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 and the use of specific N-nutrients.

Keywords

reduced alcohol wine, aerobic fermentation, non-Saccharomyces, sensory analysis

Highlights

Aerated fermentation with non-Saccharomyces strains for reduced alcohol wine was scaled up.

Sensory analysis of wines in comparison with standard (S. cerevisiae, non aerated) was performed.

Wines elaborated with different strains had different aroma profiles.

Volatile compound analysis identifies the compounds responsible for differences in aroma nuances.
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 (Andorrá 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., 2001; 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 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
hours 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 hours 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 SO₂, 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 (Looke 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 with 1.5 mM H₂SO₄ as mobile phase, at a flow rate of 0.6 mL/min. Prior to injection, samples were filtered through 0.22-μm-pore-size nylon filters and diluted 10-fold.

One way analysis of variance was carried out on the main fermentation metabolites. Means of biological replicates were compared using Tukey’s test, with significance level set at 5%. All analyses were performed using SPSS Statistics v. 23 program (IBM, Armonk, NY).

2.6. Sensory analysis of wines

Sensory analysis was performed one month after bottling. The starting point was a sorting task to select exemplars representative for sensory differences observed in the sensory space. These
samples were further characterized (flash profile) by a panel of semi-trained panelists and their aroma quality was finally evaluated by a panel of wine experts. In the three tasks, samples were presented simultaneously attending to a random order different for each assessor. Twenty-mL samples were poured in dark wine glasses (ISO 3591, 1977) labelled with 3-digit random codes and covered by plastic Petri dishes. All samples were served at room temperature and evaluated in individual booths. Panelists were not informed about the nature of the samples to be evaluated.

2.6.1. Sorting task

The sorting task consisted in grouping wines by similarity and generating descriptors to differentiate the wines. A total of eleven wines (9 vats + 2 duplicates) were evaluated. Vats Sc1, Sc2 and Sc3, were elaborated with *S. cerevisiae*; Mp4, Mp5 and Mp6, elaborated with *M. pulcherrima*; Td7, Td8 and Td9, elaborated with *T. delbrueckii*. The sorting task was carried out by a panel of eighteen wine experts (11 women and 7 men, ranging from 23 to 63 years of age, average = 35) in two independent sessions. In a first session, the panel was asked to group samples by orthonasal aroma; and in a second session, according to in-mouth sensations (aroma, mouthfeel and taste). No limits to number of groups were given. Panelists were asked to write a maximum of 3 words describing each group of wines.

2.6.1.1. Sorting task data analysis

An individual similarity binary matrix (11 wines x 11wines) was built with data of each panelist, where 1 means similar and 0 means different. A co-occurrence matrix, obtained by sum of all panelists, was submitted to a non-parametric Multidimensional Scaling (MDS) analysis (absolute model) in order to obtain a spatial representation of wines. The quality and the reliability of representations were evaluated by Shepard diagrams and Kruskal`s stress value. Finally, Hierarchical cluster analysis (HCA) with the Ward criterion was performed on the matrix consisting
of wines x coordinates of the retained MDS dimensions. All analyses were carried out with XLSTAT (2015 version).

A list of 12 descriptors for these wines was made with terms generated by panel members, avoiding hedonic and quantity adjectives, and grouping words belonging to the same category (Franco-Luesma et al., 2016). Descriptors are listed in Table 1.

2.6.2. Aroma characterization: flash profile

A flash profile was carried for wine aroma characterization. The panel was formed by 13 semi-trained assessors (8 women and 5 men, ranging from 25 to 39 years old, average = 31) with experience in sensory description of wine. The task was similar to classical flash profile, with some modifications carried out with the aim of facilitating the interpretation of attributes, which deems difficult in this methodology given the absence of consensus and training of participants. Therefore, references for the 12 terms obtained in sorting task (Table 1) were built and presented to participants. This familiarization task finished when panelists could correctly match terms with reference standards. Afterwards, they were presented with the six samples, four representing each group formed in previous task, and 2 duplicates. In a first session, assessors were asked to provide the descriptors differentiating each wine. In a second session, they were asked to rank the six wines attending to each one the terms chosen to differentiate among samples. A non-structured 10 cm continuous length scale anchored with the words “absence” and “high intensity” on the left and right ends was provided for each descriptor.

2.6.2.1. Flash profile data analysis

Principal Component Analysis (PCA) was performed with the mean intensity scores of descriptors that were individually discriminant in a two-way ANOVA (participants as random and
wines as fix factors) and that were used by more than half of panelists. Analyses were carried out with XLSTAT software (version 2015).

2.6.3. Aroma quality evaluation

Evaluation of aroma quality was carried out by a panel of 12 wine experts (7 women, ranging from 27 to 62 years old, average = 38). They were all oenologists, who had attended wine-tasting classes and had relevant professional experience in winemaking (Parr et al., 2002). Assessors were presented with seven wines: the four representing each group formed in sorting task and three control samples. The control wines comprised one young white wine (elaborated with Viura) of high quality (C_hq) and two white wines of low quality representing reduction (C_Red) and oxidation (C_ox) defects. Reduction defect was generated by spiking wines with hydrogen sulfide (60 µg L⁻¹) and methanethiol (20 µg L⁻¹) and oxidation with methional (90 µg L⁻¹) and phenylacetaldehyde (180 µg L⁻¹). Participants were asked to smell each sample from left to right and to score their aroma quality on a nine-point scale (1=very poor; 3=poor; 5=average; 7=good and 9=very good) based on orthonasal olfaction.

2.6.3.1. Aroma quality data analysis

A two-way ANOVA was carried on quality scores with assessors as random factor and wines as fixed factor, followed by Fischer post-hoc pairwise comparison (95%) test.

2.7. Volatile compounds analysis

Major volatile compounds were isolated by liquid-liquid extraction and analyzed in a gas chromatograph with flame ionization detector (GC-FID) as described (Ortega et al., 2001). Minor and trace volatile compounds were isolated through solid-phase extraction (SPE) and analyzed by
gas chromatography coupled to a mass spectrometry detection system (GC-MS), as described by Lopez et al. (2002).

Polyfunctional mercaptans were analyzed and quantified by GC-MS with negative chemical ionization (NCI) after SPE derivatization with 2,3,4,5,6-pentafluorobenzylbromide (PFBBr) (Mateo-Vivaracho et al., 2008).

Free Volatile Sulfur Compounds (VSCs) were determined by direct static headspace analysis using a GC coupled with a pulsed flame photometric detection system (GC-PFPD) (Franco-Luesma and Ferreira, 2014).

Free forms of aldehydes (methional, isobutyraldehyde, isovaleraldehyde, and phenylacetaldehyde) were quantified by SPME followed by GC-MS as described (Bueno et al., 2014).

### 2.7.1. Volatile compound data analysis

Quantitative data of volatile compounds were transformed into Odor Activity Values (OAV) by dividing them by their corresponding sensory thresholds (ST). The OAV of the limits of detection and quantification was also calculated and used as minimal value when that of compound was lower (San Juan et al., 2011). Odorants with similar chemical and sensory properties were grouped in aroma vectors (Loscos et al., 2007, Saenz_Navajas et al., 2015). Table 2 shows the composition of the fourteen aroma vectors constructed. To rank compounds or families of compounds in accordance to the differentiation ability, the quotient between the maximum OAV and minimum OAV was worked out for each compound or family. Value max/min = 1.5 was established as threshold.

### 2.8. Multivariate analysis
Principal Component Analysis (PCA) was calculated with sensory descriptors as active variables and chemical compounds (expressed as OAVs) as supplementary variables. Only chemical compounds presenting OAV > 1.5 in at least one wine were considered. The statistical analyses were carried out with XLSTAT software (Version 2014.2.02).

3. Results and discussion

3.1. Selection of a Metschnikowia pulcherrima strain

Some strains of *M. pulcherrima* had shown good properties to be used in aerobic fermentation for alcohol level reduction (Quirós et al., 2014) and one of them was successfully used at laboratory scale in co-inoculation with *S. cerevisiae* (Morales et al., 2015). For that reason, we decided to make a screening among different grape isolates of *M. pulcherrima* to select a good candidate for further development.

The screening involved 11 *M. pulcherrima* recent isolates, in addition to *M. pulcherrima* CECT 12841, from the previous work (Morales et al., 2015), as a reference. Strains were grown in a synthetic must with vigorous agitation for 4 days at 18°C and parameters considered important for the correct behavior of strains in aerated fermentation were measured. Results are presented in Figure 1. The strain with the lowest ethanol yield was Mp274, but it also showed the highest acetic acid yield (see plot) and ethyl acetate production (data not shown). Strain Mp440 had the lowest acetic acid yield and very low ethanol yield, but the amount of sugars consumed was lower than other strains. There was a group of 5 strains with a low acetic acid yield and similar ethanol yield: Mp374, Mp395, Mp411, Mp416 and Mp711. Among them, Mp395 and Mp711 showed the highest amounts of consumed sugars and glycerol production. All strains could ferment a synthetic must
with 400 g/L sugars, and consumed between 86 g/L (Mp594) and 138 g/L (Mp395) in 4 days at 25ºC (data not shown).

3.2. Preliminary pilot-scale tests

Two fermentation assays were run during the 2015 harvest season (prior to M. pulcherrima strain selection). Non-Saccharomyces strains were grown in YPD for 48 h, centrifuged and then inoculated in must at initial OD600 of 0.4. An aeration regime of 60 L/h (3 VVH) was maintained for 48 h in vats inoculated with non-Saccharomyces. After this time, aeration was stopped, and S. cerevisiae was added as dry yeast at 30 g/HL. Nitrogen supplementation was performed at the beginning and after inoculation of S. cerevisiae, as described in Materials and Methods. Room temperature was set at 20ºC. The wines produced in aerated conditions contained less alcohol than the control (see Table 4), but acetic acid was over the limits of acceptability (data not shown). In addition, microbiological analyses showed that in these conditions, native must microbiota prevailed over the inoculated non-Saccharomyces strains 24 h after inoculation.

Considering these results, a second trial including reduced airflow (12 L/h or 0.6 VVH) and a step of adaptation of strains to must conditions was run. For the latter, strains were grown for 48 h in YPD, cells were then collected and suspended in 1 L of pasteurized must at OD 8. Cells were incubated for 3 days with vigorous agitation and then used to inoculate 20 L fresh grape must. Room temperature was set at 20ºC. In these conditions, inoculated non-Saccharomyces strains prevailed over wild microbiota at least until S. cerevisiae inoculation. Reduction in alcohol levels was moderate (see Table 4), but still significant, and acetic acid produced was very low in all conditions. This fermentation was performed at the very end of the 2015 harvest season, and counts of S. cerevisiae in must were high. Must contained 0.5 % ethanol (v/v) just before inoculation. For that reason, we decided to repeat this assay under more suitable conditions.
3.3. Optimized pilot-scale aerobic fermentation

This experiment was carried during the 2016 harvest season with a white must containing 21% sugars, pH 3.43, and 237 mg/L total assimilable nitrogen. *T. delbrueckii* NSTD and *M. pulcherrima* Mp395 were conditioned as previously described. Room temperature was set at 18ºC and aeration in non-*Saccharomyces* vats at 12 L/h. Must was racked overnight at 4ºC, just before inoculation, and was still cold at inoculation time. After 2 days, the temperature increase in vats indicated microbial activity in non-*Saccharomyces* vats (see Figure 2). For that reason, aeration was kept till day 4, longer than in previous assays. Potassium bisulfite was added just after aeration stopping, and 1 hour later *S. cerevisiae* added in non-*Saccharomyces* vats, as active dry yeast at 30 g/HL. On day 5, total nitrogen was below 15 mg/L and an extra addition of nitrogen supplements was done in all vats to help *S. cerevisiae* activity. On day 9, density indicated that sugars were depleted in all vats so 90 mg/L potassium bisulfite was added in each vat, head space filled with nitrogen and vats closed and kept for 10 days at 10ºC. Then, wine was transferred into colored glass bottles and kept at 4ºC.

Microbiological analysis showed that must contained 2.6 x 10³ cells/ml just before inoculation. Maximal counts in non-*Saccharomyces* vats, higher than 10⁸ cells/ml, were found on day 2. The color of colonies in plates indicated that *M. pulcherrima* was dominant in the vats where it had been inoculated. Maximal counts in *S. cerevisiae* vats were reached on day 4, lower than 10⁷ cells/ml, and maintained constant till the end of fermentation.

On day 4, before inoculation with *S. cerevisiae*, counts in *M. pulcherrima* vats were 1 log unit lower than on day 2, and the color indicated that a third of colonies were other microorganisms. All five sequenced non-*Metschnikowia* colonies were *S. cerevisiae*. Counts in *T. delbrueckii* vats for
this time point were about 2 log units lower than on day 2. All five sequenced colonies were *T. delbrueckii*.

The must density curve followed the same pattern than residual sugars, plotted in Figure 2. Sugar consumption in non-*Saccharomyces* vats was appreciated earlier than in *S. cerevisiae* vats. On day 2, there were 203, 176 and 153 g/L residual sugars for *Saccharomyces, Metschnikowia* and *Torulaspora* vats respectively. On day 4, before addition of *S. cerevisiae*, residual sugars in *S. cerevisiae* and in *M. pulcherrima* vats were similar, around 50% of initial sugars, while in *T. delbrueckii* vats the 75% of initial sugars had been consumed. Sugars had been exhausted on day 7 in *S. cerevisiae* vats and on day 8 in non-*Saccharomyces* vats. The aerated process had taken only one day more than the traditional one.

Table 5 shows metabolites found at the end of fermentation. A moderate reduction in ethanol content, but still significant, was achieved by the end of fermentation. The levels of acetic acid were low in all samples. Moreover, levels were significantly lower in non-*Saccharomyces*, aerated fermentations than in *S. cerevisiae* fermentations. *M. pulcherrima* produced the highest levels of glycerol.

### 3.4. Sensory analysis

Results of the sorting task based exclusively on orthonasal aroma perception are summarized in the dendrogram shown in Figure 3. Samples group in three stable clusters perfectly matching the yeast used. Wines belonging to the same cluster were grouped together at least 10 times (56% of participants), except the Mp4 wine which was grouped with Mp5 and Mp6 six (33%) and four (22%) times, respectively, which suggests that is the least similar to the other two replicates. For that reason, this cluster containing *M. pulcherrima* wines was split into two for wine characterization. Results of the sorting task based on the overall flavor (aroma, taste and mouth-feel
properties) produced similar results (Supplementary Figure S1) which suggests that most sensory
differences are mainly driven by aroma properties.

Wines Sc1, Td7, Mp5 and Mp4 (with replicates of Sc1* and Td7* as controls) were chosen as
group representative for wine aroma characterization and were subjected to orthonasal descriptive
analysis by means of flash profile with a panel of semi-trained assessors. Training consisted in
familiarization with terms and references obtained from the sorting task and given in Table 1.

Sixteen different terms were generated including the 12 attributes in Table 1, together with meat,
grain, lemon (cited by just one participant) and red fruit (cited by two participants). The more cited
terms (at least 7 out of 13 panelists) were: oxidation, spirit-like, dried fruit, nuts-walnut, reduction,
white fruit-pear and tropical fruit-banana. As the pairs dried fruits/nuts-walnuts and
oxidation/spirit-like were strongly correlated ($r>0.90$) they were further considered as single terms
under the labels dried fruit/nuts and oxidation/spirit-like.

Figure 4 shows the projection of wines on the graph obtained with the first and second principal
components of PCA analysis, representing respectively 58% and 38% of variance. Duplicate
samples group together in the plot, indicating the reliability of panel. Three groups of wines can be
observed in the graph, coinciding with yeasts used. This result suggests that even if wine Mp4
seems to be relatively different from Mp5, they present aroma commonalities that make them to be
more similar to each other than to $S. \text{cerevisiae}$ or $T. \text{delbrueckii}$ wines. The first PC confronts the
terms white fruit-pear and tropical fruit-banana, mainly attributed to wines elaborated with $S.\text{cerevisiae}$ yeasts, to dried fruit/nuts and reduction, which characterize $T. \text{delbrueckii}$ wines. The
second PC is basically driven by the term oxidation/spirit-like, which seems to be predominant in
$M. \text{pulcherrima}$, especially in Mp4 and to a lesser extent in Mp5. This can be clearly seen in the
spider plot shown in Figure 5, which confirms that $S. \text{cerevisiae}$ wines have maxima scores for
white fruit-pear and tropical fruit-banana, $T. \text{delbrueckii}$ wines for dried fruit/nuts and reduction
and, $M. \text{pulcherrima}$ wines for oxidation/spirit-like.
Aroma quality was also assessed and results are summarized in Figure 6. As seen in the Figure, scores for experimental wines ranged from 3.9 (poor-average quality) for both *M. pulcherrima* wines to 6.7 (good quality) for *S. cerevisiae* wine. *T. delbrueckii* wine was classified as average quality.

### 3.5. Volatile compound analysis

Table 6 shows the quantitative data of more than 80 volatile compounds found in the 4 exemplars analyzed used in sensory analyses. Concentrations are within the normal range of occurrence in wines (San Juan et al., 2012; Swiegers et al., 2005) with some exceptions, since levels of ethyl dihydroxycinnamate, methionol and β-phenylethanol are unusually high in *T. delbrueckii* sample, and those of 2-methyl-1-propanol (isobutanol) in *M. pulcherrima* samples.

Data of aroma compound concentration were converted into OAVs and further grouped with other aroma molecules with similar odors into aroma vectors, as shown in Table 3. The biplot with the two first components of the PCA made on sensory data and aroma vectors is given in Figure 7. The plot makes it possible to identify the aroma vectors potentially responsible for the sensory differences observed between samples. The fruity character of wines elaborated with *S. cerevisiae* is consistent with the higher levels of acetates, especially 3-mercaptohexyl acetate (MHA), and ethyl esters. The lowest aroma quality of *Metschnikowia* wines is no doubt related to their oxidation and spirit/like character and to their negligible fruity character. These sensory notes can be attributed to the highest levels of aliphatic fusel alcohols, which have been found to impair the perception of fruitiness and give a spirit note (de-la-Fuente-Blanco et al., 2017), and to the highest levels of Strecker aldehydes and of acetaldehyde, which are responsible for the oxidative notes. Finally, the reductive odor note found in *Torulaspora* wines should be related to their highest levels in VSCs (Franco-Luesma et al., 2016), while the dry fruit/nut character may be related to the highest levels of methional (San-Juan et al., 2011) and of cinnamates. The fact that the oxidation notes were found...
only in Metschnikowia wines and not in Torulaspora wines indicates that this defect is related to the strain used, rather than to the process of aeration on its own. Strain selection for commercial purposes would require the analysis of volatile compounds produced under aerated conditions.

It is noteworthy that many compounds explaining aroma differences are related to the amino acid metabolism of the different yeast strains. This is the case of fusel alcohols and their acetates, of Strecker aldehydes, and of the most important VSCs: $\text{H}_2\text{S}$ and methanethiol. Attending to present data, it seems that some of these compounds are most likely responsible for some of the aromatic problems detected in Metschnikowia wines (oxidation, lack of fruitness) and Torulaspora wines (reduction). Thus, it can be hypothesized that a specific reengineering of the nitrogen supplementations provided to the yeast may produce wines with much improved sensory characters and yet reduced levels of ethanol.

4. Conclusions

In summary, we have shown the feasibility of scaling up aerated fermentation conditions, and the use of non-$\text{Saccharomyces}$ yeast strains, for reducing ethanol content of wines. One key point in the optimization process has been the improvement of the inoculum preparation step, to warrant pre-adaptation of non-$\text{Saccharomyces}$ cells to grape must, as well as an active metabolism ever since the inoculation time. Aeration conditions could not be extrapolated directly from the relative air flows (vvh) under laboratory conditions, and probably increasing the depth of the tanks would require further reduction in air flows. Since we have previously shown the increased production of acetic acid by $\textit{S. cerevisiae}$ under aerated conditions, sequential inoculation, with $\textit{S. cerevisiae}$ being inoculated after aeration is stopped, seems to be a better choice than co-inoculation with non-$\textit{Saccharomyces}$ strains. The secondary problem of nutrient depletion by the non-$\textit{Saccharomyces}$ starter, before inoculation of standard wine yeasts, has been easily addressed by a rational use of yeast nutrients in key moments of the process. However, results of sensory and aroma analysis
suggest that those nutrients should be specifically formulated to limit the formation of problematic
compounds such as VSCs, Strecker aldehydes or fusel alcohols. While the current protocol allowed
circumventing the problem of acetic acid production, further optimization will be required to
develop an industrially feasible protocol for aerated fermentation with non-\textit{Saccharomyces} yeast
strains. Topics to be further addressed are the problem of adjusting oxygenation levels to improve
alcohol reduction, the non-\textit{Saccharomyces} strain selection, and the formulation of specific nutrients
to limit the formation of aroma compounds of demonstrated negative character.

\textbf{Abbreviation}

\textbf{HCA}, Hierarchical Cluster Analysis; \textbf{MDS}, Multidimensional Scaling; \textbf{OAV}, Odor Activity Value;
\textbf{PCA}, Principal Component Analysis; \textbf{YAN}, Yeast Assimilable Nitrogen.

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\textbf{Supplementary data}

Figure S1. Tree diagram obtained from Hierarchical Cluster Analysis (HCA) with the Ward
criterion performed on data from sorting task based on aroma taste and mouth-feel properties.
List S1. References for Odor threshold in Table 6.

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Figure Captions

Figure 1. Sugars consumed and metabolites produced by Metschnikowia pulcherrima strains in synthetic must (200 g/L sugars) at 18°C.
**Figure 2.** Monitoring of fermentation parameters.

**Figure 3.** Tree diagram obtained from Hierarchical Cluster Analysis (HCA) with the Ward criterion of wines performed with data from MDS of orthonasal aroma descriptors as variables.

**Figure 4.** Projection of wines used in flash profile (4 wines + 2 replicates) and discriminant attributes on the two first dimensions (PC1 and PC2) of the PCA performed with selected aroma descriptors.

**Figure 5.** Sensory description of wine samples (average for duplicate samples Sc1 and Td7).

**Figure 6.** Mean aroma quality ratings of studied wines (including controls: C_ox, C_red, C_hq. Different letters indicate the existence of a significant difference between samples ($\alpha<0.05$) (Fischer post-hoc test). Error bars are calculated as $s/(n)^{1/2}$; $s$, standard deviation; $n$, number of assessors.

**Figure 7.** Projection of sensory descriptors (blue color), chemical vectors (red color), and wines on the two first dimensions (PC1 and PC2) of the PCA performed with sensory descriptors as active variables and chemical variables (expressed as OAVs) as supplementary variables.