

THE PRODUCTION OF LOW-ALCOHOL WINES BY SEQUENTIAL SEMI-ANAEROBIC MUST FERMENTATION

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ABSTRACT. Nowadays, consumers go towards products that provide food security and nutritional richness, consumption of highly alcoholic beverages not complying with these health-related requirements. This study aimed to obtain low-alcohol wines by performing sequential alcoholic fermentation of grape must (Muscat Ottonel). Thus, 11 commercial yeast strains (10 *Saccharomyces cerevisiae* and 1 *Torulaspora delbrueckii*) were preliminarily tested in anaerobic and semi-anaerobic conditions. Based on laboratory tests, grape must fermentation was sequentially initiated in semi-anaerobic conditions (loose cotton plugs; intermittent homogenization), with the *T. delbrueckii* strain (10⁶ CFU/mL) and the addition of preliminarily selected *S. cerevisiae* strains (10⁴ CFU/mL), to the consumption of 50% of the sugars in the must, to complete the fermentation. By applying this technology, dry wines were obtained with an alcohol content lower by up to 1.10% vol., but with a

lower concentration of volatile terpenes, due to additional must oxidation. Also, semi-anaerobiosis enhanced glycerol synthesis by yeasts (< 35%), with a positive impact on the sensory characteristics of the wine.

Keywords: alcoholic fermentation; glycerol; low-alcohol wine; non-*Saccharomyces* yeast; *Torulaspora delbrueckii*.

INTRODUCTION

Over time, the alcoholic concentration of wines has evolved progressively, a trend that was initially attributed to climate change, i.e. a gradual increase in temperatures, which led to high accumulation of carbohydrates in grapes (Canonico *et al.*, 2019; Garcia *et al.*, 2020). This trend was later correlated with the improvement of agrotechnical practices,



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selection of genotypes with high capacity to accumulate sugars in berries or the use of osmotolerant, highly alcoholic yeasts (Filimon *et al.*, 2020).

Microorganisms that produce alcoholic fermentation constitute a very large group of organisms (mainly yeasts and bacteria), different in nature, form and biological activity. Yeasts are responsible for the alcoholic fermentation of grape must, converting sugars into alcohol, carbon dioxide and various concentrations of other by-products (Romano *et al.*, 2020). Nowadays, the use of selected yeasts for the initiation and control of alcoholic fermentation has become a current practice, even for producers with limited material possibilities.

Saccharomyces cerevisiae is generally recognized as the main agent involved in the alcoholic fermentation of grape must (Nadai *et al.*, 2019). However, strains belonging to other yeast genera, including *Hanseniaspora*/*Kloeckera*, *Pichia*, *Candida*, *Lachancea*, *Torulaspora* or *Metschnikowia*, predominate during the first stages of alcoholic fermentation (< 3–4% vol.) (Fleet and Heard, 1993). Among these, *Torulaspora delbrueckii* is the most studied and most frequently isolated and tested yeast for use in winemaking (Ramirez and Velázquez, 2018). This is because *T. delbrueckii* can be used to optimize some chemical parameters of wine, such as obtaining low volatile acidity, reducing the alcoholic concentration of wines, producing a higher amount of glycerol, releasing mannoproteins and polysaccharides, forming higher amounts of aromatic compounds that enrich the aromatic profile of the wine (fruity aromas –

esters, lactones, thiols and terpenes) and reducing the concentrations of undesirable compounds (Mehlomakulu *et al.*, 2021).

Depending on the concentration of oxygen in the environment, the metabolic degradation of carbohydrates can follow two paths: that of oxidation (*aerobic*), with the formation of caloric energy, CO₂ and H₂O, and that of alcoholic fermentation (*anaerobic*), with the formation of alcohol, CO₂, caloric energy and various secondary compounds. The aerobic metabolism of carbohydrates is associated with the respiration process ('Pasteur' effect), but some yeasts may produce ethanol even in aerobic conditions at high exogen glucose concentrations ('Crabtree' effect) (Gonzalez *et al.*, 2013). Although yeasts can utilize sugars either through fermentation or respiration, aeration of culture media induces an increase in the amount of biomass and a decrease in alcohol production (Meiser and Vazquez, 2020). When the dissolved oxygen concentration increases, yeast switch from a fully fermentative metabolism to a mixed respiro-fermentative mechanism, ethanol production remaining the main metabolic direction (Aceituno *et al.*, 2012). Moreover, Gonzalez *et al.* (2013) proposed the use of respiratory metabolism of non-*Saccharomyces* yeasts as a tool for reducing the alcohol content in wine. Although non-*Saccharomyces* yeasts are generally characterized by low fermentation vigour, from a physiological point of view *T. delbrueckii* is the closest species to *S. cerevisiae* (Mehlomakulu *et al.*, 2021). For these reasons, *T. delbrueckii* was the first non-*Saccharomyces* yeast

proposed for industrial grape must fermentation (Castelli, 1955).

Considering all these aspects, the purpose of this study was to obtain low-alcohol wines by manipulating the fermentative metabolism of some laboratory-selected commercial yeast strains, and to identify the conditions that induced the modification in the sugar/ethanol conversion rate, as an alternative approach to avoiding excess ethanol production during winemaking.

MATERIALS AND METHODS

Ten commercial *Saccharomyces* sp. yeast strains and a non-*Saccharomyces* strain, *Torulaspora delbrueckii* (Table 1), were tested in monoculture, in anaerobic and semi-anaerobic conditions. To obtain the inoculum, 0.1 g of each commercial yeast was rehydrated in water (20 min, 30°C), pre-cultured in 5 mL of liquid YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose; autoclaved 20 min at 121°C) and incubated for 48 h at 30 °C (Sadoudi *et al.*, 2012).

Two hundred millilitres of sterile (10 min, 100°C) Muscat Ottonel must (171 g/L sugars, total acidity 3.82 g/L as tartaric acid, pH 3.52, 203 mg/L assimilable nitrogen), was separately inoculated with each yeast strain, at a cell density of 10^6 CFU (colony-forming units)/mL, and incubated at $20 \pm 2^\circ\text{C}$ (Biobase, Firlabo) for alcoholic fermentation. The Erlenmeyer flasks for anaerobic fermentation (500 cm³) were closed with a two-chamber unidirectional bubbler glass airlock, while the flasks for semi-anaerobic fermentation were closed with loose

cotton plugs and homogenized for 20 s, once a day at sampling.

Yeast characteristics during the fermentation process (fermenting power, fermentation kinetics, growth in liquid medium) were assessed according to Resolution OIV-OENO 370-2012 (OIV, 2012). Cell growth was determined by measuring the absorbance at 600 nm and by weighing the cell biomass (ATX 224 analytical balance, Shimadzu, Japan) after centrifugation (7500 rpm, 10 min).

Based on laboratory experiments, non-sterile clarified Muscat Ottonel must (186 g/L sugars, total acidity 4.61 g/L as tartaric acid, pH 3.46, 166 mg/L assimilable nitrogen) was sequentially fermented at micropilot level (in 25 L glass demijohns), in semi-anaerobic conditions (loose cotton plugs), by initial inoculation with *T. delbrueckii* (10^6 CFU/mL), followed by the addition of the preliminarily selected *S. cerevisiae* strain inoculum (10^4 CFU/mL) to the consumption of 50% of the sugars in must. The Muscat Ottonel must, fermented in anaerobic conditions (fermentation airlocker) by inoculation with *S. cerevisiae* (10^6 CFU/mL) in monoculture, represented the control sample. Prior to this, active cells were pre-cultured for 48 h at 28°C, in grape must diluted 50% with distilled water, in order to shorten the lag phase of the non-*Saccharomyces* yeast. Also, the *S. cerevisiae* starter cells were acclimated for 1 h in 1:1 water:must medium before inoculation.

In order to ensure the antioxidant and antimicrobial protection of the wines, 100 mg/L of SO₂ was added after racking wine off the lees. The main physicochemical parameters of grape

musts and wines, including total polyphenolic content (TPC), were analysed according to the OIV *Compendium of International Methods of Wine and Must Analysis* (OIV, 2019). Glycerol concentrations were determined by enzymatic kit no. 12812 (BioSystems, Spain). Free volatile terpenes (FVT) and precursors (PVT) were evaluated using the method proposed by Dimitriadis and Williams (1984) ($\mu\text{g/L}$ as linalool equivalent). Wine colour components (colour intensity, colour hue and the proportions of red, yellow and blue) were analysed according to Glories (1984), the absorbance of the samples being determined at 420, 520 and 620 nm. All spectral determinations were performed using a Specord 200 plus double-beam UV-vis spectrophotometer (Analytik Jena, Germany).

Sensory analysis of the wine was carried out by a panel of 13 initiated and expert tasters, scoring from 1 (poorest)

to 10 (best) each of the 19 characteristics analysed.

Data are reported as the mean of a minimum of two replicates, with standard deviation ($\pm$).

Analysis of variance (ANOVA test) (Microsoft® Excel, Data Analysis) was initiated to investigate significant differences between data. p -values < 0.05 were considered to be significant (●).

RESULTS AND DISCUSSION

The preliminary tests performed with the 11 commercial yeasts tested in monoculture provided important information on their growth and development. Fermenting power (FP) in anaerobic conditions (20 °C), determined by gravimetric analysis as the daily difference (Δ) in flask weight ($\text{FP} = 2.5 \cdot \Delta$), was high, varying between 60.00 (T8) and 63.61 (T7) (*Figure 1*).

Table 1 – Commercial yeast strains used in the experiments

Strain code	Product name	Producer	Yeast species
T1	Fermactive CB	Sodinal, France	<i>Saccharomyces cerevisiae</i> var. <i>bayanus</i>
T2	Fermactive Thiols	Sodinal, France	<i>S. cerevisiae</i>
T3	Zymasil Bayanus	AEB, France	<i>S. bayanus</i> (oviformis)
T4	SP39	Sofralab, France	<i>S. cerevisiae</i> galactose + <i>S. cerevisiae</i>
T5	Zymaflore X5	Laffort, France	<i>S. cerevisiae</i>
T6	Zymaflore X16	Laffort, France	<i>S. cerevisiae</i>
T7	Viniferm	Agrovin, Spain	<i>S. cerevisiae</i>
T8	Vinoferm Basic Cerevisiae	Essedielle, Italy	<i>S. cerevisiae</i>
T9	Vinoferm Basic Bayanus	Essedielle, Italy	<i>S. bayanus</i>
T10	Vinoferm Aroma	Essedielle, Italy	<i>S. cerevisiae</i>
T11	Zymaflore Alpha	Laffort, France	<i>Torulaspora delbrueckii</i>

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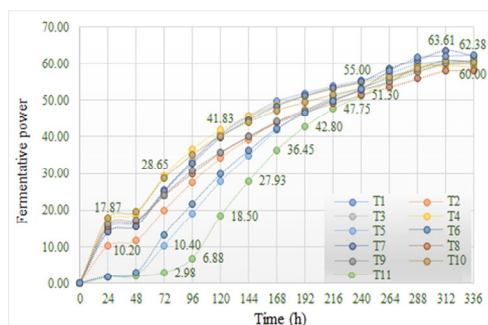


Figure 1 – Fermentative power of tested yeast strains in anaerobic conditions

Note: T1–T11 represent yeast codes as shown in Table 1.

After 14 days of anaerobic fermentation a different evolution of the FP was observed, in relation to sugar metabolization, ethanol synthesis and CO₂ release. Strains T4 (SP39, *S. cerevisiae*) and T7 (Viniferm, *S. cerevisiae*) showed high values of FP throughout the fermentation (Figure 1).

In the case of strain T11 (*T. delbrueckii*), although it showed average final values of FP, in the first 3 days, the values for this parameter were the lowest compared to *Saccharomyces* yeast strains. *Torulaspora* cells have a spherical shape, smaller than those of *S. cerevisiae*, but its life cycle has not yet been fully elucidated. The delay in ethanol synthesis was partially explained by Alves-Araujo *et al.* (2007). Thus, unlike *S. cerevisiae*, that switches to a respiro-fermentative metabolism as the amount of oxygen decreases, *T. delbrueckii* was still able to sustain full respiration under low-oxygen conditions.

Similar to anaerobic fermentation, in semi-anaerobic conditions (limited gas circulation), all yeasts managed to consume over 99% of the sugars in the must, with residual sugar concentrations

between 0.32 and 1.60 g/L (data not shown).

During the semi-anaerobic fermentation, the daily values of the cell density (OD 600 nm) increased gradually. The highest values were registered for strain T4 (SP39) (14.00 absorbance units), much higher than for the non-*Saccharomyces* strain (T11, *T. delbrueckii*), which showed the lowest values after 14 days of semi-anaerobic fermentation (10.24) (Figure 2).

A massive increase in the optical density at 600 nm was observed in the first 7 days of the semi-anaerobic process (exponential growth phase), after a lag phase of 12–48 h, depending on the strain. The *T. delbrueckii* strain (T11) showed different kinetics than did *Saccharomyces* strains, respectively a longer adaptation phase to environmental conditions (3 days) and a shorter exponential growth phase (approx. 4 days), the plateau phase being reached at the same time as in the *Saccharomyces* strains.

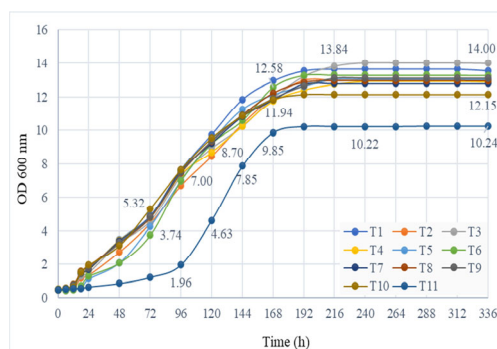


Figure 2 – Optical density at 600 nm of the yeast cultures during semi-anaerobic fermentation

Note: T1–T11 represent yeast codes as shown in Table 1.

Regarding the cell biomass at the end of the fermentation processes, it was

observed that semi-anaerobiosis induced the formation of larger amounts of biomass for all strains: in anaerobiosis the biomass yield varied between 1.10 and 1.78 g/100 mL, while in semi-anaerobiosis cell biomass increased by between 5.41% (T3) and 38.20% (T4) (Figure 3).

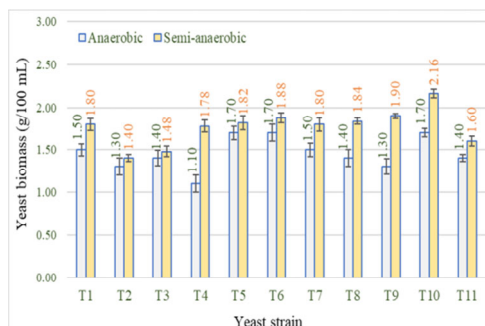


Figure 3 – Cell biomass formed by the tested yeasts in anaerobic and semi-anaerobic conditions

Note: T1–T11 represent yeast codes as shown in Table 1; error bars indicate the standard deviations (n = 2).

T. delbrueckii produced average amounts of cell biomass (1.40–1.60 g/100 mL), showing a good response to semi-anaerobiosis. Ramirez and Velázquez (2018) concluded that *T. delbrueckii* has less fermentation vigour and a slower growth rate than *S. cerevisiae*. Also, CO₂ production and O₂ consumption were higher in *T. delbrueckii*, a fact that generated lower amounts of biomass.

The alcohol yield was calculated according to the amount of sugar metabolized. Thus, in the anaerobiosis the mean values varied between 16.41 (T6) and 16.89 g (T11), while in semi-anaerobic conditions, the tested yeasts consumed between 17.44 (T4) and 19.90 g (T11) of sugar to produce 1% vol. of alcohol (Figure 4).

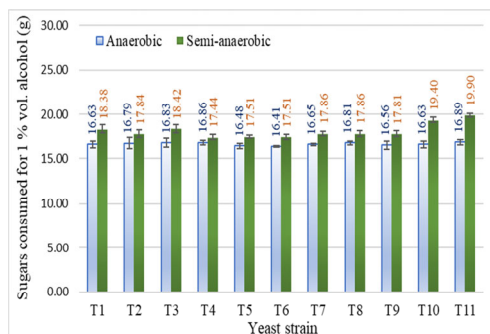


Figure 4 – Amount of sugar consumed to produce a volume of alcohol by tested yeasts

Note: T1–T11 represent yeast codes as shown in Table 1; error bars indicate the standard deviations (n = 2).

Compared to anaerobiosis, in semi-anaerobic fermentation, the *Saccharomyces* strains additionally consumed between 3.32% (T4) and 14.25% (T10) more sugars to produce a volume of alcohol, while the non-*Saccharomyces* strain (T11) consumed over 15.00% more sugar for each volume of alcohol produced.

Fermentation dominated by *T. delbrueckii* took longer to complete, a situation that can lead to additional wine oxidation (Ramirez and Velázquez, 2018). Values of the browning index of the wines obtained by anaerobic and semi-anaerobic fermentations, evaluated as the absorbance at 420 nm, are shown in Figure 5.

Although the recorded values were not high, a significant increase in the degree of oxidation was observed through semi-anaerobic fermentation (+35.38–54.87%), in comparison to anaerobiosis, due to the intermittent homogenization and limited gas exchange.

The complex laboratory experiments allowed the selection of two

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yeast strains which developed well in semi-anaerobic conditions, showed good fermentation power and consumed a larger amount of sugar for a volume of alcohol. Thus, in the micropilot experiment, the Muscat Ottonel grape must was sequentially fermented under semi-anaerobic conditions, by initial inoculation with the *T. delbrueckii* strain (T11) and the subsequent addition of the *S. cerevisiae* strain T10 (Vinoferm Aroma, Essedielle), when 50% of the sugars were consumed, in order to ensure completion of the sugar fermentation, as previously shown by Contreras *et al.* (2015). The control sample was anaerobically fermented in monoculture with the *S. cerevisiae* strain. Analysing the dynamics of yeast development (OD 600 nm), a shorter lag phase of the non-*Saccharomyces* strain was noticed, due to the pre-adaptation phase. After 6 days, strain T11 managed to consume 50% of the sugars, the must being inoculated with *S. cerevisiae* (T10), cell density increasing accordingly (Figure 6).

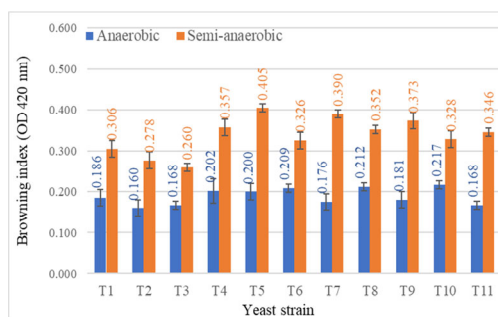


Figure 5 – Browning index of wines obtained by anaerobic and semi-anaerobic fermentations

Note: T1–T11 represent yeast codes as shown in Table 1; error bars indicate the standard deviation (n = 2).

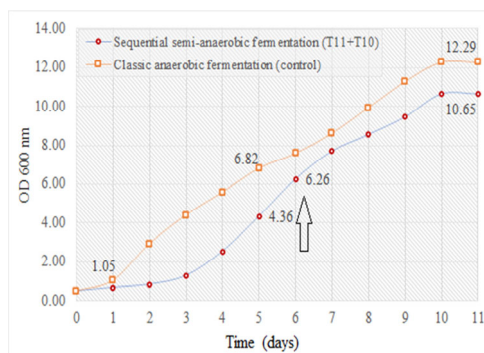


Figure 6 – Dynamics of yeast development (OD 600 nm) in sequential semi-anaerobic fermentation

Note: T10 and T11 represent yeast codes as shown in Table 1; the arrow indicates the time of *S. cerevisiae* strain inoculation in sequential fermentation.

After 11 days of sequential semi-anaerobic fermentation, the optical density at 600 nm was 15% lower than that of the control fermentation, probably due to microorganisms' interactions. As known, grape must and wine constitute very complex microbial ecosystems containing a mixture of different species and strains that interact intensively during must fermentation (competition, commensalism, antagonism, parasitism) (Liu *et al.*, 2017).

The dynamics of alcohol accumulation was different in the case of sequential semi-anaerobic fermentation, due to the distinct characteristics of the process and species involved. With the addition of *S. cerevisiae*, rapid completion of the alcoholic fermentation occurred (Figure 7).

The alcoholic concentration of the wines obtained by sequential fermentation in semi-anaerobiosis was lower by up to 1.10% vol. compared to classic anaerobic fermentation, meaning

a decrease of about 10%, at similar residual sugar levels (dry wines).

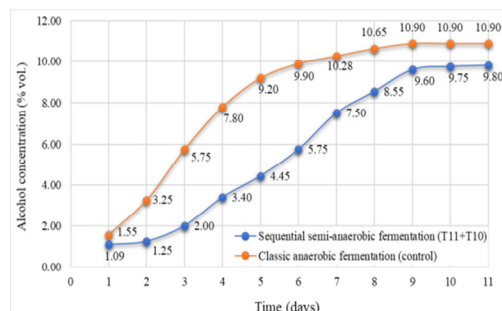


Figure 7 – Dynamics of alcohol synthesis in sequential semi-anaerobic fermentation

Note: T10 and T11 represent yeast codes as shown in Table 1.

The sugar/ethanol conversion rate varied from 16.85 (anaerobic) to 18.80 g sugar/vol. alcohol (semi-anaerobic sequential fermentation). The total acidity of the sequentially fermented wines was higher compared than that of the control wines (+0.31 g/L), while the

pH was slightly lower (Table 2). The volatile acidity values of wines were very similar (0.42–0.45 g/L as acetic acid), non-significantly higher values being found in sequentially semi-anaerobic fermented wine samples. Similar results were reported by Ramirez and Velázquez (2018). The TPC of wines was low, confirmed by the absorbance at 280 nm (5.88–6.19), non-significantly lower values being recorded in wines with a lower alcohol content (Table 3). Terpenes, as aroma compounds, are present in grapes, must and wines both as free (FVT) and combined volatile compounds, especially as glycosidic aroma precursors (PVT) (Lengyel, 2012). Due to semi-anaerobic conditions, a significant reduction in FVT (–10%) was registered (Table 3).

Table 2 – Physico-chemical characterization of wines obtained by semi-anaerobic sequential fermentation

Experimental variant	Alcohol (% vol.)	Total acidity (g/L tartaric acid)	Volatile acidity (g/L acetic acid)	Free SO ₂ (mg/L)	Total SO ₂ (mg/L)	Residual sugars (g/L)	pH
Sequential fermentation	9.80 ± 0.05	4.96 ± 0.10	0.45 ± 0.02	22 ± 1	107 ± 2	1.04 ± 0.08	3.11 ± 0.02
Control	10.90 ± 0.05	4.59 ± 0.11	0.43 ± 0.02	30 ± 2	110 ± 4	0.94 ± 0.10	3.16 ± 0.02
Significance	●	n.s.	n.s.	●	n.s.	n.s.	n.s.

Note: n.s. and ● - non-significant and significant at $p < 0.05$ (ANOVA test).

Table 3 – Phenolic compounds, terpenes and glycerol in wines obtained by semi-anaerobic sequential fermentation

Experimental variant	TPC (g GAE/L)	TPI (OD 280 nm)	FVT (µg LE/L)	PVT (µg LE/L)	Glycerol (g/L)
Sequential fermentation	0.26 ± 0.09	4.84 ± 0.08	298.18 ± 2.40	669.09 ± 6.32	9.32 ± 0.12
Control	0.30 ± 0.11	5.47 ± 0.14	330.91 ± 6.16	744.24 ± 10.42	5.97 ± 0.17
Significance	n.s.	●	●	●	●

Note: TPC (GAE) - total phenolic compounds (gallic acid equivalent); FVT - free volatile terpenes (linalool equivalent); PVT - precursor volatile terpenes; TPI - total polyphenolic index (OD at 280 nm); n.s and ● - non-significant and significant at $p < 0.05$ (ANOVA test).

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Previous studies showed that *T. delbrueckii* generates wines with high concentrations of terpenols, compared with wines fermented with *S. cerevisiae* (Sadoudi *et al.*, 2012).

Glycerol is a component of the wine matrix closely related to yeast metabolism and contributing to the sweetness and viscosity of the wine, with a positive impact on its sensory characteristics (Nieuwoudt *et al.*, 2002).

Semi-anaerobiosis enhanced yeast glycerol synthesis by up to 35% (Table 3). Previous studies also showed that under limited aerobic conditions the use of *T. delbrueckii* favours glycerol production (Contreras *et al.*, 2015).

In the choice and consumption of food, colour plays an important role, being associated with food quality and safety. Chromatic parameters can be used in the objective description of wine

The wine obtained by semi-anaerobic fermentation showed a non-significantly higher proportion of red hue, probably due to a slight oxidation of the phenolic compounds, while the control wine showed a higher colour intensity, and higher yellow (%Y) nuances (Table 4).

For the sensory analysis of the experimental wines, 19 characteristics were assessed by the panel of wine tasters (Figure 8).

Table 4 – Chromatic parameters of wines obtained by semi-anaerobic sequential fermentation

Experimental variant	Colour intensity (I)	Colour hue (H)	d 420 % (%Y)	d 520 % (%R)	d 620 % (%B)
Sequential fermentation	7.9 ± 0.32	6.2 ± 0.30	79.28 ± 0.29	15.25 ± 0.34	3.53 ± 0.30
Control	8.0 ± 0.21	5.4 ± 0.24	81.02 ± 0.22	14.98 ± 0.28	4.00 ± 0.19
<i>Significance</i>	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>

Note: %Y, %R, %B - percentage of yellow, red and blue colour; n.s. and • - non-significant and significant at $p < 0.05$ (ANOVA test).

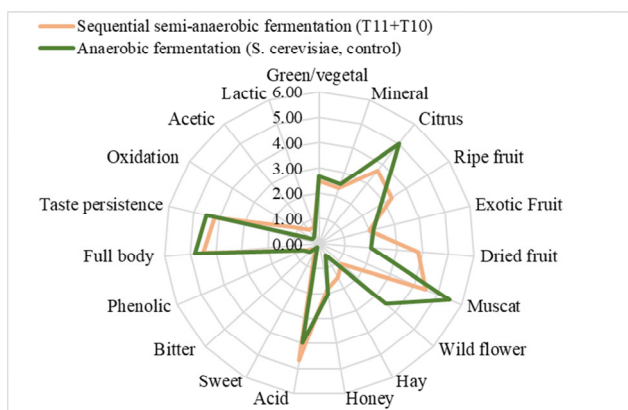


Figure 8 – Sensory analysis of wines obtained by anaerobic and semi-anaerobic fermentation

Low-alcoholic wines obtained by semi-anaerobic sequential fermentation of Muscat Ottonel must were appreciated as more acidic, with good aromas of muscat and dried fruits, and with a taste persistence and full body perception similar to those of control wine. The early oxidation of must was associated with the disappearance of green colour shades, resulting in yellow-gold tones, the lessening of some primary aromas, appearing aromas of ripe fruit and a sensation of dryness.

CONCLUSIONS

Microbiological strategies for the production of low-alcohol wines may represent a promising alternative to the currently used post-fermentation processes. By applying sequential fermentation, initiated with a *T. delbrueckii* yeast strain and completed with selected *S. cerevisiae* strains, in semi-anaerobic conditions, dry wines were obtained with an alcohol content lower by 1.10% vol., with a lower concentration of volatile terpenes and a higher amount of glycerol (< 35%). Nevertheless, sequential semi-anaerobic fermentation can be recommended for a reduction in ethanol content of 1% to 3% vol., based on the ability of non-*Saccharomyces* strains to perform in partial aeration conditions, maintaining an analytical and sensorial profile very similar to that of wine obtained by conventional winemaking technology. Further studies are necessary in order to select or to isolate new yeast strains capable of consuming more sugar per volume of produced alcohol, and better adapted to the semi-anaerobic or even aerobic conditions.

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