proceedings

MDPI

Proceedings

Base wine and traditional sparkling wine making using *Torulaspora delbrueckii* killer yeasts†

Alberto Martínez[1], Rocío Velázquez[1], Emiliano Zamora[2], María L. Franco[2], Camille Garzo[3] Patricia Gil[1], Luis M. Hernández[1], and Manuel Ramírez[1].

[1] Departamento de Ciencias Biomédicas, Facultad de Ciencias, Universidad de Extremadura, 06071 Badajoz, Spain; mramirez@unex.es
[2] Estación Enológica, Junta de Extremadura, 06200 Almendralejo, Spain;
[3] IUT Génie Biologique, Université de Bourgogne BP 17867 21078 DIJON CEDEX, France;

* Correspondence: albertomb@unex.es.
† Presented at the 1st International Electronic Conference on Food Science and Functional Foods, 10–25 November 2020; Available online: https://foods_2020.sciforum.net/

Submitted: date; Accepted: date; Published: date

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 (base wine and cava). *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 the 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 inappropriate 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.

1. Introduction

Torulaspora delbrueckii is probably the non-*Saccharomyces* yeast most frequently used for wine fermentation. This yeast can improve wine complexity, decrease volatile acidity and acetaldehyde content, and increase dried-fruit and pastry aromas [1]. 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 [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, a VECPRD according to the European Union CEE 1993/1999). It has been described that the foam of cava depends to a great extent on its content of proteins and mannoproteins, mainly the foam stability [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 yeasts autolysis is to use mixtures of killer and sensitive yeasts as inocula in the second cava fermentation. Killer toxin 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 nor the consequent increase in pressure, improving the cava foam an dits organoleptic quality [7]. In order to complement these results, it is interesting to analyze the usefulness of killer *T. delbrueckii* yeast strains, which can dominate must fermentation [1,8], to produce base wine and cava. Furthermore, since the killer effect can improve 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 base-wine making, the capacity of *T. delbrueckii* to carry out the second fermentation at high CO_2 pressure, the aromatic profile and the foaming properties of base wine and cava made with *T. delbrueckii* compared 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 cava-wine making, an assemblage of *S. cerevisiae* base wines was used. Before base wine inoculation, yeasts were adapted to growth in this base wine as described previously [9] and was supplemented with 2.4% sucrose and 0.02% diammonium phosphate. Thereafter, the base wine was single (with *S. cerevisiae* EX229, killer Klus and sensitive to cycloheximide, cyh^S; or *T. delbrueckii* EX1180-2K$^-$), and mixed (EX229+EX1180-2K$^-$) inoculated in 0.75 L capped bottles resistant to high pressure, inoculating about 1-4×10^6 cells/mL for *S. cerevisiae*, or 2-4×$10^7$for *T. delbrueckii*. And incubating at 18-19^{o}C during the first 15 days, to increase the killer effect, which is more effective at this temperature, and then at 12-14^{o}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 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 determined by staining with methylene blue, wine mannan (mannoproteins) content, polysaccharides and proteins as described previously [9]. The wine aroma compounds were measured by GC-MS, and the foaming parameters using a Mosalux system as described previously [9]. The principal analytical parameters were determined according to EC recommended methods and the organoleptic analysis was carried out by wine taster's expert 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. Influence 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 that 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 foam ability (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 foam ability, and its higher quantity of glycerol the lower stability of the foam. The higher amount of alcohols in *T. delbrueckii* wines can explain its lower foam ability, and its higher amount of polysaccharides that the little foam that is formed is more stable (Table 1).

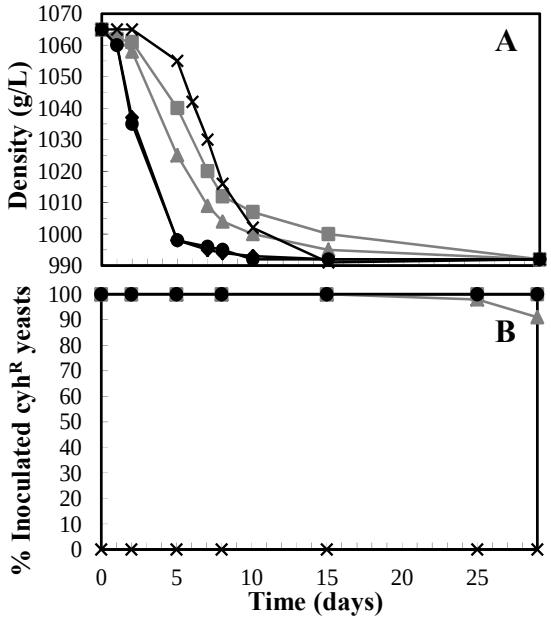

Figure 1. A: Evolution of must/wine density. B: Evolution of the percentage of each inoculated yeast (cyh^R) during the must fermentation. Symbols: non-inoculated control, (—×—), *Sc* E7AR1 (—♦—), *Sc* EX85R (—●—), *Td* EX1180-11C4 (—■—), and *Td* EX1180-2K$^-$ (—▲—).

Table 1. White must fermentation parameters and results of the corresponding base wine 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 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 level; EF, end of fermentation; 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.

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 other wine compounds than polysaccharides and proteins may be importantly involved in the wine's foaming quality. To continue with the elaboration of cava-wine making, an assemblage of *S. cerevisiae* base wines was used for this purpose. The organoleptic quality of *T. delbrueckii* base wines was considered atypical for this purpose, nonetheless these wines were considered of good quality and without defects.

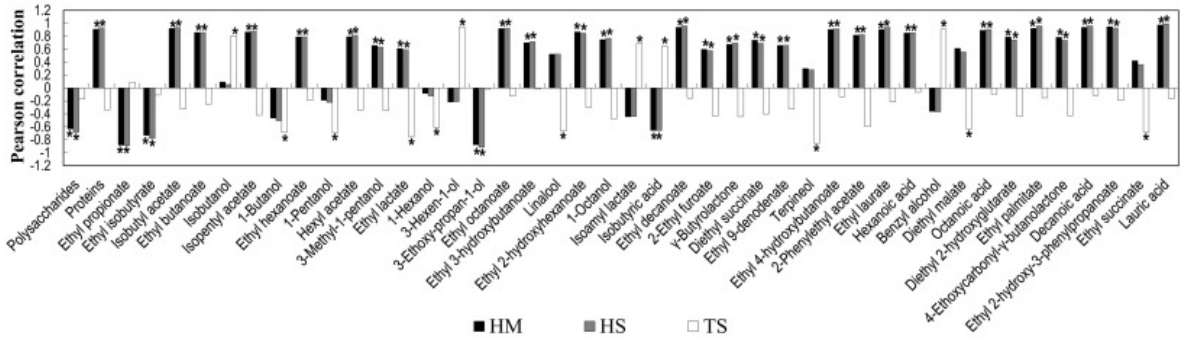

Figure 2. Pearson correlation coefficients between each of the foaming parameters (HM, HS, and TS) and the concentrations of polysaccharides, proteins, and 42 aroma compounds of the base wines.*Compounds for which the correlation (two-tailed) was statistically significant at the $p<0.05$ level.

3.2. Influence of T. delbrueckii on the second fermentation and the quality of the sparkling wine

Fermentations with *S. cerevisiae* (single or mixed with *T. delbrueckii*) were very efficient, reaching 6 or more atm of pressure at 60 days. In contrast, single yeasts *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* fermentations, 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 appreciated for their complexity, better mouth feel, notes of dried fruit, and pleasant 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 explains its low score in the organoleptic analysis (Table 2).

Table 2. Some relevant parameters and the organoleptic quality of cava wines made by single or mixed inoculating base wine 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

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 level; EF, end of fermentation; 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 three types of inoculum. Different lower-case letters (a, b, and c) in a given row mean significantly different homogeneous groups found with Duncan test at p<0.05.

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 assemblage base wine. On the other hand, there were significant differences in 15 of the 75 volatile compounds analyzed: 7 compounds more abundant in *T. delbrueckii* cava wines, and 8 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 similar to what was previously observed for still wines [1, 8].

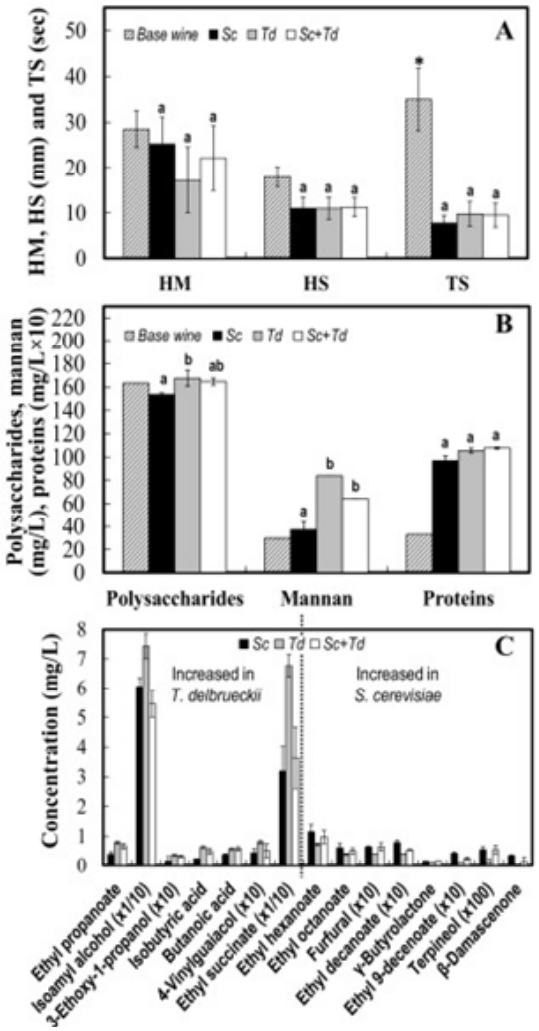

Figure 3.A: Foaming parameters – HM, maximum height; HS, foam stability height; TS, foam stability time. *Sc*, *S. cerevisiae*; Td, *T. delbrueckii*; *Sc+Td*, *S. cerevisiae* + *T. delbrueckii*. * TS value of base wine divided by ten. B: Mean polysaccharide, mannan (measured as mannose), and protein 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* cava wines.

4. Conclusions

The killer phenotype allowed *T. delbrueckii* to reduce or eliminate 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 option to improve the organoleptic quality of the cava wine, mainly because *T. delbrueckii* increased the amounts of some interesting compounds and improved the foam stability.

Funding: This research was funded by Extremadura Regional Government and Spanish Ministry of Economy, Industry and Competitiveness (co-financed with FEDER funds), grant number GR18117 and AGL2017-87635-R".

Acknowledgments: We are grateful for the technical and human support provided by the SAIUEx Facility of Elemental and Molecular Analysis (financed by UEX, Junta de Extremadura, MICINN, FEDER, and FSE).

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

References

1. Ramírez, M.; Velázquez, R.; Maqueda, M.; Zamora, E.; López-Piñeiro, A.; Hernández, L.M. Influence of the dominance of must fermentation by *Torulaspora delbrueckii* on the malolactic fermentation and organoleptic quality of red table wine. *Int. J. Food Microbiol***2016**, *238*, 311–319.
2. González-Royo, E.; Pascual, O.; Kontoudakis, N.; Esteruelas, M.; Esteve-Zarzoso, B.; Mas, A.; Canals, J.M.; Zamora, F., 2014. Oenological consequences of sequential inoculation with non-*Saccharomyces* yeasts (*Torulaspora delbrueckii* or *Metschnikowia pulcherrima*) and *Saccharomyces cerevisiae* in base wine for sparkling wine production. *Eur. Food Res. Technol***2014**, 999–1012.
3. Brissonnet, F., and A. Maujean. Characterization of foaming proteins in a champagne base wine. *American Journal of Enology and Viticulture***1993**,*44*, 297-301.
4. Vanrell, G.; R. Canals; M. Esteruelas; F. Fort; J. M. Canals; and F. Zamora. Influence of the use of bentonite as a riddling agent on foam quality and protein fraction of sparkling wines (Cava). *Food Chemistry***2007**, *104*,148-155.
5. Gallart, M.; E. López-Tamames; G. Suberbiola; and S. Buxaderas. Influence of fatty acids on wine foaming. *Journal of Agricultural and Food Chemistry***2002**, 50, 7042-7045.
6. Todd, B. E.; G. H. Fleet; and P. A. Henschke. Promotion of autolysis through the interaction of killer and sensitive yeasts: potential application in sparkling wine production. *American Journal of Enology and Viticulture***2000**, *51*, 65-72.
7. Velázquez, R.; Zamora, E.; Álvarez, M.L.; Álvarez, M.L.; Ramírez, M. Using mixed inocula of new killer strains of *Saccharomyces cerevisiae* to improve the quality of traditional sparkling-wine. *Food Microbiol***2016**, 59, 150–160.
8. Velázquez, R.; Zamora, E.; Alvarez, M.L.; Hernández, L.M.; Ramírez, M.Effects of new *Torulaspora delbrueckii* killer yeasts on the must fermentation kinetics and aroma compounds of white table wine. Front. Microbiol **2015**, 6, 1222.
9. Velázquez, R.; Zamora, E.; Álvarez, M.L.; Ramírez, M. Using *Torulaspora delbrueckii* killer yeasts in the elaboration of base wine and traditional sparkling. *Int. J. Food Microbiol***2019**, *289*, 134-144.