± 15	33 ± 3.7	0.000
HS (mm)	137 ± 8.7	19 ± 3.3	0.000
TS (sec)	111 ± 22	161 ± 33	0.248

T15, time required for fermenting 15% of sugars present in the must; T100, time required for fermenting 100% of sugars; EF, end of fermentation; HM, foam maximum height; HS, foam stability height; TS, foam stability time.[a]*p*-values from the ANOVA carried out for two types of wine. Data taken from [9].

In general, considering all the wines together, there was a significant positive correlation of HM and HS with proteins and 31 aromatic compounds, mainly C_4-C_{16} ethyl esters; and TS with various alcohols. The correlation of HM and HS with polysaccharides was negative, as was that of TS with other 35 compounds, mainly alcohols (Figure 2). Some of these foam correlations with aromatic compounds have already been previously described for sparkling wines, especially the positive correlations with C_4–C_{16} ethyl esters [5,7], indicating that wine compounds other than polysaccharides and proteins may be importantly implicated in the wine's foaming quality. To continue with the elaboration of cavawinemaking, a base wine blend of *S. cerevisiae* was used for this objective. The organoleptic properties of the base wine of *T. delbrueckii* were considered anomalous for this purpose, nonetheless, these wines were considered of good quality and without defects.

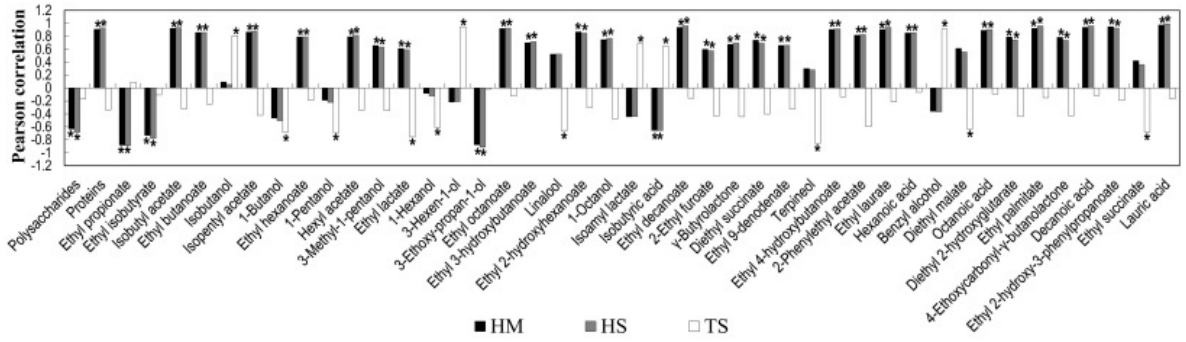

Figure 2. Pearson correlation between foaming parameters (HM, HS, and TS) and polysaccharides, proteins, and 42 aroma compounds of the base wines. *Compounds for which the correlation was statistically significant at the $p<0.05$ level. Data taken from [9].

3.2. Utility of T. delbrueckii on the Second Fermentation and the Quality of the Sparkling Wine

Fermentation with *S. cerevisiae* (single or mixed with *T. delbrueckii*) was very efficient, reaching 6 or more atm of pressure at 60 days. In contrast, single yeast *T. delbrueckii* showed little viability and did not complete the second fermentation under these conditions. The percentage of dead cells was always higher in *T. delbrueckii* fermentation, single or mixed, and *S. cerevisiae* totally replaced *T. delbrueckii* at 60 days (not shown). The *S. cerevisiae* and *S. cerevisiae* + *T. delbrueckii* cava wines were of good quality, as indicated by the physical-chemical parameters and the organoleptic analysis (Table 2). The wines with mixtures of *S. cerevisiae* + *T. delbrueckii* were also the most valued for their complexity, better mouthfeel, notes of dried fruit, and pleasing aged character. On the contrary, the *T. delbrueckii* cava wines presented low levels of pressure, alcohol, and total acidity, and higher levels of volatile acidity, reducing sugars, and pH, which explain its low score in the organoleptic analysis (Table 2).

Table 2. Some important parameters and organoleptic analyses of cava wines made by single or mixed inoculating base wines with strains of *S. cerevisiae*(*Sc*) and *T. delbrueckii* (*Td*).

Parameter	*S. cerevisiae*	*T. delbrueckii*	*Sc+Td*	*p*[a]
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

[a]*p*-values from the ANOVA carried out for the wines made with the three types of inoculum. Different lower-case letters (a, b, and c) in each row mean significantly different homogeneous groups found with the Duncan test at $p < 0.05$. Data taken from [9].

In general, the foam parameters of cava wines were worse than those of base wines (Figure 3A). *S. cerevisiae*cava wines (single or mixed with *T. delbrueckii*) had the best HM, and those of *T. delbrueckii* (single or mixed with *S. cerevisiae*) had the best TS and greater amount of total polysaccharides and mannan (Figure 3A, B). Although there were no differences in the amount of protein between the three types of cava wines, in all of them, it increased by 30% compared to the base wine (Figure 3B). These results suggest that the amount of these compounds is less relevant than previously thought [3,4], at least in our working conditions. Nor was any correlation found between the foam properties and the aromatic compounds, probably because the differences in these parameters in these cava wines were relatively small as they all came from the same base wineblend. On the other hand, there were significant differences in 15 of the 75 volatile compounds analyzed: seven compounds more abundant in *T. delbrueckii* cava wines and eight more abundant in *S. cerevisiae* and *S. cerevisiae* + *T. delbrueckii*, mainly ethyl esters responsible for fruity aromas, and with a relevant odor activity value (OAV), such as ethyl hexanoate, ethyl octanoate, and β-damascenone (Figure 3C). These results are like what was previously observed for still wines [1,8].

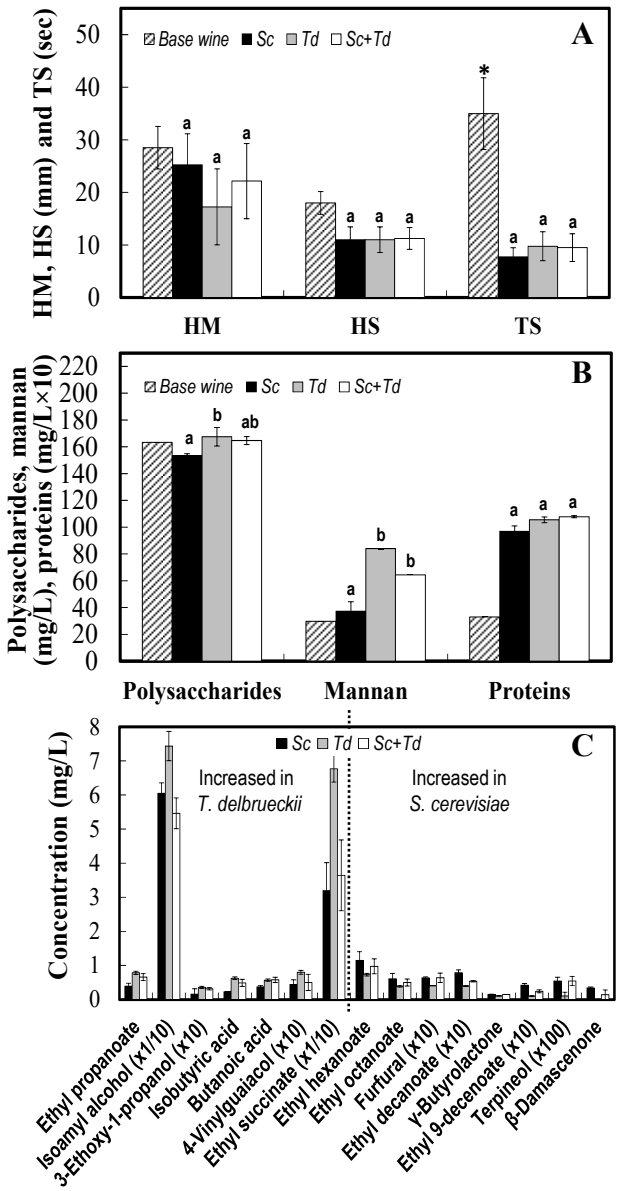

Figure 3. (**A**): Foaming parameters (HM, HS, and TS). *Sc*, *S. cerevisiae*; *Td*, *T. delbrueckii*. *TS value of base wine divided by ten. (**B**): Mean polysaccharide, mannan, and protein content. Different lower-case letters mean significantly different groups found with the Duncan test at $p < 0.05$. (**C**): Aroma compounds for which statistically significant differences were found between *Sc*,*Sc*+*Td*, and *Td*cava wines. Data taken from [9].

4. Conclusions

The killer phenotype permitted *T. delbrueckii* to reduce the presence of wild yeasts during must fermentation. Nonetheless, the lower aromatic quality and lower capacity to form foam in their base wines make this yeast unsuitable for cava winemaking, although it could be interesting to produce other types of wines. Furthermore, the exclusive inoculation of *T. delbrueckii* did not complete the second fermentation, which also discourages its use for this purpose. Nevertheless, the mixed inoculation of *S. cerevisiae* + *T. delbrueckii* in the second fermentation proved to be a good strategy to enhance the organoleptic quality of the cava wine, mainly because *T. delbrueckii* enhanced a greater amount of some compounds of interest and improved the foam stability.

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Conflicts of Interest: The authors declare no conflict of interest.

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