

AN INNOVATIVE TOOL FOR THE WINEMAKER: SEQUENTIAL INOCULATION WITH A NON-SACCHAROMYCES YEAST AND A SACCHAROMYCES CEREVISIAE YEAST

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1. Introduction

In the past, when alcoholic fermentation was carried out by wild yeasts present in the winery, the conversion of sugars into alcohol and the production of aromatic compounds occurred by sheer luck, making alcoholic fermentation a risky process. Clearly, the qualitative risks were very real. Aromatic deviations, such as *piqûre acétique*, and other problems related to fermentation, were frequent. The utilization of selected yeasts in an active dry form has gradually changed practices and made alcoholic fermentation much more reliable.

Studies comparing yeast ecologies in vineyards and cellars clearly show that the yeasts present on grapes are subject to such natural phenomenon as grape maturity and weather, as well as to human intervention and the phytosanitary treatments carried out (Guerra et al. 1999, and Cabras and Angioni 2000). These studies also show that residual indigenous yeasts are not the yeasts most appropriate for carrying out alcoholic fermentation. The utilization of selected *Saccharomyces* yeasts reduces the unreliability due to the development of uncontrolled indigenous populations, and ensures smooth alcoholic fermentation. The result is an improvement in the overall quality of the wines (Loiseau et al. 1987) and the coherence of production processes (Fleet et al. 1993, and Lambrechts and Pretorius 2000).

It is also important to consider the diversity of the yeast microflora present in the vineyards (Davenport 1974, and Mortimer and Polsinelli 1999), musts (Heard and Fleet 1986, Ganga and Martinez 2004, Torija et al. 2001, and Hierro et al. 2006) and during the first stages of winemaking (Zott et al. 2008). The involvement of non-*Saccharomyces*-type microorganisms in alcoholic fermentation has been described by scientists (Ciani 1997, Egli et al. 1998, and Soden et al. 2000).

Wines can benefit from the quantitative and qualitative diversity of the products and by-products of fermentation obtained through non-*Saccharomyces* yeasts (Ciani and Ferraro 1998, Ciani et al. 1996, and Ferraro et al. 2000). Due to this biodiversity, the winemaker can make selections to better differentiate the wine or to reveal the aromatic potential, in terms of both intensity and complexity (Egli et al. 1998, Romano et al. 2003, Rojas et al. 2003, and Viana et al. 2009). According to the latest research, certain non-*Saccharomyces* yeasts present interesting sensory impacts for winemaking. Lallemand's research on the aromatic potential of non-*Saccharomyces* yeasts, combined with the optimization of the production of these yeasts in dry form, now lets the winemaker take advantage of fermenting with non-*Saccharomyces* wine yeasts while maintaining control over the fermentation process.

These new tools combine biodiversity, reliability and qualitative advantages.

2. Production of *Torulaspota delbrueckii* yeast with high viability and vitality

2.1 WHAT MAKES THE *TORULASPORA DELBRUECKII* SPECIES INTERESTING?

Within the biodiversity naturally present on the grape and in the fermentation ecosystem, certain yeasts have been studied in terms of oenological fermentation for their renowned sensory contributions. We are particularly interested in the *Torulaspota*, *Candida*, *Debaryomyces*, *Pichia*, *Kloeckera*, *Kluyveromyces* and *Metschnikowia* genera, among others (Belancic et al. 2003, Ciani 1997, Ciani and Ferraro 1998, Egli et al. 1998, Mora et al. 1990, and Rosi et al. 1994). *T. delbrueckii* stands out, not only for revealing typicity, but for the pureness of its fermentation profile (Ciani and Picciotti 1995, Martinez et al. 1990, Mauricio et al. 1991, and Moreno et al. 1991) and its capacity to correct certain faults in wines, such as volatile acidity (Languet et al. 2005, and Bely et al. 2008). As variability is the standard in nature, whether inter- or intra-species (Renault et al. 2009), the choice of working with a precise strain of *T. delbrueckii* was made in partnership with the Institut National de la Recherche Agronomique (INRA) in France after winemaking trials and according to the yeast population and sensory analyses, while respecting all AFNOR standards (Languet et al. 2005). AFNOR is the Association française de normalisation, the French national standardization organization.

2.2 THE SURVIVAL RATE, CAPACITY TO MULTIPLY AND FERMENTATION PURENESS OF *TORULASPORA DELBRUECKII*

Different strategies for producing active dry yeast (ADY) from *T. delbrueckii* – the Lallemand yeast collection number 291 – were developed to increase the survival rate under difficult oenological conditions and to increase the yeast's capacity to multiply so that it can establish itself in grape musts.

In the following example, *T. delbrueckii* 291 yeasts resulting from different production lots were coded "ADY TD1" and "ADY TD2" and tested. The must utilized was a grape juice from the Viognier varietal, and the standard analyses are presented in Table 1. As this natural must presents low turbidity and therefore has lower concentrations of micronutrients and survival factors, it is an environment recognized as hostile to yeasts in general and to non-*Saccharomyces* yeasts in particular. The fermentation temperature applied to these fermentations was 20°C.

In parallel, and in order to compare the survival capacities of non-*Saccharomyces* yeasts, we utilized a selected *Saccharomyces cerevisiae* yeast recognized the world over for making high quality white wines. In addition, this control yeast was produced according to the YSEO® (Yeast SEcurity Optimization) process. Enjoying the latest advances in terms of production processes, this yeast was coded "ADY SC YSEO®."

Sugar g/L	Turbidity NTU	Available nitrogen mg/L	pH	Total H ₂ SO ₄ g/L
215	42	150	3.65	2.50

TABLE 1. Standard analysis of the Viognier must utilized

The viability of each of these three yeasts is presented in Table 2. The minimum viability required for reliable fermentation is 1×10^{10} cfu/g of ADY. Therefore, all yeasts utilized exceeded quality standards.

ADY	ADY TD1	ADY TD2	ADY SC YSEO®
Viability cfu/g	4×10^{10}	2×10^{10}	2×10^{10}

TABLE 2. Viability of different yeasts

The control of the viability of yeasts inoculated into the musts and the yeast count was carried out 50 hours after the maximal rate of fermentation was reached. The yeast count was carried out through spreading on Sabouraud agar plates and adding 0.055 g/L of methylene blue to permit morphologic differentiation. This method had already been checked and validated through genetic analysis. The results are presented in Figure 1. In parallel, the capacity of the ADY TD1 and ADY TD2 yeasts to multiply – their “vitality” – was compared. Those results are presented in Figure 2. The reliability of the process for controlling the hygiene of the wines is presented in Table 3, which shows the standard analysis of the resulting wines.

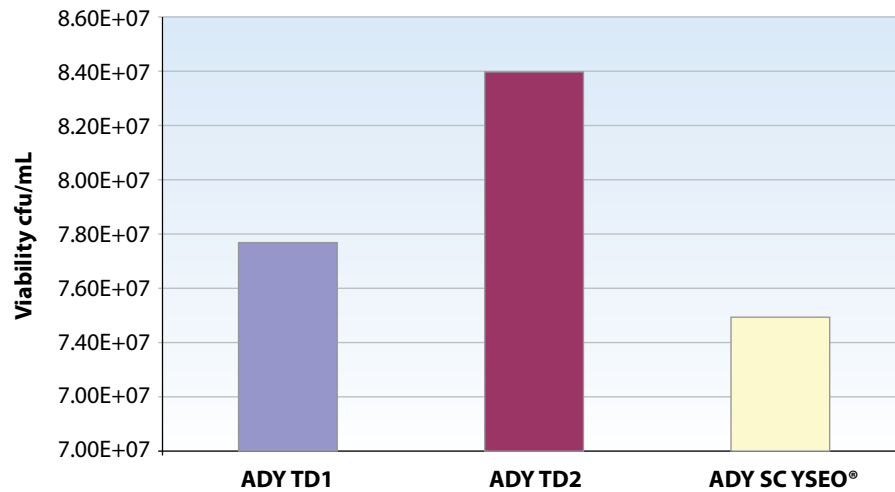


FIGURE 1. Viability in the musts of different non-*Saccharomyces* yeasts and one *Saccharomyces* yeast, after 50 hours of alcoholic fermentation

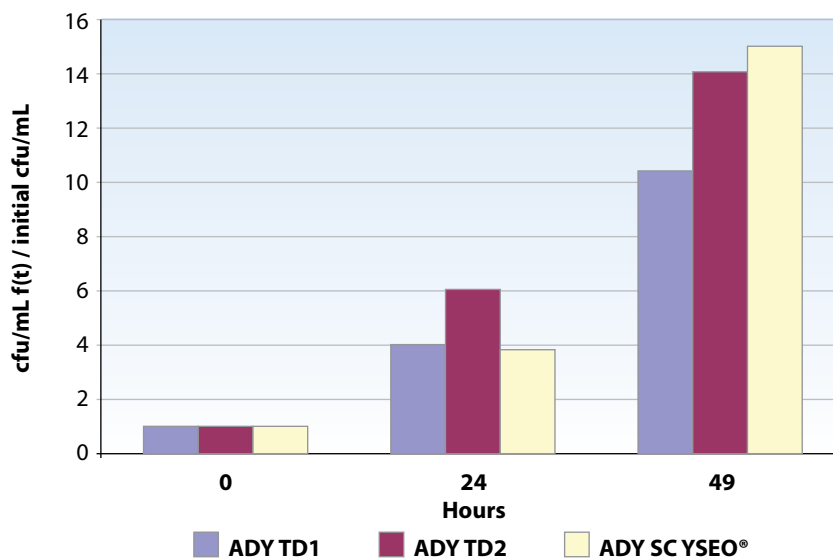


FIGURE 2. Vitality, or the capacity to multiply of different yeasts, during the first 50 hours of alcoholic fermentation

	Total alcohol % vol	Glucose-fructose g/L	Total SO ₂ mg/L	Free SO ₂ mg/L	Volatile acid H ₂ SO ₄ g/L	Total acidity H ₂ SO ₄ g/L	pH
TD1 then SC YSEO® in sequential inoculation	12.72	0.0	14	6	0.17	2.50	3.73
TD2 then SC YSEO® in sequential inoculation	12.72	0.0	14	6	0.12	2.60	3.73
SC YSEO®	12.83	0.0	16	8	0.23	3.10	3.63

TABLE 3. Standard analyses for Viognier wines

The results show that, according to the how the yeasts are produced, the viability at the time of inoculation and the capacity for multiplication (or vitality), can be predicted. The ADY TD2 yeast appears to be the better performing product in terms of vitality and viability compared to ADY TD1. The ability of *T. delbrueckii* 291 to ferment under difficult conditions when there are survival factor deficiencies (i.e., sterols, due to the low turbidity of the must), was observed.

Yeast viability is important in order to take hold and dominate over the indigenous yeasts, but the capacity to multiply is also essential, given that it is during the multiplication phase of yeasts that the main fermentation aromas are synthesized. What's more, it has already been shown that to contribute to the sensory profile of wines most of the non-*Saccharomyces* yeasts must reach a large total population, on the order of 10⁶ or 10⁷ cells per millilitre (Heard and Fleet 1986).

Today, adaptations to our ADY production processes allow us to produce non-*Saccharomyces* yeasts that meet the need for viability and vitality as reliably as active dry *Saccharomyces cerevisiae* yeasts.

3. Strategy for utilizing *Torulasporea delbrueckii* 291 in sequential inoculation

It is generally believed that utilizing non-*Saccharomyces* yeasts in a monoculture does not allow the proper and reliable completion of fermentation (with residual sugars < 2 g/L) within a timeframe compatible with current winemaking requirements, while guaranteeing the absence of organoleptic faults. In oenological conditions, these species have limited fermentation capacities compared to *Saccharomyces* yeasts, due notably to their low capacity to multiply and their particular needs for micronutrients and oxygen (Mauricio et al. 1991, and Hansen et al. 2001). However, because it is extremely adaptable to hostile conditions, *Saccharomyces* yeast manages to grow and surpass the indigenous non-*Saccharomyces* yeasts (Fleet 1993). Although it has been widely reported that the succession of yeast populations, with the sequential domination of a non-*Saccharomyces* yeast in the first phase of alcoholic fermentation then a *Saccharomyces* yeast, can contribute to the aromatic complexity of wines (Zironi et al. 1993, and Ferraro et al. 2000), we validated this theory in a previous study (Languet et al. 2005). The succession of yeast populations, such as those obtained with this selected strain of *T. delbrueckii* 291 is therefore valuable to obtain a more complex sensory profile. The result is a sequential inoculation that avoids putting the sensory and fermentation qualities of each species into competition.

The first trials consisted of optimizing the yeast pairing. Several *Saccharomyces* yeasts were tested with the *T. delbrueckii* 291 strain in sequential inoculation, on several varieties and in diverse fermentation conditions. Among the *S. cerevisiae* yeasts selected because they corresponded to set criteria, a single strain proved to be compatible with the *T. delbrueckii* 291 for obtaining wines with a distinctly positive aroma profile.

3.1 COURSE OF THE SEQUENTIAL FERMENTATION

A synthetic must coded “MS300-FA-O2 GF,” which mimics extreme deficiencies in sterols, was utilized. This must presented a glucose-fructose level of 200 g/L, and was devoid of sterol-type survival factors. In addition, it was made inert through direct bubbling with ALIGAL™ before inoculation with the *T. delbrueckii* ADY TD 291 and the control yeast, *Saccharomyces* ADY SC, so that no oxygen was dissolved in the must. Therefore, the fermentation conditions were extreme and allowed us to verify whether sequential inoculations with the active dry *T. delbrueckii* 291 and *Saccharomyces* yeasts are compatible in this type of situation. Given these particularly difficult conditions, Go-Ferm Protect® yeast rehydration protectant was added to the active dry yeasts during rehydration, following the producer’s recommendations.

The fermentation rates for both trials are presented in Figure 3. The standard wine analyses are shown in Table 4.

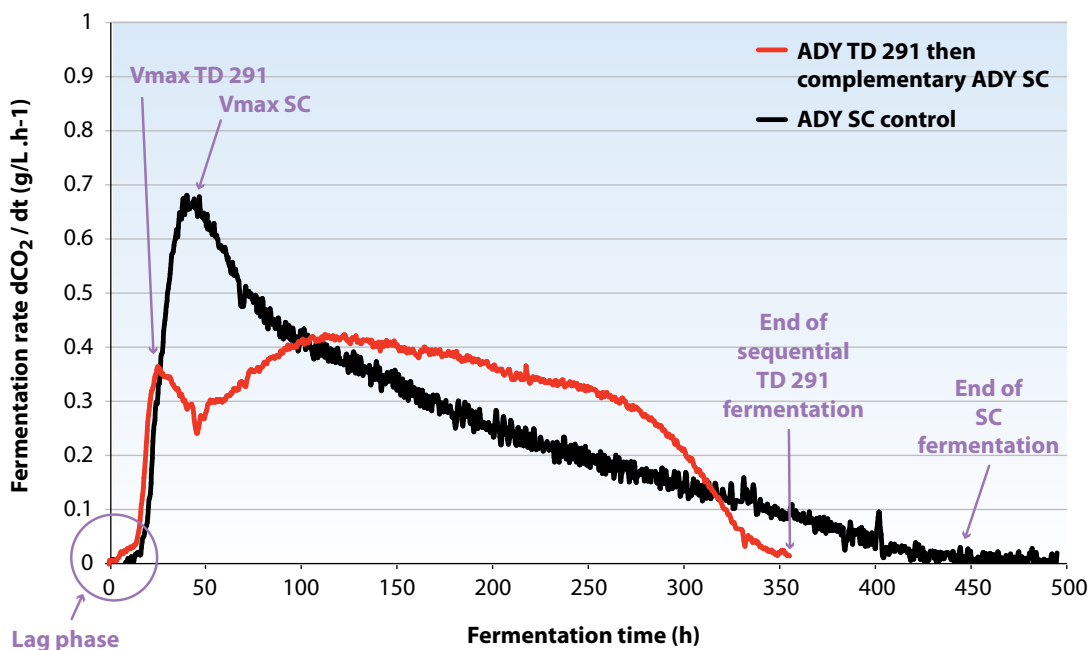


FIGURE 3. Fermentation kinetics of sequential inoculation and classic inoculation in a deficient must

The fermentation kinetics obtained (Figure 3) show that the lag phase is extremely short, both for the fermentation inoculated with ADY TD 291 and the control fermentation inoculated with ADY SC. The maximum rate of fermentation (V_{max}) is slower in the case of the sequential inoculation, but it is nearly constant in the stationary phase. The end of fermentation is more abrupt than in the control. The absence of a fermentation peak during the sequential inoculation avoids a thermal increase, often associated with alcoholic fermentation.

	Total alcohol % vol	Glucose-fructose g/L	Total SO ₂ mg/L	Free SO ₂ mg/L	Volatile acid H ₂ SO ₄ g/L	Total acidity H ₂ SO ₄ g/L	pH
TD 291 then SC YSEO® in sequential inoculation	12.09	0.0	< 25	< 5	0.33	7.67	3.53
SC YSEO®	12.83	0.1	< 25	< 5	0.79	8.35	3.45

TABLE 4. Standard analyses of wines made with MS300-FA-O2 GF synthetic must

The analysis shows that the decrease in volatile acidity is still very significant (Table 4).

4. Characterization of the *Saccharomyces cerevisiae* / *Torulaspora delbrueckii* 291 pairing in sequential inoculation

4.1. UTILIZING A PROTECTANT FOR *SACCHAROMYCES CEREVISIAE* YEAST IN SEQUENTIAL INOCULATION

To assess the fermentation capacities of sequential inoculation in extreme conditions in regards to oxygen and to study the relevance of adding a rehydration protectant to *S. cerevisiae*, several fermentation trials were conducted. The results show that under difficult fermentation conditions (i.e., a deficient must) it is preferable to utilize a yeast protectant (like Go-Ferm Protect®) for *Saccharomyces* yeast (Figure 4).

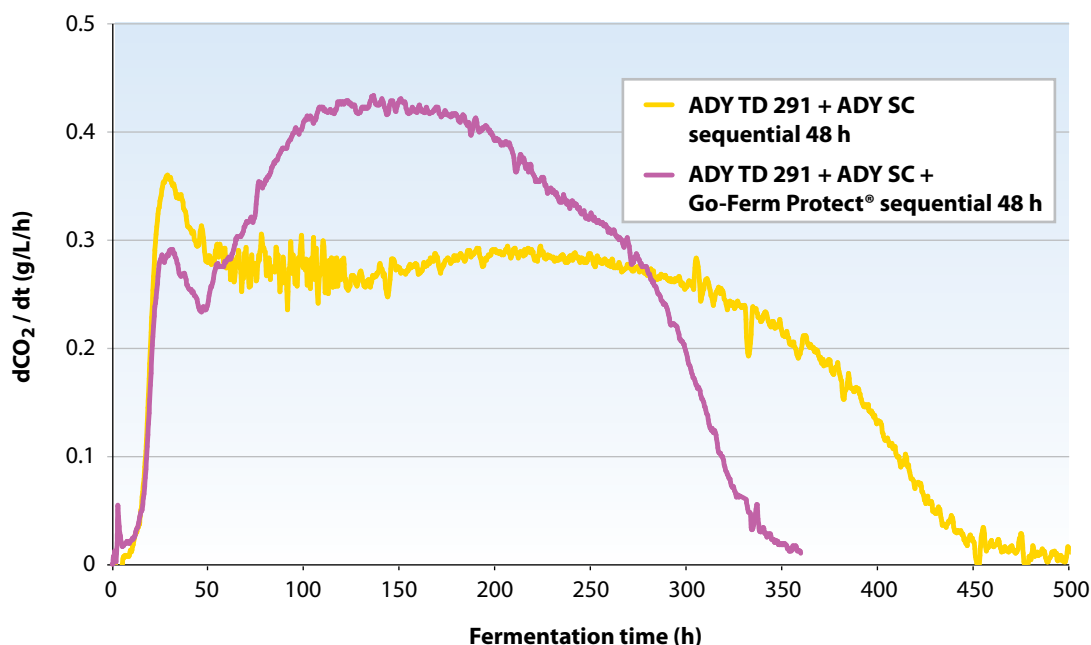


FIGURE 4. Fermentation kinetics with and without a yeast protectant during the rehydration of *Saccharomyces cerevisiae* in sequential inoculation with *Torulaspora delbrueckii* 291

The impact of the yeast protectant is visible in Figure 4 in terms of the fermentation rate. By utilizing a protectant for the rehydration of *S. cerevisiae*, the length of fermentation was reduced by 25% compared to the control fermentation (with no protectant). This presents a real advantage considering that the fermentation time is generally longer when making wine through sequential inoculation.

4.2 ASSESSING NITROGEN DEMAND FOR THE OPTIMIZED PAIRING UTILIZED IN SEQUENTIAL INOCULATION

In order to determine nitrogen demand when utilizing two yeasts in sequential inoculation, alcoholic fermentation was carried out on synthetic musts deficient in available nitrogen. According to Julien et al. 2001, the duration of fermentation in a medium deficient in nitrogen is proportional to the nitrogen necessary to maintain a constant fermentation rate in this medium. Yeasts described as having low, medium or high nitrogen demand were compared to yeast pairings utilized in sequential fermentation. The resulting fermentation kinetics place the *T. delbrueckii* 291 yeast in the category with a high demand for nitrogen.

4.3 OPTIMIZED NUTRITION FOR THE YEAST PAIR IN SEQUENTIAL INOCULATION

Laboratory results show that the high nitrogen demand of the yeast pair utilized in sequential fermentation was supported, according to observations during the winery scale trials. The adapted nutrition strategies were then studied in the laboratory and validated during winery trials.

In the laboratory, the medium utilized was lacking in anaerobic factors (survival factors) mimicking a total lack of sterols and lipids, and the yeast available nitrogen (YAN) levels were on the order of 100 mg/L. The fermentation temperature was maintained at a constant 20°C and the initial sugar level was 220 g/L.

Two different yeast nutrition strategies were carried out:

- The addition of a nutrient called “complex” for its balanced levels of organic and inorganic nitrogen. This nutrient also contains vitamins and inactivated yeasts – a source of sterols and unsaturated fatty acids (Fermaid® E).
- The addition of a 100% organic nutrient comprised only of nitrogen in the form of amino acids (Fermaid® O).

Both these nutrition strategies were carried out during fermentation with the sequential inoculation of *Saccharomyces* ADY SC and *Torulaspota* ADY TD 291 yeasts.

In Figure 5, we can see that appropriate nutrition allows a 54% reduction of the fermentation time with a complex nutrient and a 32% reduction with an organic nutrient supplement. The impact is huge in terms of fermentation reliability. These results are coherent with the high nitrogen needs assessed for the yeast pair.

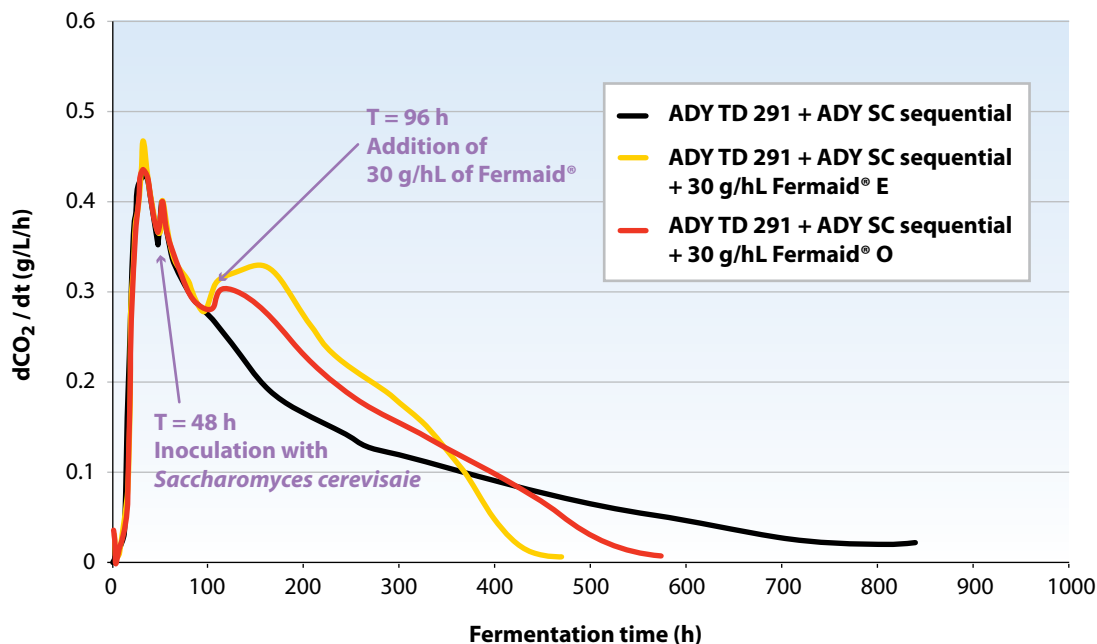


FIGURE 5. Fermentation kinetics with different nutrition strategies for the yeasts utilized in sequential inoculation

4.4 A PROMISING APPROACH FOR LOWERING VOLATILE ACIDITY

According to the results of trials utilizing sequential inoculation with *T. delbrueckii* 291 to facilitate the fermentation of late-harvest musts, this process would be particularly useful not only to improve the aromatic profile of wines, but to reduce volatile acidity (VA) as well – a problem generally associated with this type of fermentation. Indeed, during a series of trials carried out in Sauternes, France, on a Sémillon must with an alcohol potential of 21.4%, the degree of volatile acidity of the lot that underwent sequential inoculation was significantly lower than the lot that underwent classic inoculation (0.35 g/L instead of 0.70 g/L).

5. Sensory contribution of *Torulaspota delbrueckii* 291 in sequential fermentation

5.1 COMPARISON WITH CONVENTIONAL SACCHAROMYCES CEREVISIAE-TYPE YEASTS

During a trial in a Chardonnay must (Table 5) from the Mâcon Village O.A.C. in France, we obtained very minor differences in terms of alcoholic fermentation (Figure 6) between the classic inoculation (control) with an active dry *S. cerevisiae* yeast, and sequential inoculation with *T. delbrueckii* 291 and *Saccharomyces* (named the Level² TD™ kit). However, significant differences became apparent after analysis of the aromatic compounds (see Figures 7a and 7b).

	Control	Level ² TD™
Glucose + fructose g/L	202	202
Total acidity H ₂ SO ₄ g/L	6.84	6.85
pH	3.26	3.25
Free SO ₂ mg/L	11	11
Total SO ₂ mg/L	39	38
Malic acid g/L	6.6	6.7
Available nitrogen mg/L	275	276
Turbidity	62	63

TABLE 5. Characteristics of the Chardonnay must

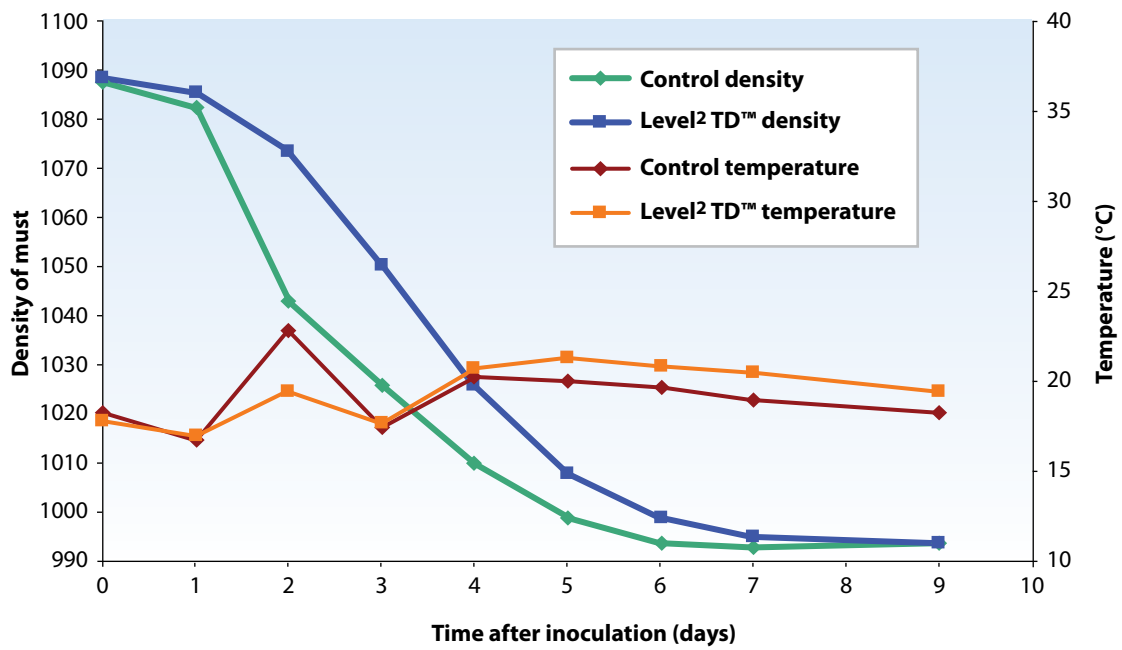
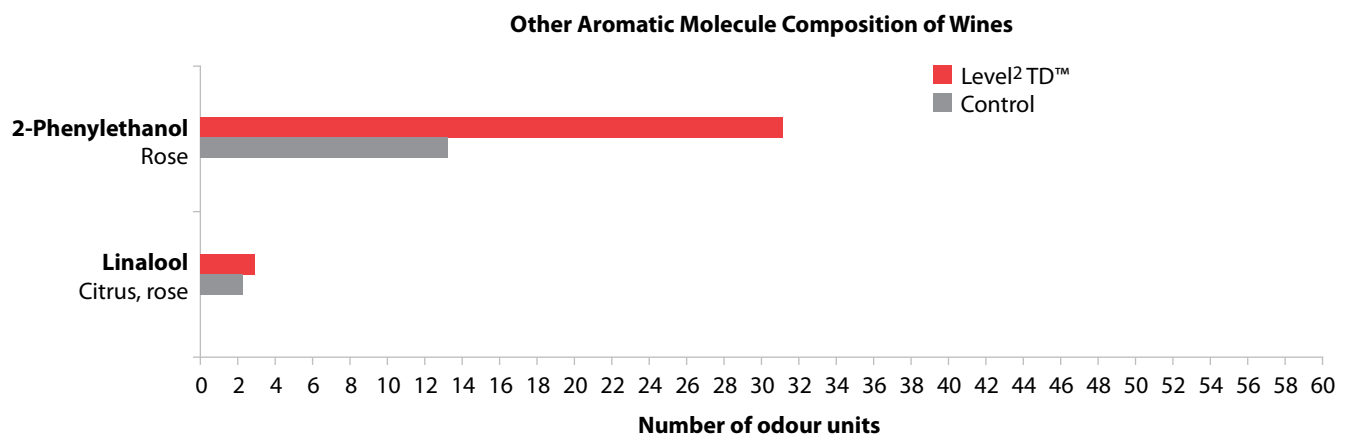
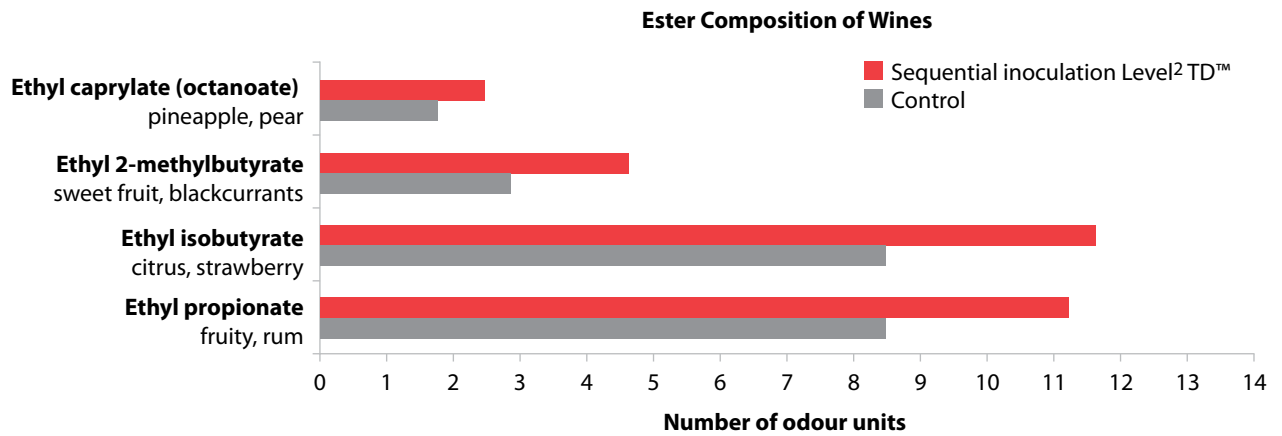


FIGURE 6. Sequential inoculation compared to classical inoculation fermentation kinetics in a Chardonnay must



FIGURES 7A AND 7B. Chardonnay: Number of olfactive units (ratio concentration / perception threshold) for different aromatic molecules for each process

Esters. At the end of alcoholic fermentation (AF), the levels of ethyl hexanoate and ethyl butyrate, which intensify the fruity perception, were significantly higher in the trial wine (with sequential inoculation) than the control wine.

Other aromatic molecules. At the end of AF, the levels of linalool (citrus and rose) and 2-phenylethanol (floral notes) were higher in the lot fermented using Level2 TD™.

Once malolactic fermentation was finished, a consumer panel expressed a clear preference for the wine made with Level2 TD™ (Figure 8), declaring that its aromatic complexity was superior to the control wine. According to some panel members, the wine obtained through sequential inoculation presented positive aromatic notes of “pastry” that were not perceived in the control wine.

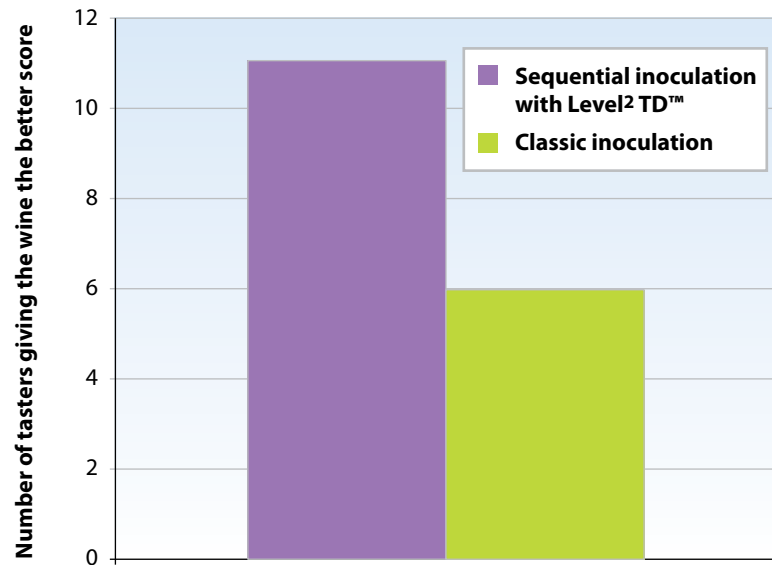
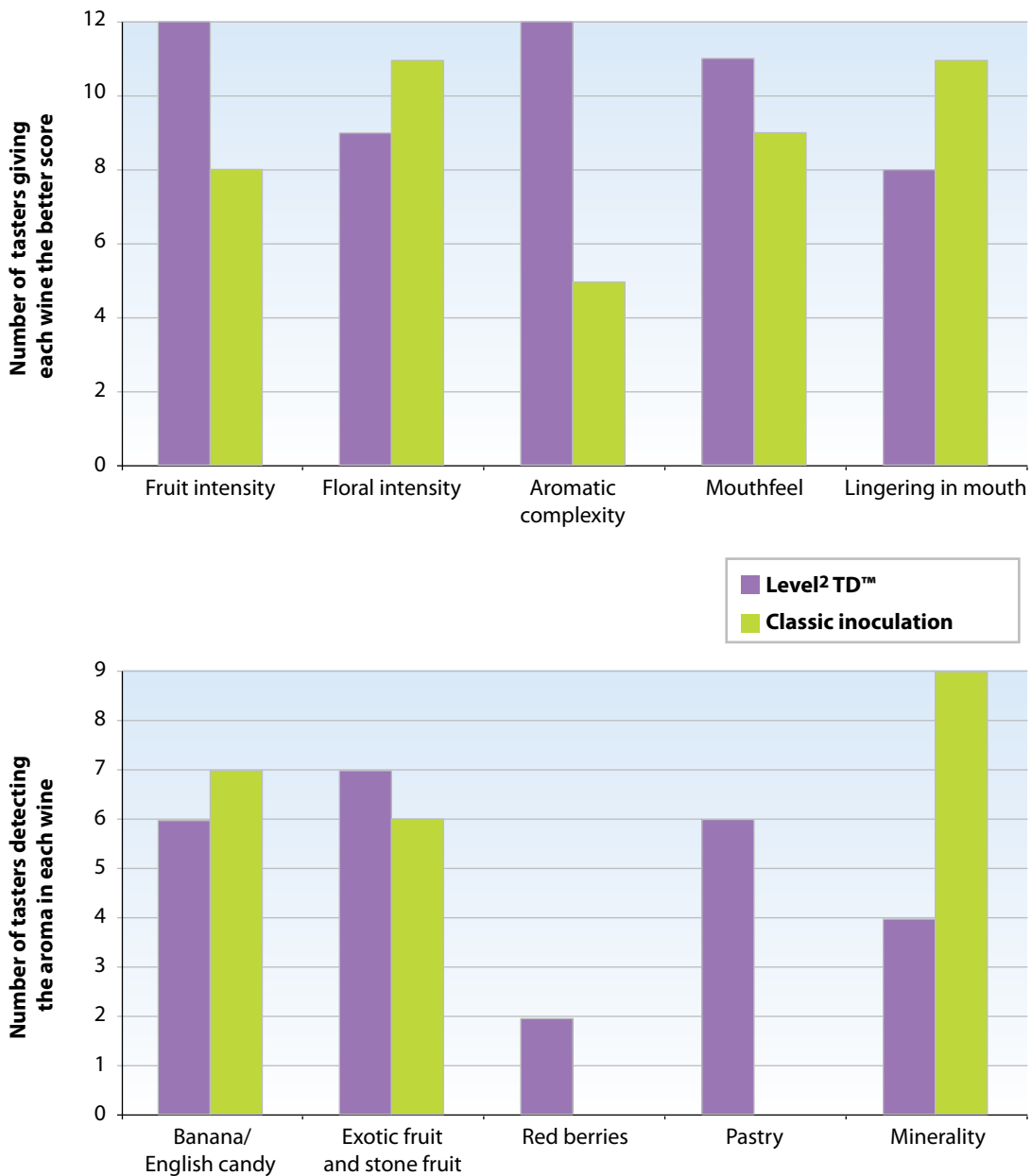


FIGURE 8. Comparative evaluation of both wines by a consumer panel after malolactic fermentation

A significant difference in descriptors appeared between the two wines tasted by a consumer panel (Figures 9a and 9b). Notably, the wine resulting from Level2 TD™ inoculation was perceived as having superior aromatic complexity, while not being less intense. It is interesting to note that some of the tasters also perceived the presence of the “pastry” aromatic range in this wine, while no one detected it in the control wine.



FIGURES 9A AND 9B. Chardonnay: Results of descriptive sensory analysis carried out by a non-professional consumer panel

5.2 COMPARISON WITH OTHER AVAILABLE NON-CONVENTIONAL YEASTS

In this example, three non-conventional yeasts products (NSC1, NSC2 and NSC3) promoting the presence of non-*Saccharomyces* yeasts using classic inoculation were prepared following their recommendations for rehydration and utilization of the different producers, and were compared to *Torulasporea/Saccharomyces* sequential inoculation with yeast from our own production (NSC TD 291). Figure 10 presents the viability obtained for the different yeasts according to the method described above. The dominance of non-*Saccharomyces* yeasts obtained to initiate AF is represented by the percentage of non-*Saccharomyces* (% NSC) yeast present compared to total live *Saccharomyces* (SC) and non-*Saccharomyces* yeasts.

In this example, the must is a blend of several grape juices. The resulting wines were analyzed by an independent laboratory specialized in aroma analysis (the DICTUC aroma centre in Chile) through gas chromatography combined with mass spectrometry (GC-MS); 4-nonanol was utilized as an internal standard. Immediately after the completion of fermentation, the wines were sent for aroma analysis, without any contact with wood.

The results in Figure 10 show that for the three combinations of non-*Saccharomyces* and *Saccharomyces* yeasts there was no dominance by non-conventional yeast populations; the *Saccharomyces cerevisiae* species is clearly in the majority at the time of inoculation into the must. One can therefore surmise that the non-*Saccharomyces* yeasts had a very small impact – even no impact – on the aroma of the wines resulting from those fermentations.

Inversely, within the framework of our inoculation with *Torulasporea* yeast alone at the the start of fermentation, we observed more than 90% implantation. This very high percentage was possible due to the good viability and vitality of the NSC TD 291 4.14×10^{14} yeast.

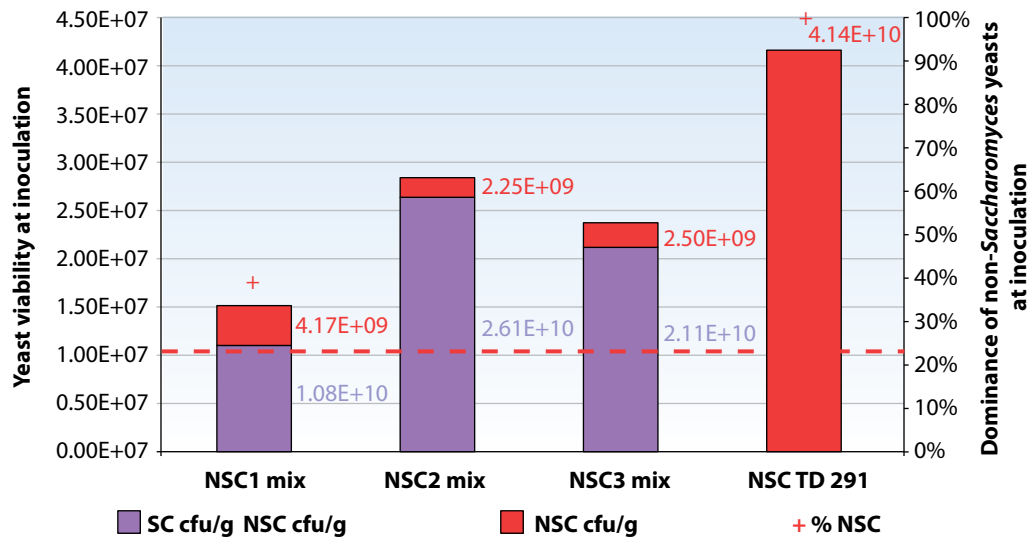
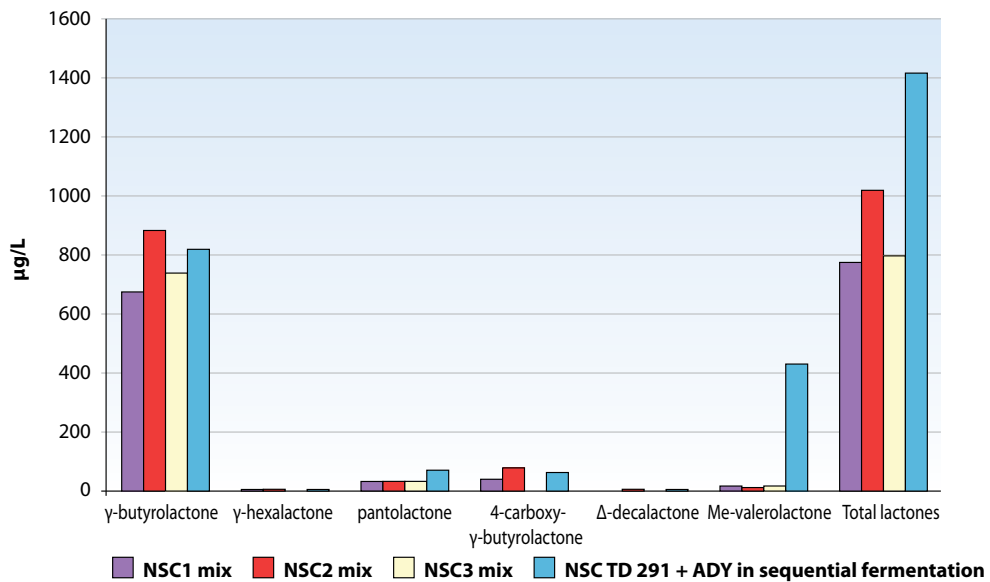
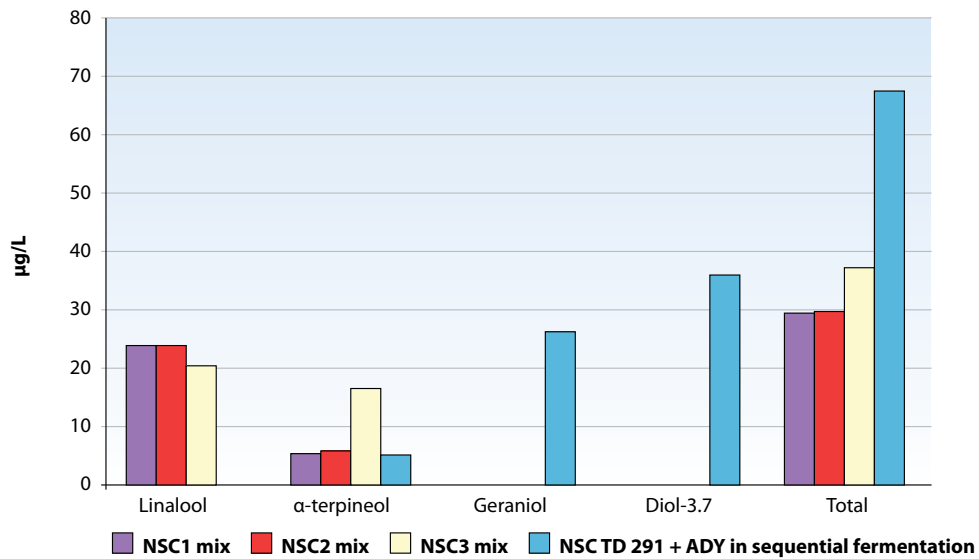
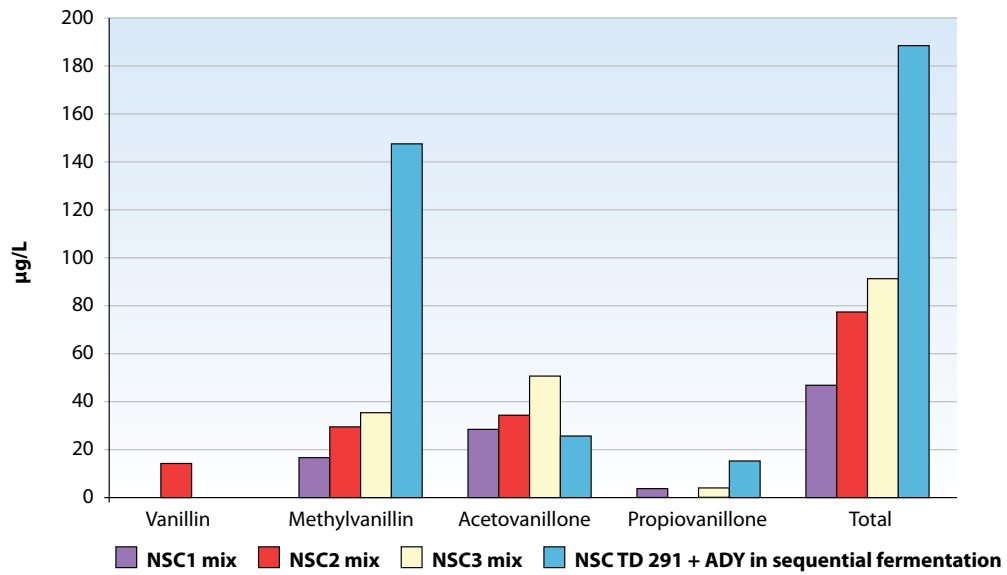


FIGURE 10. Viability according to the *Saccharomyces* and non-*Saccharomyces* yeasts present and the percentage of viable non-*Saccharomyces*



FIGURES 11, 12 AND 13. The level of vanilla derivatives, the terpene concentrations and the lactone levels.

No matter which family of aromatic compounds was studied, the analyses show that the final concentrations in the wines are very significantly higher when the fermentation was carried out according to the sequential inoculation protocol with Level² TD™.

6. Conclusion

Five years of Lallemand research and development in the laboratory, yeast production facility and at winery scale confirm the winemaking interest of a pair of complementary non-*Saccharomyces* and *Saccharomyces* yeasts, utilized in sequential inoculation and called "Level² TD™." Due to the numerous efforts to optimize the non-*Saccharomyces* yeast production process, the *T. delbrueckii* 291 yeast selected by Lallemand has an excellent survival rate after must inoculation. During alcoholic fermentation, after a 15-point drop in density, the must is then inoculated with a *Saccharomyces cerevisiae* yeast selected specifically for its synergy with the *T. delbrueckii* 291.

The most striking impact of the synergy between these two yeasts is the complexity, aromatic intensity and mouth-feel contribution they give to wines from many white grape varieties. It is this sequential development during the alcoholic fermentation of the non-*Saccharomyces* and *Saccharomyces* yeast populations that contributes to the intensity and complexity of the wines.

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