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# Aroma profiling of an aerated fermentation of natural grape must with selected yeast strains at pilot scale

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ABSTRACT

The use of non-*Saccharomyces* strains in aerated conditions has proven effective for alcohol content reduction in wine during lab-scale fermentation. The process has been scaled up to 20 L batches, in order to produce lower alcohol wines amenable to sensory analysis. Sequential instead of simultaneous inoculation was chosen to prevent oxygen exposure of *Saccharomyces cerevisiae* during fermentation, since previous results indicated that this would result in increased acetic acid production. In addition, an adaptation step was included to facilitate non-*Saccharomyces* implantation in natural must. Wines elaborated with *Torulaspora delbrueckii* or *Metschnikowia pulcherrima* in aerated conditions contained less alcohol than control wine (*S. cerevisiae*, non-aerated). Sensory and aroma analysis revealed that the quality of mixed fermentations was affected by the high levels of some yeast amino acid related byproducts, which suggests that further progress requires a careful selection of non-*Saccharomyces* strains and the use of specific N-nutrients.

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## 1. Introduction

*Saccharomyces cerevisiae*, the yeast carrying alcoholic fermentation of grape must, constitutes a minor fraction of the microbiota found on sound ripe grapes (Wang et al., 2015). Other yeast species, collectively known as non-*Saccharomyces* in this field, are much more abundant and considered to play an important role during the first hours of grape must fermentation (Fleet and Heard, 1993). Cell counts of the yeast genera *Hanseniaspora*, *Pichia*, *Metschnikowia* or *Torulaspora* can be moderately high during a short time when alcohol levels are still low, before *S. cerevisiae* takes over the fermentation process. There are many evidences that some non-*Saccharomyces* yeast species can positively contribute to the aroma profile, sensory complexity, and color stability of wines (Andorrà et al., 2012; Comitini et al., 2011; Gobbi et al., 2013; Viana et al., 2008; Sadoudi et al., 2012). Many authors have suggested the controlled use of these strains in combination with *S. cerevisiae* in order to improve aromatic complexity of wine (Ciani et al., 2010; Fleet, 2008; Padilla et al., 2016).

Nowadays, most yeast-producing companies have non-*Saccharomyces* yeast starters in their catalogs, and among them, *Torulaspora delbrueckii* is the most represented in the market. Mixed cultures of *T. delbrueckii*/*S. cerevisiae* have been proposed to reduce the acetic acid content and to enhance organoleptic profiles of wines (Moreno et al., 1991; Jolly et al., 2003; Bely et al., 2008).

The competitive advantage of *S. cerevisiae* over all the other yeast species during grape must fermentation translates into a small variability in alcohol yield between different isolates of this

*Abbreviation:* HCA, Hierarchical Cluster Analysis; MDS, Multidimensional Scaling; OAV, Odor Activity Value; PCA, Principal Component Analysis; YAN, Yeast Assimilable Nitrogen.

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species. For that reason, the alcohol yield variability of non-*Saccharomyces* wine yeasts has been explored by several authors (Ciani et al., 2016; Ciani and Maccarelli, 1998; Comitini et al., 2011; Domizio et al., 2011).

A recent proposal to reduce the ethanol content of wine considers the use of aerobic conditions in order to allow for respiro-fermentative metabolism of grape juice sugars. Non-*Saccharomyces* yeast strains are used in order to overcome the limitations due to the Crabtree positive character of *S. cerevisiae* (Gonzalez et al., 2013). Relevant parameters to assess the potential usefulness of yeast strains for this purpose were not only their respiratory capacity under high sugar conditions, but the production of acetic acid and the amount of sugars consumed during the aerobic stage (Quirós et al., 2014). The feasibility of the process was proven at the laboratory scale by co-inoculation of *Metschnikowia pulcherrima* and *S. cerevisiae*, and controlled aeration during the first 48 h (Morales et al., 2015). A maximal reduction of 3.7% ABV (alcohol by volume) was achieved for the fermentation of a natural grape must (260 g/L sugars), as compared to anaerobic fermentation with *S. cerevisiae*. Considering additional parameters, like keeping dissolved oxygen levels as low as possible, and avoiding excess volatile acidity, a 2.2% ABV reduction was achieved under optimized conditions. The aim of this work was to scale-up this process to pilot scale in order to identify potential bottlenecks outside the controlled conditions of the laboratory, and to produce wines amenable to sensory analysis. A strain of *M. pulcherrima* and a commercial strain of *Torulaspora delbrueckii* were used.

The effect of the commercial strain *T. delbrueckii* Viniferm NSTD on wine quality had been previously analyzed under standard fermentation conditions (Belda et al., 2015). The mouthfeel properties of wine produced at semi-pilot scale in a sequential inoculation with *S. cerevisiae* were preferred by a sensory panel, and correlated with an increase in the mannoprotein content.

## 2. Materials and methods

### 2.1. Strains and laboratory media

Strain *M. pulcherrima* Mp591, used in preliminary winemaking experiments, was provided by Agrovin S.A. (Alcázar de San Juan, Spain). *M. pulcherrima* strains used in the screening were isolated from grapes in La Rioja, Spain, and belong to the Microwine group strain collection (Instituto de Ciencias de la Vid y del Vino, Logroño, Spain). *M. pulcherrima* CECT 12841 (Morales et al., 2015) was used as a reference for the screening. *M. pulcherrima* Mp395, used in the final fermentation trial, was selected in the screening among other isolates of this species, based on the amount of sugars consumed, ethanol yield, and low aroma impact in a synthetic must. *S. cerevisiae* Viniferm Carácter and *T. delbrueckii* Viniferm NSTD are commercial strains from Agrovin S.A. (Alcázar de San Juan, Spain).

Synthetic grape must contained: 100 g/L glucose, 100 g/L fructose, 6 g/L citric acid, 6 g/L malic acid, 0.764 g/L ammonium chloride, 1.7 g/L Yeast Nitrogen Base without ammonium sulphate and amino acids, and 18 mg/L myo-inositol, pH adjusted to 3.5 with NaOH.

### 2.2. Screening of *M. pulcherrima* strains

*M. pulcherrima* strains were grown on YPD (2% glucose, 1% yeast extract, 2% peptone) for 48 h at 25 °C and 200 rpm. Cells were washed 2 times and resuspended in water to $OD_{600} = 10$. Then, 250 ml Erlenmeyer flasks containing 50 ml synthetic grape must were inoculated with 1 ml preculture, covered with an aluminium foil, and incubated for 4 days at 200 rpm at 18 °C. After this time, consumed sugars and metabolites produced were determined by HPLC as described in section 2.5. Experiments were carried out in duplicate.

### 2.3. Non-*Saccharomyces* inoculum preparation for winemaking

Non-*Saccharomyces* strains were grown in YPD for 48 h at 25 °C and 200 rpm. After centrifugation, aliquots of 8000 units $OD_{600}$ were suspended in 1 L pasteurized natural white must, and incubated for 3 days at 150 rpm and 22 °C to adapt them to grape must. Natural must was pasteurized in the autoclave by heating up to reach 105 °C and leaving to cool down inside. The whole culture was then used to inoculate 20 L of fresh natural non-sterile grape must (see below).

### 2.4. Scaled-up aerated winemaking procedure

Natural Viura-Malvasía white must was racked overnight at 4 °C. It contained 21% sugars, 237 mg/L total assimilable nitrogen, and 35 mg/L total $SO_2$, pH 3.43. Batches of 20 L in 30 L vats (36 cm diameter, resulting in a column of liquid about 20 cm high) were inoculated with 1 L conditioned inoculum of *M. pulcherrima* or *T. delbrueckii*. Batches of 21 L were inoculated with *S. cerevisiae* following the instructions of manufacturer (30 g/HL). In this way, the input volume of grape must in the whole process was the same for all conditions (21 L). Each tank was supplemented with 1.4 g/L tartaric acid, and 0.3 g/L Actimax Natura (Agrovin S.A., Spain). Three vats were fermented for each condition, using independent inocula. Vats inoculated with non-*Saccharomyces* were sparged with compressed air at 200 ml/h through submerged ceramic spargers. Gas flow was controlled with MFC17 mass flow controllers (Aalborg Instruments and Controls, Inc.; Orangeburg, NY), previously calibrated with an electronic precision flowmeter (Agilent Technologies, Santa Clara, CA). Room temperature was maintained at 18 °C.

Temperature and density were monitored daily. Density was measured with a portable digital densitometer (Densito 30PX, Mettler Toledo GmbH, Analytical, Schwerzenbach, CH). At day 4, air flow was stopped, 50 mg/L potassium bisulfite was added and, one hour later, vats were inoculated with *S. cerevisiae*, following the instructions of manufacturer (30 g/HL). At day 5, 0.3 g/L Actimax Plus (Agrovin S.A., Spain) was added in all vats, control vats included. After sugar depletion, on day 9, 90 mg/L potassium bisulfite was added in each vat, headspace filled with nitrogen and vats closed and kept 10 days at 10 °C. Finally, wine was transferred into colored glass bottles and kept at 4 °C.

Implantation of yeast starter cultures was monitored along the fermentation. Samples of days 0, 4 and 8 were plated on YPD, and DNA of 5 isolated colonies extracted (Lõoke et al., 2011). The presence of *M. pulcherrima* or *T. delbrueckii* was confirmed by PCR amplification of d1/d2 LSU 26S DNA and sequencing (Kurtzman and Robnett, 1998). Amplification of interdelta elements (Legras and Karst, 2003) was used to verify implantation at the *S. cerevisiae* strain level.

Production and consumption of the main fermentation-related metabolites in daily samples was determined by HPLC.

### 2.5. HPLC analysis of main fermentation metabolites

Production and consumption of the main fermentation-related metabolites in daily samples, (glucose, fructose, glycerol, acetic acid and ethanol) were determined in duplicate using a Surveyor Plus chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with a refractive index and a photodiode array detector (Surveyor RI Plus and Surveyor PDA Plus, respectively). Hyper REZ XP carbohydrate H+8 μm column and guard (Thermo Fisher Scientific) were used and maintained at 50 °C. Elution was performed

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