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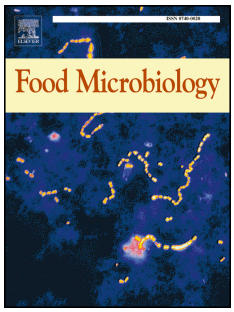

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PII: S0740-0020(17)30588-9

DOI: 10.1016/j.fm.2017.10.008

Reference: YFMIC 2885

To appear in: *Food Microbiology*

Received Date: 21 June 2017

Revised Date: 5 October 2017

Accepted Date: 16 October 2017

Please cite this article as: Tronchoni, J., Curiel, J.A., Sáenz-Navajas, Marí.Pilar., Morales, P., de-la-Fuente-Blanco, A., Fernández-Zurbano, Purificació., Ferreira, V., Gonzalez, R., Aroma profiling of an aerated fermentation of natural grape must with selected yeast strains at pilot scale, *Food Microbiology* (2017), doi: 10.1016/j.fm.2017.10.008.

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 25ºC 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 18ºC. 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 25ºC 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 22ºC to adapt them to grape must. Natural must was

pasteurized in the autoclave by heating up to reach 105ºC 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 4ºC. 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 18ºC.

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 10ºC. Finally, wine was transferred into colored glass bottles and kept at 4ºC.

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 H_2SO_4 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 $\mu g\ L^{-1}$) and methanethiol (20 $\mu g\ L^{-1}$) and oxidation with methional (90 $\mu g\ L^{-1}$) and phenylacetaldehyde (180 $\mu g\ L^{-1}$). 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 (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 stablished 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 18ºC 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.

320

321 *3.3. Optimized pilot-scale aerobic fermentation*

322

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

336 Microbiological analysis showed that must contained 2.6 x 10^3 cells/ml just before inoculation.
337 Maximal counts in non-*Saccharomyces* vats, higher than 10^8 cells/ml, were found on day 2. The
338 color of colonies in plates indicated that *M. pulcherrima* was dominant in the vats where it had been
339 inoculated. Maximal counts in *S. cerevisiae* vats were reached on day 4, lower than 10^7 cells/ml,
340 and maintained constant till the end of fermentation.

341 On day 4, before inoculation with *S. cerevisiae* , counts in *M. pulcherrima* vats were 1 log unit
342 lower than on day 2, and the color indicated that a third of colonies were other microorganisms. All
343 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

368 properties) produced similar results (Supplementary Figure S1) which suggests that most sensory
369 differences are mainly driven by aroma properties.

370 Wines Sc1, Td7, Mp5 and Mp4 (with replicates of Sc1* and Td7* as controls) were chosen as
371 group representative for wine aroma characterization and were subjected to orthonasal descriptive
372 analysis by means of flash profile with a panel of semi-trained assessors. Training consisted in
373 familiarization with terms and references obtained from the sorting task and given in Table 1.
374 Sixteen different terms were generated including the 12 attributes in Table 1, together with *meat*,
375 *grain*, lemon (cited by just one participant) and *red fruit (*cited by two participants). The more cited
376 terms (at least 7 out of 13 panelists) were: *oxidation*, *spirit-like*, *dried fruit*, *nuts-walnut*, *reduction*,
377 *white fruit-pear* and *tropical fruit-banana*. As the pairs *dried fruits/nuts-walnuts* and
378 *oxidation*/*spirit-like* were strongly correlated (r>0.90) they were further considered as single terms
379 under the labels *dried fruit/nuts* and *oxidation/spirit-like.*

380 Figure 4 shows the projection of wines on the graph obtained with the first and second principal
381 components of PCA analysis, representing respectively 58% and 38% of variance. Duplicate
382 samples group together in the plot, indicating the reliability of panel. Three groups of wines can be
383 observed in the graph, coinciding with yeasts used. This result suggests that even if wine Mp4
384 seems to be relatively different from Mp5, they present aroma commonalities that make them to be
385 more similar to each other than to *S. cerevisiae* or *T. delbrueckii* wines. The first PC confronts the
386 terms *white fruit-pear* and *tropical fruit-banana*, mainly attributed to wines elaborated with *S.*
387 *cerevisiae* yeasts, to *dried fruit/nuts* and *reduction*, which characterize *T. delbrueckii* wines. The
388 second PC is basically driven by the term *oxidation/spirit-like*, which seems to be predominant in
389 *M. pulcherrima*, especially in Mp4 and to a lesser extent in Mp5. This can be clearly seen in the
390 spider plot shown in Figure 5, which confirms that *S. cerevisiae* wines have maxima scores for
391 *white fruit-pear* and *tropical fruit-banana*, *T. delbrueckii* wines for *dried fruit/nuts* and *reduction*
392 *and*, *M. pulcherrima* wines for *oxidation/spirit-like*.

393 Aroma quality was also assessed and results are summarized in Figure 6. As seen in the Figure,
394 scores for experimental wines ranged from 3.9 (poor-average quality) for both *M. pulcherrima*
395 wines to 6.7 (good quality) for *S. cerevisiae* wine. *T. delbrueckii* wine was classified as average
396 quality.
397

398 *3.5. Volatile compound analysis*

399 Table 6 shows the quantitative data of more than 80 volatile compounds found in the 4
400 exemplars analyzed used in sensory analyses. Concentrations are within the normal range of
401 occurrence in wines (San Juan et al., 2012; Swiegers et al., 2005) with some exceptions, since levels
402 of ethyl dihydroxycinnamate, methionol and β-phenylethanol are unusually high in *T. delbrueckii*
403 sample, and those of 2-methyl-1-propanol (isobutanol) in *M. pulcherrima* samples.
404 Data of aroma compound concentration were converted into OAVs and further grouped with
405 other aroma molecules with similar odors into aroma vectors, as shown in Table 3. The biplot with
406 the two first components of the PCA made on sensory data and aroma vectors is given in Figure 7.
407 The plot makes it possible to identify the aroma vectors potentially responsible for the sensory
408 differences observed between samples. The fruity character of wines elaborated with *S. cerevisiae* is
409 consistent with the higher levels of acetates, especially 3-mercaptohexyl acetate (MHA), and ethyl
410 esters. The lowest aroma quality of *Metschnikowia* wines is no doubt related to their oxidation and
411 spirit/like character and to their negligible fruity character. These sensory notes can be attributed to
412 the highest levels of aliphatic fusel alcohols, which have been found to impair the perception of
413 fruitiness and give a spirit note (de-la-Fuente-Blanco et al., 2017), and to the highest levels of
414 Strecker aldehydes and of acetaldehyde, which are responsible for the oxidative notes. Finally, the
415 reductive odor note found in *Torulaspora* wines should be related to their highest levels in VSCs
416 (Franco-Luesma et al., 2016), while the dry fruit/nut character may be related to the highest levels
417 of methional (San-Juan et al., 2011) and of cinnamates. The fact that the oxidation notes were found

418 only in *Metschnikowia* wines and not in *Torulaspora* wines indicates that this defect is related to the
419 strain used, rather than to the process of aeration on its own. Strain selection for commercial
420 purposes would require the analysis of volatile compounds produced under aerated conditions.
421 It is noteworthy that many compounds explaining aroma differences are related to the amino acid
422 metabolism of the different yeast strains. This is the case of fusel alcohols and their acetates, of
423 Strecker aldehydes, and of the most important VSCs: H_2S and methanethiol. Attending to present
424 data, it seems that some of these compounds are most likely responsible for some of the aromatic
425 problems detected in *Metschnikowia* wines (oxidation, lack of fruitness) and *Torulaspora* wines
426 (reduction). Thus, it can be hypothesized that a specific reengineering of the nitrogen
427 supplementations provided to the yeast may produce wines with much improved sensory characters
428 and yet reduced levels of ethanol.
429
430 **4. Conclusions**
431 In summary, we have shown the feasibility of scaling up aerated fermentation conditions, and the
432 use of non-*Saccharomyces* yeast strains, for reducing ethanol content of wines. One key point in the
433 optimization process has been the improvement of the inoculum preparation step, to warrant pre-
434 adaptation of non-*Saccharomyces* cells to grape must, as well as an active metabolism ever since
435 the inoculation time. Aeration conditions could not be extrapolated directly from the relative air
436 flows (vvh) under laboratory conditions, and probably increasing the depth of the tanks would
437 require further reduction in air flows. Since we have previously shown the increased production of
438 acetic acid by *S. cerevisiae* under aerated conditions, sequential inoculation, with *S. cerevisiae*
439 being inoculated after aeration is stopped, seems to be a better choice than co-inoculation with non-
440 *Saccharomyces* strains. The secondary problem of nutrient depletion by the non-*Saccharomyces*
441 starter, before inoculation of standard wine yeasts, has been easily addressed by a rational use of
442 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-*Saccharomyces* yeast strains. Topics to be further addressed are the problem of adjusting oxygenation levels to improve alcohol reduction, the non-*Saccharomyces* strain selection, and the formulation of specific nutrients to limit the formation of aroma compounds of demonstrated negative character.

Abbreviation

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

Acknowledgements

Agrovin S.A. is acknowledged for providing yeast strains and enological products.

This work was supported by grants MINECO/FEDER RTC-2014-2186-2 (SOUNDWINE) developed in collaboration with Agrovin S.A., and AGL2015-63629-R, and EU project YeSVitE. (7FP-IRSES-GA n° 612441). JAC and PSN are the recipients of a MINECO Formación Postdoctoral contract from the Spanish Government.

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.

References

Andorrà, I., Berradre, M., Mas, A., Esteve-Zarzoso, B., Guillamón, J.M., 2012. Effect of mixed culture fermentations on yeast populations and aroma profile. LWT Food Sci. Technol. 49, 8–13.

Belda, I., Navascues, E., Marquina, D., Santos, A., Calderon, F., Benito, S., 2015. Dynamic analysis of physiological properties of *Torulaspora delbrueckii* in wine fermentations and its incidence on wine quality. Appl. Microbiol. Biotechnol. 99, 1911–1922

Bely, M., Stoeckle, P., Masneuf-Pomarède, I., Dubourdieu, D., 2008. Impact of mixed *Torulaspora delbrueckii–Saccharomyces cerevisiae* culture on high-sugar fermentation. Int. J. Food Microbiol. 122, 312–320.

Bueno, M., Zapata, J., Ferreira, V., 2014. Simultaneous determination of free and bonded forms of odor-active carbonyls in wine using a headspace solid phase microextraction strategy. J. Chromatogr. A 1369, 33-42. doi: 10.1016/j.chroma.2014.10.004

Ciani, M,; Comitini, F., Mannazzu, I., Domizio, P., 2010. Controlled mixed culture fermentation: a new perspective on the use of non-*Saccharomyces* yeasts in winemaking. FEMS Yeast Res. 10,123–133.

Ciani, M., Maccarelli, F., 1998. Oenological properties of non-*Saccharomyces* yeasts associated with wine-making. World J. Microbiol. Biotechnol. 14, 199– 203. doi:10.1023/A:1008825928354

Ciani, M., Morales, P., Comitini, F., Tronchoni, J., Canonico, L., Curiel, J.A., Oro, L., Rodrigues, A.J., Gonzalez, R., 2016. Non-conventional yeast species for lowering ethanol content of wines. Review. Front. Microbiol. 7, art 642. DOI:10.3389/fmicb.2016.00642

Comitini, F., Gobbi, M., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., Ciani, M., 2011. Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with Saccharomyces cerevisiae. Food Microbiol. 28, 873–882.

de-la-Fuente-Blanco, A., Sáenz-Navajas, M. P., Ferreira, V., 2017. Levels of higher alcohols inducing aroma changes and modulating experts' preferences in wine model solutions. Aust. J. Grape Wine Res. 23, 162-169. doi: 10.1111/ajgw.12273

Domizio, P., Romani, C., Lencioni, L., Comitini, F., Gobbi, M., Mannazzu, I., Ciani, M., 2011. Outlining a future for non-*Saccharomyces* yeasts: selection of putative spoilage wine strains to be used in association with *Saccharomyces cerevisiae* for grape juice fermentation. Int. J.Food Microbiol. 147, 170–180.doi: 10.1016/j.ijfoodmicro.2011.03.020

Fleet, G.H., 2008. Wine yeasts for the future. FEMS Yeast Res. 8, 979–995.

Fleet, G. H., Heard, G. M., 1993. Yeasts-growth during fermentation. In: Fleet, G.H. (Ed), Wine Microbiology and Biotechnology. Harwood Academic Publishers: Chur, Switzerland, pp. 27-54.

Franco-Luesma, E., Ferreira, V., 2014. Quantitative analysis of free and bonded forms of volatile sulfur compouds in wine. Basic methodologies and evidences showing the existence of reversible cation-complexed forms. J. Chromatogr. A 1359, 8-15. doi: 10.1016/j.chroma.2014.07.011

Franco-Luesma, E., Sáenz-Navajas, M.P., Valentin, D., Ballester, J., Rodrigues, H., Ferreira, V., 2016. Study of the effect of H_2S, MeSH and DMS on the sensory profile of wine model solutions by Rate-All-That-Apply (RATA). Food Res. Int. 87, 152-160. doi: 10.1016/j.foodres.2016.07.004

Gobbi, M., Comitini, F., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., Ciani, M., 2013. *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in simultaneous and sequential co-

515 fermentation: a strategy to enhance acidity and improve the overall quality of wine. Food
516 Microbiol. 33, 271–281.

517 Gonzalez, R., Quirós, M., Morales, P., 2013. Yeast respiration of sugars by non-*Saccharomyces*
518 yeast species: a promising and barely explored approach to lowering alcohol content of
519 wines. Trends Food Sci. Technol. 29, 55–61.

520 Jolly, N.P., Augustyn, O.P.H., Pretorius, I.S., 2003. The effect of non-*Saccharomyces* yeasts on
521 fermentation and wine quality. S. Afr. J. Enol. Vitic. 24, 55-62.

522 Kurtzman, C.P., Robnett, Ch.J., 1998. Identification and phylogeny of ascomycetous yeasts
523 from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Ant. Leeuw.
524 73, 331-371.

525 Legras, J.L., Karst, F., 2003. Optimization of interdelta analysis for *Saccharomyces cerevisiae*
526 strain characterization. FEMS Microbiol. Lett. 221, 249-255.

527 Lõoke, M., Kristjuhan, K., Kristjuhan, A., 2011. Extraction of genomic DNA from yeasts for
528 PCR-based applications. Biotechniques 50, 325-328.

529 Lopez, R., Aznar, M., Cacho, J., Ferreira, V., 2002. Determination of minor and trace volatile
530 compounds in wine by solid-phase extraction and gas chromatography with mass
531 spectrometric detection. J. Chromatogr. A 966(1-2), 167-177. doi: 10.1016/s0021-
532 9673(02)00696-9

533 Loscos, N., Hernandez-Orte, P., Cacho, J., Ferreira, V., 2007. Release and formation of varietal
534 aroma compounds during alcoholic fermentation from nonfloral grape odorless flavor
535 precursors fractions. J. Agric. Food Chem. 55(16), 6674-6684. doi: 10.1021/jf0702343

536 Mateo-Vivaracho, L., Cacho, J., Ferreira, V., 2008. Improved solid-phase extraction procedure
537 for the isolation and in-sorbent pentafluorobenzyl alkylation of polyfunctional mercaptans

538 Optimized procedure and analytical applications. J. Chromatogr. A 1185(1), 9-18. doi:
539 10.1016/j.chroma.2008.01.037
540 Morales, P., Rojas, V., Quirós, M., Gonzalez, R., 2015. The impact of oxygen on the final
541 alcohol content of wine fermented by a mixed starter culture. Appl. Microbiol. Biotechnol.
542 99, 3993–4003.
543 Moreno, J.J., Millán, C., Ortega, J.M., Medina, M., 1991. Analytical differentiation of wine
544 fermentations using pure and mixed yeast cultures. J. Ind. Microbiol. 7, 181–190.
545 Ortega, C., Lopez, R., Cacho, J., Ferreira, V., 2001. Fast analysis of important wine volatile
546 compounds. Development and validation of a new method based on gas chromatographic-
547 flame ionisation detection analysis of dichloromethane microextracts. J. Chromatogr. A
548 923(1-2), 205-214. doi: 10.1016/s0021-9673(01)00972-4
549 Padilla, B., Gil, J.V., Manzanares, P., 2016. Past and future of non-Saccharomyces yeasts: from
550 spoilage microorganisms to biotechnological tools for improving wine aroma complexity.
551 Review. Front. Microbiol. 7, art 411. DOI: 10.3389/fmicb.2016.00411
552 Parr, W. V., Heatherbell, D., White, K. G., 2002. Demystifying wine expertise: Olfactory
553 threshold, perceptual skill and semantic memory in expert and novice wine judges. Chem.
554 Senses 27(8), 747-755.
555 Quirós, M., Rojas, V., Gonzalez, R., Morales, P., 2014. Selection of non-*Saccharomyces* yeast
556 strains for reducing alcohol levels in wine by sugar respiration. Int. J. Food Microbiol. 181,
557 85–91.
558 Sadoudi, M., Tourdot-Maréchal, R., Rousseaux, S., Steyer, D., Gallardo-Chacón, J.J., Ballester,
559 J., Vichi, S., Guérin-Schneider, R., Caixach, J., Alexandre, H., 2012. Yeast– yeast
560 interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by
561 single or co-culture of non-*Saccharomyces* and *Saccharomyces* yeasts. Food Microbiol. 32,
562 243–253.

563 Sáenz-Navajas, M. P., Avizcuri, J. M., Ballester, J., Fernández-Zurbano, P., Ferreira, V.,
564 Peyron, D., Valentin, D., 2015. Sensory-active compounds influencing wine experts' and
565 consumers' perception of red wine intrinsic quality. LWT Food Sci. Technol. 60, 400-411.
566 San Juan, F., Cacho, J., Ferreira, V., Escudero, A., 2012. Aroma Chemical Composition of Red
567 Wines from Different Price Categories and Its Relationship to Quality. J. Agric. Food Chem.
568 60(20), 5045-5056. doi: 10.1021/jf2050685
569 San Juan, F., Ferreira, V., Cacho, J., Escudero, A., 2011. Quality and aromatic sensory
570 descriptors (mainly fresh and dried fruit character) of Spanish red wines can be predicted
571 from their aroma-active chemical composition. J. Agric. Food Chem. 59(14), 7916-7924.
572 Swiegers, J. H., Bartowsky, E. J., Henschke, P. A., Pretorius, I. S., 2005. Yeast and bacterial
573 modulation of wine aroma and flavour. Aust. J. Grape Wine Res. 11(2), 139-173. doi:
574 10.1111/j.1755-0238.2005.tb00285.x
575 Viana, F., Gil, J.V., Genovés, S., Vallés, S., Manzanares, P., 2008. Rational selection of non-
576 *Saccharomyces* wine yeasts for mixed starters based on ester formation and enological traits.
577 Food Microbiol. 25, 778–785.
578 Wang, C., García-Fernández, D., Mas, A., Esteve-Zarzoso, B., 2015. Fungal diversity in grape
579 must and wine fermentation assessed by massive sequencing, quantitative PCR and DGGE.
580 Front. Microbiol. 6, 1156. DOI: 10.3389/fmicb.2015.01156
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582
583 **Figure Captions**
584
585 **Figure 1.** Sugars consumed and metabolites produced by *Metschnikowia pulcherrima* strains in
586 synthetic must (200 g/L sugars) at 18ºC.

587 **Figure 2.** Monitoring of fermentation parameters.

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

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

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

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

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

600

601

Table 1. List of descriptors used for aroma descriptive analysis (flash profile), with the corresponding odor reference standards presented during familiarization task.

	Descriptor	**Odor reference**
1	Solvent/spirit-like	isoamyl alcohol
2	Dried fruit. Dried prune	4,5-dimethyl-3-hydroxy-2,5-dihydrofuran-2-one , linalool, methional, β-damascenone, phenylacetaldehyde
3	Alcohol/ethanol	ethanol
4	Tropical fruit. Passion fruit	3-mercaptohexyl acetate
5	Tropical fruit. Banana	isoamyl acetate
6	Yellow fruit. Peach	γ-decalactone
7	White fruit. Pear	isobutyl acetate
8	Nuts. Walnut	4,5-Dimethyl-3-hydroxy-2,5-dihydrofuran-2-one, 2-methoxyphenol
9	Medicinal/chemist	4-vinylphenol, 2-methoxy-4-vinylphenol
10	Oxidation. Potato, honey	methional, phenylacetaldehyde, acetaldehyde
11	Vegetal. Green	2-isobutyl-3-methoxypirazine
12	Reduction. Rotten eggs	Hydrogen sulfide, methanethiol

Table 2. Family vectors constructed by combining the OAV of similar odorants in both structures and odor properties.

Family vector	Chemical compounds
Acetates	2-methylpropyl acetate, butyl acetate, phenylethyl acetate, isoamyl acetate, hexyl acetate
Acetic/ethyl acetate	Acetic acid, ethyl acetate
Acids	butyric, 2-methylpropanoic, 2-methylbutanoic, hexanoic, octanoic, and decanoic acids
Isoamyl/isobutanol	Isoamyl alcohol, isobutanol
Norisoprenoids	β-damascenone, α-ionone, β-ionone
Ethyl esters	ethyl propanoate, butyrate, hexanoate, octanoate, decanoate, lactate, 2-methylpropanoate, 2-methylbutyrate, 3-methylbutyrate, diethyl succinate
Cinnamates	ethyl cinnamate, ethyl dihydroxycinnamate
Volatile phenols	guaiacol, o-cresol, m-cresol, 4-propylguaiacol, eugenol, E-isoeugenol, 2,6-dimethoxyphenol, 4-allyl-2,6-dimethoxyphenol
Vanillas	vanillin, acetovanillone, methyl vanillate, ethyl vanillate, syringaldehyde
Vinyl phenols	4-vinylphenol, 4-vinylguaiacol
Lactones	γ-nonalactone, γ-decalactone, γ-butyrolactone
Terpenols	linalool, α-terpineol, β-citronellol, geraniol
Volatile sulfurs	hydrogen sulfide, methanethiol
Isovaleraldehyde	2- and 3-methylbutyraldehyde

Table 3. OAV values of aroma vectors and differentiation ability calculated as the quotient between maximum and minimum concentrations (Max/Min) for the four wines studied.

	Sc 1	**Mp 4**	**Mp 5**	**Td 7**	**max/min**
2-Furfurylthiol (FFT)	0.0	0.0	7.7	0.0	39
Cinnamates	0.3	0.2	0.2	5.8	29
Acetates	68.4	19.0	27.5	2.5	27
Vinylphenols	12.5	5.2	6.5	0.5	26
3-Mercaptohexyl acetate (MHA)	3.2	0.9	0.7	0.0	16
Methionol	1.6	1.4	1.4	9.2	6.7
Ethyl esters	14.2	6.3	7.9	2.7	5.4
Acetic acid/ethyl acetate	3.2	6.9	7.3	1.8	4.0
β-phenylethanol	2.5	3.6	3.5	8.5	3.4
Methional	4.4	7.5	5.5	13.6	3.1
Isobutyraldehyde	1.1	2.8	2.4	2.1	2.6
Lactones	1.6	1.2	0.7	0.6	2.6
Benzylmercaptane (BM)	5.5	5.5	14.0	6.6	2.6
Acids	67.2	30.8	31.2	29.3	2.3
2-methyl-3-furanthiol (MF)	296	656	641	632	2.2
Norisoprenoids	63.0	34.7	40.4	28.4	2.2
Isoamyl alcohol/isobutanol	8.0	13.5	13.5	6.7	2.0
2- and 3-methylbutanal	2.4	3.9	3.3	2.1	1.9
Acetaldehyde	20.0	36.9	25.5	23.4	1.8
3-Mercaptohexanol (MOH)	1.3	1.9	2.2	2.5	1.8
Phenylacetaldehyde	13.0	19.5	21.6	18.8	1.7
Volatile Sulfur Compounds (VSCs)	3.5	3.0	2.6	4.0	1.6
Terpenols	0.4	0.6	0.5	0.5	1.6

Table 4. Metabolites produced in wines during scaling-up assays. Means followed by the same letter within the same column are not significantly different (P > 0:05)

	Assay 1 (12 VVH)	Assay 2 (0.6 VVH)		
	Ethanol (% vol/vol)	Ethanol (% vol/vol)	Acetic acid (g/L)	Glycerol (g/L)
S. cerevisiae	12.49 ± 0.05 a	12.55 ± 0.17 a	0.15 ± 0.01 b	7.27 ± 0.06 c
M. pulcherrima 591	11.20 ± 0.09 b	11.95 ± 0.08 b	0.15 ± 0.00 b	8.77 ± 0.06 a
T. delbrueckii	10.63 ± 0.49 b	12.04 ± 0.21 b	0.23 ± 0.04 a	7.70 ± 0.10 b

Table 6. Volatile compounds quantification (µg/L) in the 4 wines representing each group formed by sensory analysis (Figure 3).

compounds	sensory threshold[a]	Sc 1	Mp4	Mp5	Td7
ACETATES					
2-methylpropyl acetate	1600 [1]	14.2	9.43	14.0	11.8
butyl acetate	1800 [2]	15.6	12.1	11.5	8.99
phenylethyl acetate	250 [3]	289	66.3	88.8	110
ethyl acetate	12300 [4]	27793	78723	83590	17765
isoamyl acetate	30 [3]	2017	562	814	62.5
hexyl acetate	1500 [2]	<10	<10	<10	<10
ACIDS					
acetic acid	300000 [3]	269220	148380	147093	114398
butyric acid	173 [5]	658	641	687	445
2-methylpropanoic acid	2300 [6]	1370	2056	1926	5664
2-methylbutanoic acid	33 [5]	1125	669	652	746
hexanoic acid	420 [5]	3210	1087	1181	256
octanoic acid	500 [5]	9522	1292	1519	317
decanoic acid	1000 [5]	1081	227	330	48
ALCOHOLS					
2-methyl-1-propanol	40000 [3]	25994	164899	166987	58577
1-butanol	150000 [2]	460	311	313	754
3-methyl-1-butanol	30000 [3]	220696	281809	278665	156544
1-hexanol	8000 [3]	548	272	328	937
Z-3-hexenol	400 [3]	202	209	207	209
Methionol	1000 [5]	1589	1384	1406	9237
benzyl alcohol	200000 [7]	731	573	553	555
β-phenylethanol	14000 [5]	35182	50721	48651	118983
CARBONYLIC COMPOUNDS					
benzaldehyde	2000 [8]	26.6	18.4	20.2	21.4
β-damascenone	0.05 [3]	2.98	1.59	1.88	1.30
α-ionone	2.6 [2]	0.30	0.34	0.32	0.27
β-ionone	0.09 [5]	0.29	0.24	0.24	0.21
acetaldehyde	500 [3]	10001	18434	12771	11689
Diacetyl	100 [3]	<50	<50	<50	<50
Acetoin	150000 [2]	511	479	426	996
syringaldehyde	50000 [6]	0.56	0.40	0.75	0.60
isobutyraldehyde	6.0 [9]	6.4	16.5	14.5	12.6
2-methylbutanal	16 [9]	2.7	3.6	5.1	2.1
3-methylbutanal	4.6 [9]	10.2	17.0	13.9	9.1
Methional	0.5 [10]	2.2	3.8	2.8	6.8
β-phenylacetaldehyde	1.0 [9]	13.0	19.5	21.6	18.8
ESTERS					
ethyl propanoate	5500 [11]	<50	107	121	295
ethyl butyrate	125 [11]	149	116	151	66.8
ethyl hexanoate	62 [11]	603	236	305	42.6

ethyl octanoate	580 [2]	703	59.9	99.2	20.6
ethyl decanoate	200 [5]	51.6	<17	<17	<17
ethyl lactate	154000 [2]	2327	2798	3066	2613
diethyl succinate	200000 [2]	67.9	119.8	94.4	53.9
ethyl 2-methylpropanoate	15 [5]	12.0	10.8	11.5	10.7
ethyl 2-methylbutyrate	18.0 [5]	1.17	0.13	0.31	0.17
ethyl 3-methylbutyrate	3.0 [5]	2.86	2.00	2.21	1.80
ethyl cinnamate	1.1 [5]	0.36	0.22	0.22	0.34
ethyl dihydrocinnamate	1.6 [5]	<0.01	<0.01	<0.01	8.75
methyl vanillate	3000 [12]	2.36	2.24	2.53	2.18
ethyl vanillate	990 [12]	0.77	<0.02	0.73	0.53
VOLATILE PHENOLS					
guaiacol	9.5 [5]	10.0	3.77	5.21	3.45
o-cresol	31.0 [2]	0.36	0.34	0.25	0.30
4-ethylguaiacol	33.0 [5]	0.64	0.36	0.44	0.10
m-cresol	68.0 [13]	0.66	0.94	0.83	1.37
4-propylguaiacol	10.0 [12]	<0.02	<0.02	<0.02	<0.02
eugenol	6.0 [5]	0.40	0.58	0.42	0.74
4-ethylphenol	35.0 [11]	0.67	0.48	0.56	0.33
4-vinylguaiacol	40.0 [3]	357	145	184	15.9
E-isoeugenol	6.0 [14]	2.05	2.65	2.52	4.22
2,6-dimethoxyphenol	570.0 [12]	12.5	2.65	6.11	2.70
4-vinylphenol	180.0 [15]	652	288	337	14.8
4-allyl-2,6-dimethoxyphenol	1200.0 [6]	<0.01	<0.01	<0.01	<0.01
vanillin	995.0 [14]	3.91	2.48	2.48	2.59
acetovanillone	1000.0 [14]	80.0	62.2	71.2	46.8
LACTONES					
E-whiskylactone	790.0 [2]	<0.02	<0.02	<0.02	<0.02
Z-whiskylactone	67.0 [2]	<0.01	<0.01	<0.01	<0.01
γ-nonalactone	25.0 [6]	3.17	3.64	3.76	2.38
γ-nonalactone	25.0 [6]	3.17	3.64	3.76	2.38
γ-decalactone	0.7 [6]	13.7	8.8	4.3	4.2
γ-butyrolactone	35000 [14]	2098	5682	5238	3050
TERPENOLS					
linalool	25.0 [5]	3.88	4.74	4.09	3.92
α-terpineol	250.0 [5]	0.88	1.22	1.00	1.19
β-citronellol	100.0 [2]	<0.15	2.64	2.44	1.65
geraniol	20.0 [14]	3.97	6.97	5.42	7.04
VOLATILE SULFUR COMPOUNDS (VSCs)					
hydrogen sulfide (H2S)	1.1-1.6 [16]	3.05	2.75	2.20	3.42
methanethiol (MeSH)	1.8- 3.1 [17]	1.37	0.96	1.04	1.64
ethanethiol (EtSH)	1.1 [18]	<2.00	<2.00	<2.00	<2.00
dimethyl sulfide (DMS)	25 [18]	<2.00	<2.00	<2.00	<2.00
diethyl sulfide (DES)	0.9 [18]	<5.00	<5.00	<5.00	<5.00
dimethyl disulfide (DMDS)	29 [18]	<5.00	<5.00	<5.00	<5.00
POLYFUNCTIONAL MERCAPTANS					
2-methyl-3-furanthiol (MF)	0.004 [19]	1.19	2.63	2.56	2.52
2-furfurylthiol (FFT)	0.0004 [20]	<0.00014	<0.00014	0.0031	<0.00014

4-methyl-4-mercapto-2-pentanone (MP)	0.0008 [21]	<0.001	<0.001	<0.001	<0.001
3-mercaptohexyl acetate (MHA)	0.004 [21]	0.013	0.0035	0.0028	<0.0014
3-mercaptohexanol (MOH)	0.06 [21]	0.081	0.115	0.134	0.148
benzylmercaptane (BM)	0.0003 [22]	0.0016	0.0017	0.0042	0.0020

[a]Odour thresholds (calculated in red wine if available; otherwise threshold in synthetic wine is given). Reference in which the odour threshold value has been calculated is given in brackets. [1] Ferreira et al., (2002), [2] Etievant (1991), [3] Guth (1997), [4] Escudero et al., (2004), [5] Ferreira et al., (2000), [6] Gemert (2003), [7] Aznar et al., (2003), [8] Peinado et al., (2004), [9] Culleré et al., (2007), [10] Escudero et al., (2000), [11] San Juan et al., (2011), [12] López et al., (2002), [13] Ferreira et al., (2009), [14] Escudero et al., (2007), [15] Boidron et al., (1988), [16] Siebert et al., (2009), [17] Solomon et al., (2010), [18] Goniak and Noble (1987), [19] Tominaga et al., (2006), [20] Tominaga et al., (2000), [21] Tominaga et al., (1998), [22] Tominaga et al., (2003). These references are available as Supplementary material.

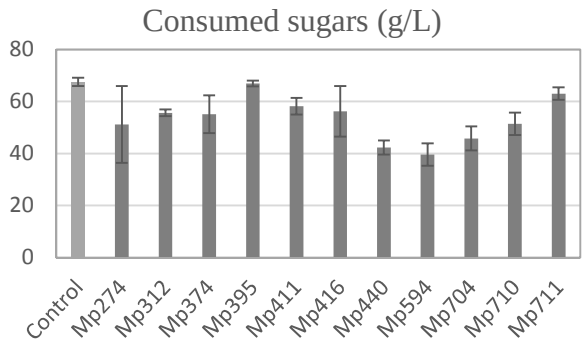
*H_2S was produced by addition of an Air-bubbled water solution of Na_2S (supplied by Sigma–Aldrich, St. Louis,MO, USA) at pH 9.6

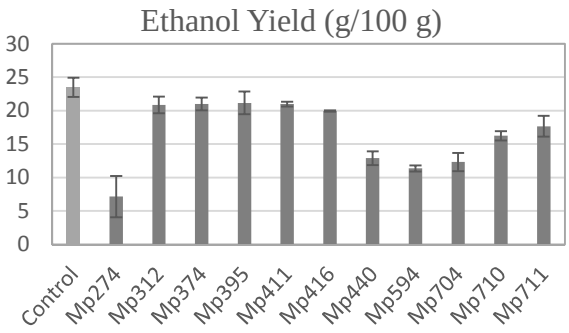
**concentration of MF expressed as micrograms per liter of furfurylthiol (FT)

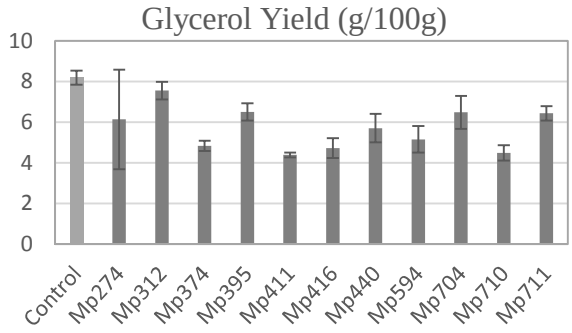
Table 5. Metabolites produced in wines during optimized pilot-scale aerobic fermentation. Means followed by the same letter within the same row are not significantly different (P > 0.05)

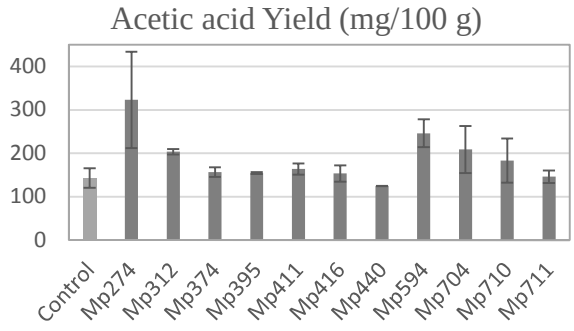
	S. cerevisiae	*M. pulcherrima*	*T. delbruekii*
Residual sugars (g/L)*	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.06
Glycerol (g/L)	7.20 ± 0.00 b	9.07 ± 0.06 a	6.33 ± 0.35 c
Ethanol (% vol/vol)	11.78 ± 0.10 a	10.90 ± 0.20 c	11.32 ± 0.20 b
Acetic acid (mg/L)	300.44 ± 5.47 a	166.99 ± 28.84 b	131.24 ± 6.85 b
Glycerol Yield (mg/g)	34.32 ± 0.00 b	43.22 ± 0.27 a	30.19 ± 1.67 c
Ethanol Yield (g/g)	0.44 ± 0.00 a	0.41 ± 0.01 c	0.43 ± 0.01 b
Acetic acid Yield (mg/g)	1.43 ± 0.03 a	0.80 ± 0.14 b	0.63 ± 0.03 b

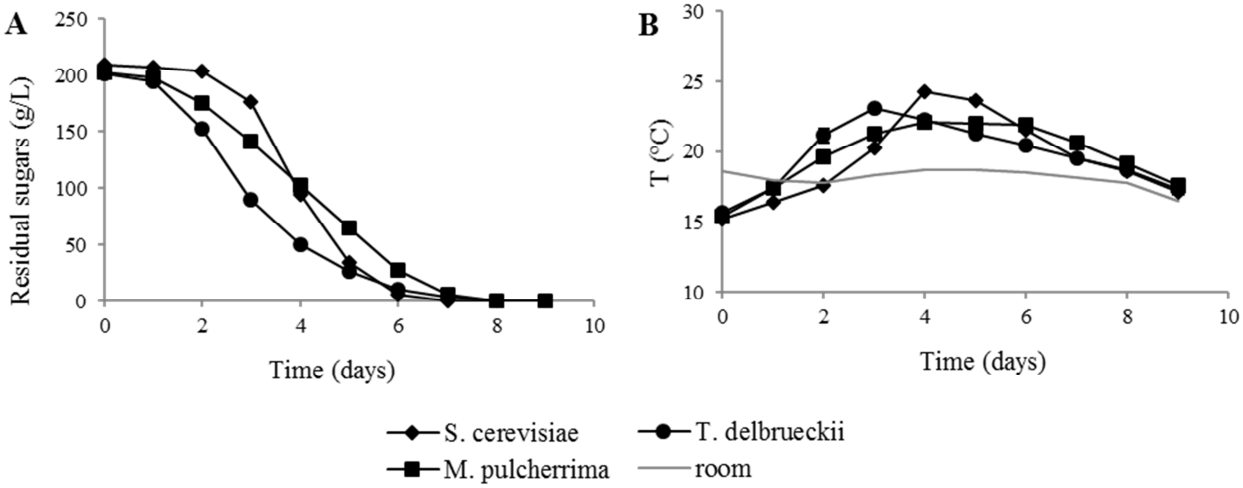
*0.00 indicates below the limit of quantification (0.03 g/L)

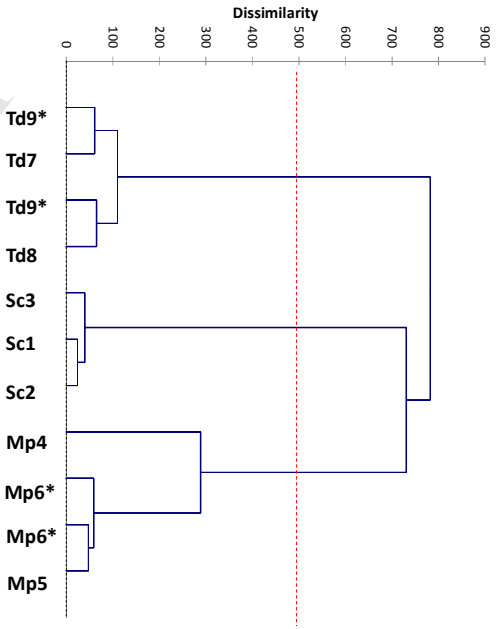


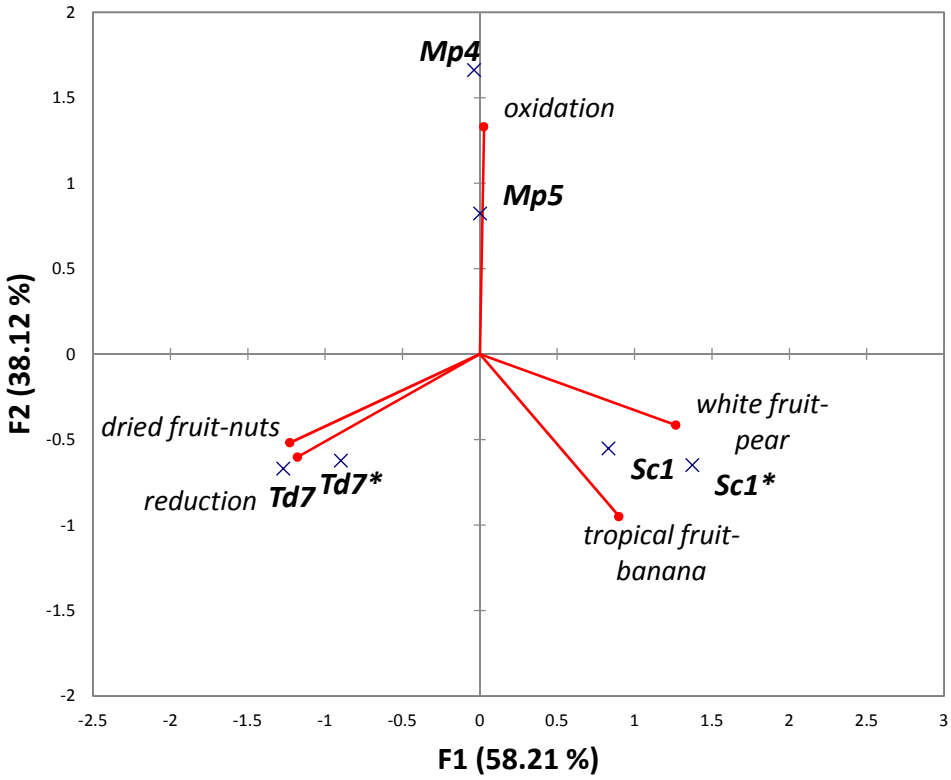


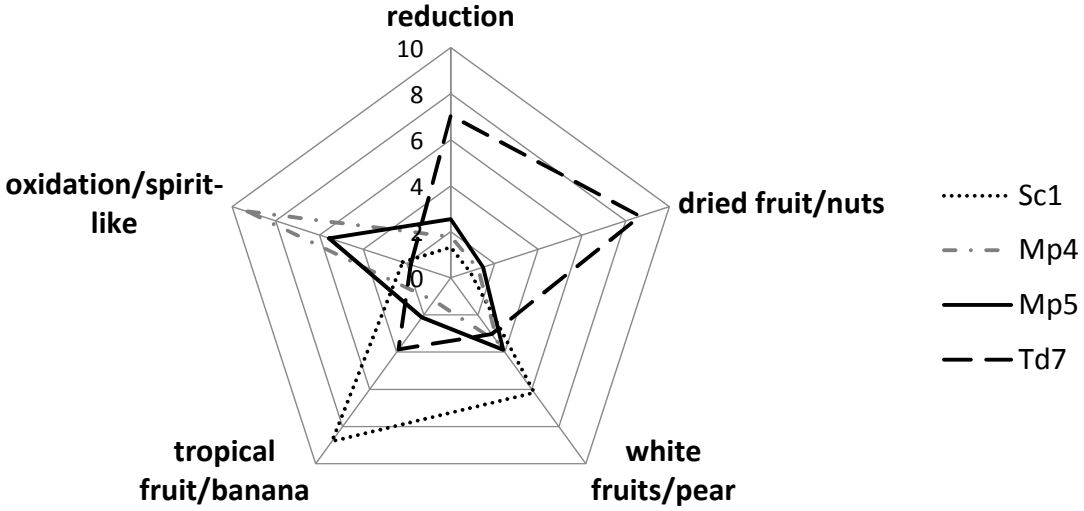


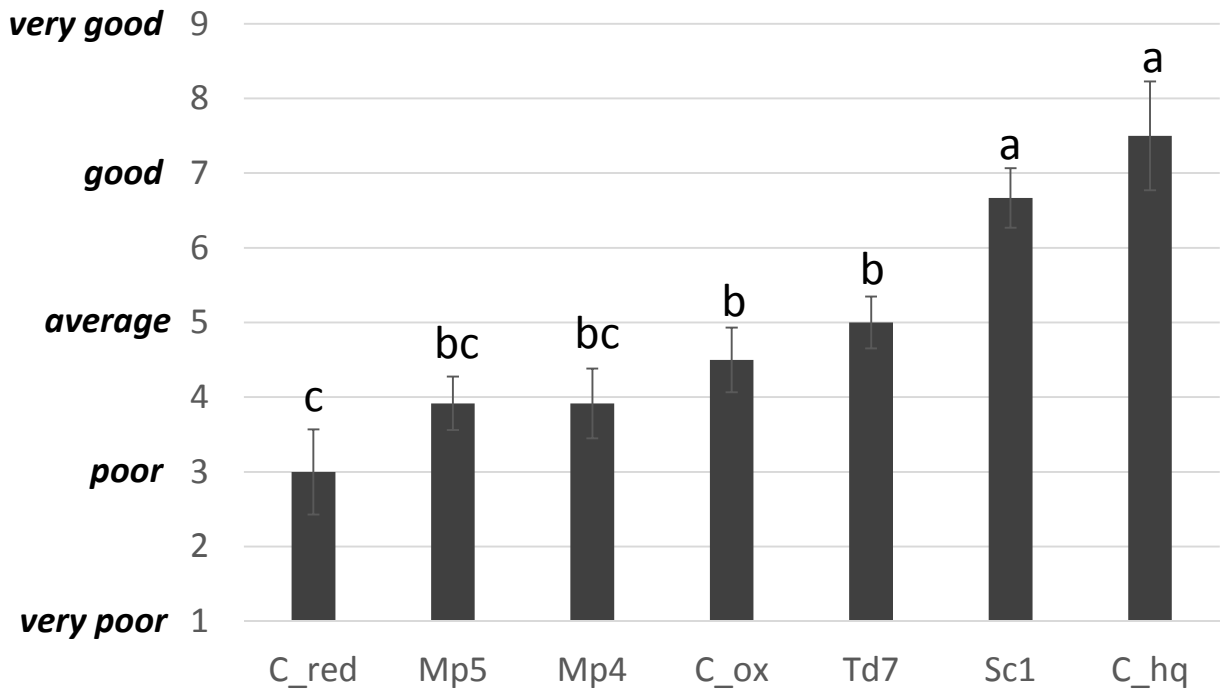


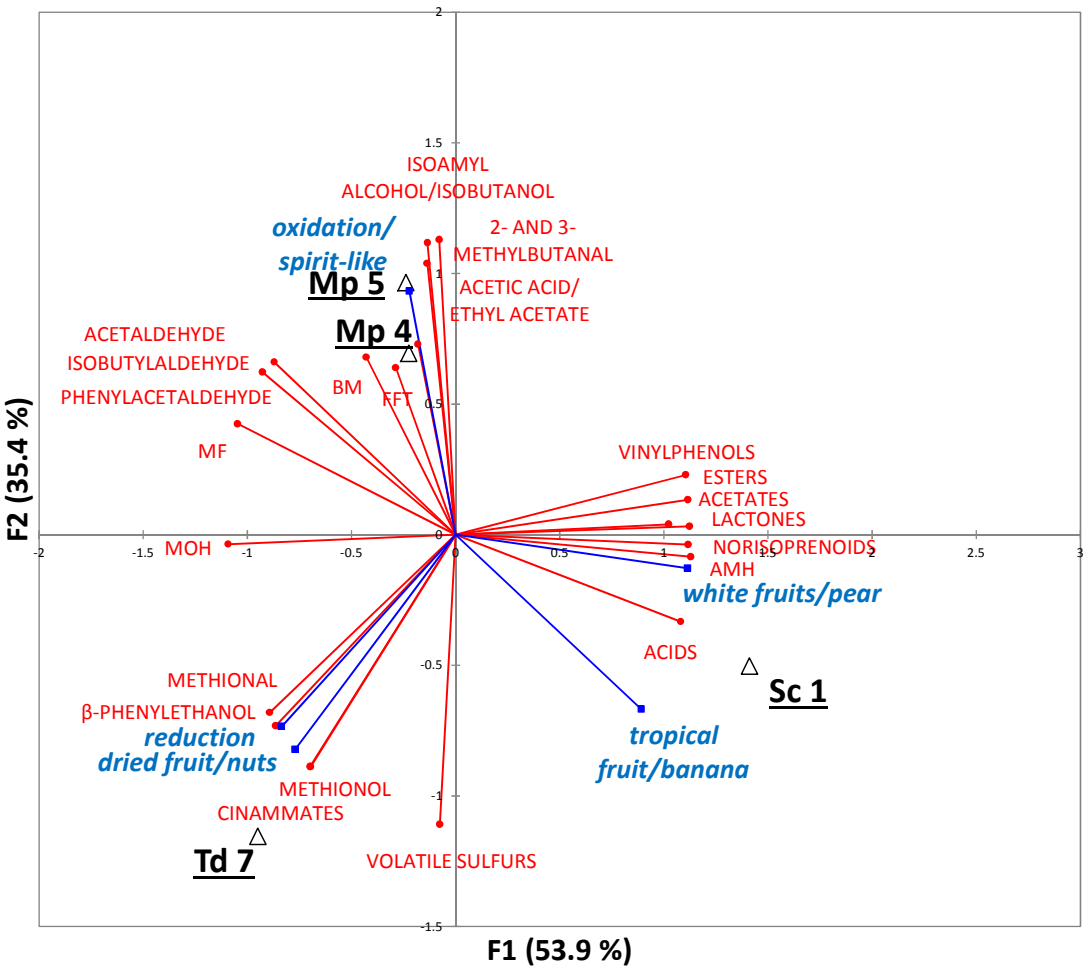







reduction
10
8
6
4
2
0
dried fruit/nuts
white fruits/pear
tropical fruit/banana
oxidation/spirit-like
Sc1
Mp4
Mp5
Td7





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.