Cold pre-fermentative maceration in presence of non-*Saccharomyces* strains: effect on fermentation behavior and volatile composition of a red wine

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Abbreviation title: *Metschnikowia* in cold pre-fermentative maceration

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Abstract

Background and Aims

This study evaluated the impact of cold pre-fermentative maceration (CPM), in presence of two non-*Saccharomyces* yeasts (*Metschnikowia pulcherrima* MP 346 or *Metschnikowia fructicola* MF 98-3) or of a commercial pectinolytic enzyme, on fermentation kinetics and on volatile composition of a Sangiovese red wine.

Methods and Results

Sangiovese grape must was inoculated with MP 346 or MF 98-3 or treated with enzyme preparation during CPM, at 5°C for 24 or 72 hours. A control wine was produced by a pure culture of *S. cerevisiae*. Both non-*Saccharomyces* strains affected the initial yeast population dynamics and the persistence of *S. cerevisiae* at the end of malolactic fermentation (MLF). Irrespective of CPM duration, the inoculum of *Metschnikowia* strains did not influence the rate of sugar consumption or the kinetics of MLF. The final wines were subjected to solid-phase extraction, followed by gas chromatography-mass spectrometry to evaluate their volatile composition. The levels of some terpenes and C13-norisoprenoids (i.e. nerol, geraniol, 8- hydroxy-linalool (*cis*) and 3-oxo-α-ionol) and of some esters (i.e. isoamyl lactate and ethyl isoamyl succinate) were higher in wines inoculated with *Metschnikowia* strains than in control and wine treated with enzyme.

Conclusions

MP 346 and MF 98-3 yeasts affect wine volatile composition.

Significance of the Study

This study shows for the first time that inoculum of *Metschnikowia* strains (MP 346 and MF 98-3) during CPM is effective in modulating the volatile composition of a Sangiovese red wine.

Keywords: non-*Saccharomyces* yeasts; *Metschnikowia* strains; cold pre-fermentative maceration; fermentation kinetics; volatile composition; Sangiovese wine

Introduction

Grape must fermentation is a complex ecological and biochemical process involving the sequential development of several microbial species. The process includes the interaction of fungi, yeasts, lactic acid bacteria, and acetic acid bacteria (Lambrechts and Pretorius, 2000). As regards the role of yeasts, commercial *Saccharomyces cerevisiae* active dry yeast (ADY) are usually inoculated to conduct the fermentation process, although less commonly, non-*Saccharomyces* strains can also been used. Indeed, the role of non-*Saccharomyces* in grape must fermentation has been recently re-evaluated, due to their contribution to wine aroma complexity and improved quality even if they do not necessarily play a role in sugar fermentation (Azzolini et al. 2015; Belda et al. 2015; Benito et al. 2015; Jolly et al. 2014; reviewed by Padilla et al. 2016).

Non-*Saccharomyces* yeasts can influence both the primary and secondary aroma through the production of enzymes and metabolites, respectively, and also impact directly or indirectly on wine color (Capozzi et al. 2015; Padilla et al. 2016). As regards the former class of molecules, most primary aroma compounds are found in grapes in bound non-odorant forms and their hydrolysis can occur during fermentation through the action of wine yeasts (Benito et al. 2015). The main yeast enzymes involved in the release of aroma compounds from odorless grape precursors are glycosidases (Gunata et al. 1988), carbon-sulfur lyases (Tominaga et al. 1998), and exo-glucanases (Gil et al. 2005).

Since it was demonstrated that the aromatic components of certain grape varieties are present in the grape berry both in free form and bound non-odorant form, there has been continuous research to find non-microbial techniques that are capable of releasing varietal aromas from precursors. These include contact with extracellular purified enzymes such as glycosidases and other lyases that are often found as side activities in pectinase enzymatic preparations, which are mainly used in red wine production for breaking down the cell walls of red grape skins, thus improving overall color intensity and color stability (Fia et al. 2005, 2016; Gil and Vallés, 2001; Gunata et al. 1988). All of these winemaking practices have enhanced interest in pre-fermentative maceration stages and have

recently attracted considerable attention from researchers (Baiano et al. 2016; Gil-Muñoz et al. 2009; González-Neves et al. 2015; Mihnea et al. 2015).

Despite the growing interest in the effects of microbial dynamics during non-*Saccharomyces*/*Saccharomyces* mixed fermentations, no studies have yet considered the specific case of non-*Saccharomyces* application during pre-fermentative maceration, nor do they compare it with the use of specific purified enzymes under the same conditions or with the sole temperature effect in pre-fermentative maceration. Nowadays *Metschnikowia* yeasts are among the most studied and promising non-*Saccharomyces* due to their impact on wine profile and quality, as reported in numerous publications over the last 2 years (Contreras et al. 2015; Lu et al. 2015; Varela et al. 2016). The aim of this study was to evaluate the effect of cold pre-fermentative maceration (CPM), carried out in presence of two different *Metschnikowia* strains (*M. pulcherrima* MP 346 or *M. fructicola* MF 98-3) or of a commercial pectinolytic enzyme preparation (Cuvée Rouge), on the fermentation kinetics and the volatile composition of a type of Sangiovese red wine. A control wine was produced by the pure culture of *S. cerevisiae* in order to evaluate the sole temperature impact during cold pre-fermentative maceration.

Materials and methods

Experimental design

Four different Sangiovese wines were produced with different treatments: i) CPM with *M. pulcherrima* (MP 346); ii) CPM with *M. fructicola* (MF 98-3); iii) CPM with commercial pectinolytic enzyme preparation; iv) CPM without addition (control). *S. cerevisiae* was inoculated in all tanks at the end of CPM, which was carried out for 24 hours (vintage 2014, CPM 24h) and 72 hours (vintage 2015, CPM 72h).

Microorganisms and media

Metschnikowia pulcherrima MP 346 and *Metschnikowia fructicola* MF 98-3 strains (in ADY preparation), the *S. cerevisiae* Lalvin RC212® strain (in ADY preparation), the *O. oeni* PN4® strain (in MBR lyophilized preparation) and the pectolytic enzyme LALLZYME® Cuvée Rouge

(containing pectinases with glucosidases side activities) used for this study were kindly provided by Lallemand SAS (Blagnac, France). Stock cultures of yeast strains were maintained at 4°C on YEPD agar (20 g/L glucose, 10 g/L yeast extract, 20 g/L peptone and 20 g/L agar; BD Difco, Italy). *O. oeni* PN4® MBR strain was maintained frozen at –20°C in Man Rogosa Sharpe (MRS) broth (BD Difco, Italy) containing 20% glycerol (v/v).

In order to evaluate the total yeast population, must and wine samples were serially diluted in 1% (w/v) peptone solution (pH 7.0) and spread in duplicate onto WL Nutrient Agar (Thermo Scientific Oxoid, Italy) plates. After 5 days of incubation at 25°C, the colonies present on each plate were counted and selected by colony morphology (form and color, elevation and margins).

In order to confirm the presence of *Metschnikowia*, either inoculated or endogenous strains, colonies of each morphotype were re-streaked on WL medium to obtain pure cultures and then streaked onto Lysine medium supplemented with 10% (v/v) lactic acid (Thermo Scientific Oxoid, Italy) to confirm growth.

Winemaking procedure

Grapes from *Vitis vinifera* L. cv. Sangiovese (2014 and 2015 vintages) were harvested at commercial maturity in a vineyard located in Cetona (Siena, Tuscany - Italy). For both vintages, four vinifications (each performed in duplicate) were carried out using the same experimental procedure, at the "Azienda Agricola Ciucci" in a micro-vinification plant.

Healthy grapes were destemmed/crushed and the resulting must was divided in aliquots (80 L) which were distributed into eight 100 L stainless-steel fermentation tanks. The composition of Sangiovese grape musts was: 23 and 24.3 °Brix, titratable acidity 7.48 g/L and 6.11 g/L of tartaric acid, pH 3.30 and 3.32 in 2014 and 2015 vintages, respectively.

A portion of the must, 20% v/w, was bled off (*saignée*). Before alcoholic fermentation (AF), pre-fermentative maceration (CPM) was performed at 5°C for 24 hours (vintage 2014) or 72 hours (vintage 2015). The set of experiments consisted in: two trials subjected to CPM in presence of 0.25 g/L *M. pulcherrima* MP 346 (Tank 1-2) or 0.5 g/L *M. fructicola* MF 98-3 (Tank 3-4),

previously rehydrated according to the manufacturer's instructions in water containing 0.3 g/ L of the yeast protectant Go-Ferm Protect® (Lallemand SAS, Blagnac, France); one trial subjected to cold maceration with 3 g/hL of the pectolytic enzyme LALLZYME® Cuvée Rouge (Tank 5-6); a control trial (Tank 7-8) subjected to cold maceration to which neither yeasts nor enzymes were added at this stage.

At the end of cold maceration, each tank was heated to 18 °C via an outer-tank heat exchanger and inoculated with 0.25 g/L of a commercial *S. cerevisiae* yeast strain (Lalvin RC212®, Lallemand SAS, Blagnac, France). The amount of yeast assimilable nitrogen (YAN) after pre-fermentative maceration during 2014 and 2015 vintages was 145 mg N/L and 110 mg N/L. YAN was supplemented by adding 30 g/hL of FERMAID E® (Lallemand SAS, Blagnac, France) at 1/3 of alcoholic fermentation in 2014 samples. Otherwise, during 2015 vintage 20 g/hL of FERMAID E® (Lallemand SAS, Blagnac, France) was added after 12 hours from the inoculum of *S. cerevisiae* and at 1/3 of alcoholic fermentation.

Once the fermentation of sugars was complete, 3 days of post-fermentation extended maceration was carried out. After devatting, all wines were inoculated with malolactic bacteria *Oenococcus oeni* PN4® MBR (Lallemand) in lyophilized preparation, rehydrated according to the manufacturer's instructions at the final inoculation of 0.01 g/L and kept at 20 °C. At the end of malolactic fermentation (MLF), the wines were transferred into stainless steel tanks and sodium metabisulphite was added in order to obtain similar free SO_2 concentrations (20 mg/L) in all tanks. Following the post-fermentation stabilization process, 60 liters of each wine sample were bottled and volatile compounds were analysed after 4 months.

Estimation of the parameters of alcoholic fermentation

The kinetics of sugar consumption during AF was fitted by means of a sigmoid or altered Gompertz decay function as previously described by other authors (Tronchoni et al. 2009; Crépin et al. 2012), applying the following equation:

$$Y=A+C*e-e\text{^}(K*(t-M)) \quad (1)$$

Where Y is the residual sugar concentration (g/L) still present in must at time t (days); A is the lower asymptote, representing the lowest residual sugar concentration when t tends to infinity ($t \rightarrow \infty$); K is the fermentation rate; C is the distance between the upper and lower asymptote and M is the half-time of sugar consumption. Eq. (1) was fitted to the experimental data by a non-linear regression procedure (GraphPad Prism 5.0, GraphPad software, Inc.) and the quality of the regression was evaluated by the coefficient of determination (R^2).

Analytical procedure

pH, tritable acidity and soluble solid content (°Brix) were determined on the Sangiovese grape at harvest. pH was measured potentiometrically with a Mettler Toledo pH meter (Steroglass, Perugia, Italy). Titrable acidity was determined as g tartaric acid/l of juice sample by titrating 10 mL of juice with NaOH 0.1 M reaching pH 7 and °Brix measurements were taken at 20°C with a digital refractometer HI 96801 (Hanna Instruments, Milan, Italy). During AF sugar consumption was evaluated by measuring the decrease in the medium density with a standard wine densimeter. L-lactic acid and malic acid contents were determined with K-LATE and K-LMALR kits (Megazyme International Ireland Ltd., Wicklow, Ireland).

Volatile compounds were analyzed by gas chromatography-mass spectrometry (GC–MS) after solid-phase extraction (SPE), carried out by ENV+ cartridge (IST Ltd., Mid Glamorgan, UK). The process was performed by an Aspec XL Sample Processor for SPE (Gilson Inc. Middleton, WI, USA). Cartridges were sequentially conditioned with methanol (9.5 mL) and distilled water (19 mL). A total of 38 mL of wine sample diluted 1:2 (by volume) with distilled water, and 1-Heptanol added as internal standard (500 µg/L) was loaded onto the cartridge. The residue was washed with 19 ml of distilled water. The free aroma compounds were eluted with 9 mL of dichlorometane. The solution was dried with Na_2SO_4 and concentrated to 0.4 mL by nitrogen flow stream.

GC–MS analysis was performed with 6980N Network GC System coupled with a 5975 XL EI/CI MSD (Agilent Technologies, Santa Clara, CA, USA), equipped with DB-Wax Bonded PEG fused silica capillary column (60m x 320 µm i.d. x 0.25 µm film thickness; Agilent Technologies).

Instrumental conditions were: electron impact (EI) mode 70 eV; injector temperature 200 °C; He carrier flow 1.5 mL/ min; column temperature 50 °C for 4 min, rising to 240 at 4 °C/min, then 20 min at 240 °C; and injection volume 2.0 µL in Splitless mode. The analyses were performed in SCAN mode. NIST data bank and co-injection of pure reference standards were used to identify the compounds. All compounds were quantified using 1-Heptanol as Internal Standard with RF = 1.

Odor activity value (OAVs)

The odor activity values (OAVs), a parameter used to evaluate the contribution of the volatiles to wine aroma, were calculated as the ratio between the concentration of individual volatile and the corresponding odor threshold found in the literature (Cai et al. 2014; Ferreira et al. 2000).

Statistical analysis

Data of wine composition and flavour compounds were analysed for statistical significance by one-way analysis of variance (ANOVA) in order to test for significant differences between treatments. When significance was reached, a Tukey (HSD) post-hoc test (confidence interval: 95%) was performed using EXCEL® Add-in macro DSAASTAT program.

Results and discussion

Yeast population dynamics

One grape variety (Sangiovese) and two vintages (2014 and 2015) were analysed to evaluate the combined effect of cold pre-fermentative maceration and the addition of selected *Metschnikowia* strains (*M. pulcherrima* MP 346 and *M. fructicola* MF 98-3) or enzyme preparations containing pectinolytic activities.

Minor differences were observed in the initial population of total yeasts present in the must, which varied between 4.1(±0.9)*10^5 (2014 vintage) and 8.8(±0.6)*10^5 CFU/mL (2015 vintage), although the yeast population dynamics showed a similar trend during the fermentation process for both the vintages analyzed. Figure 1 shows the typical evolution of the total yeast population observed throughout alcoholic fermentation during the 2015 vintage. Comparing tanks inoculated (Tanks 1-4)

and non-inoculated with *Metschnikowia* (Tanks 7-8), significant differences were observed in total yeast population both in CPM and AF phase. Similar differences were not observed when comparing the yeast population of control tanks (7 and 8) and tanks treated with the enzyme preparation (Tank 5 and 6; Figure 1).

During the first two-hours of cold pre-fermentative maceration (CPM 2H; Figure 1), the total yeast population increased fifty-fold [from 8.8(±0.6)*$10^5$to 4.7(±0.6)*10^7 CFU/mL] in tanks inoculated with *Metschnikowia*, and decreased about four-fold [up to 1.7(±0.7)*10^5 CFU/mL] in the non-inoculated tanks (control and enzyme preparation). With both *M. pulcherrima* MP 346 and *M. fructicola* MF 98-3, the total yeast population remained high up to the Draining-off (Figure 1) and, up to AF 12H, it was mainly composed (96-99%) by *Metschnikowia* cells (supplementary materials, Table 1S). The observations that an increase in the yeast cells count occurred only in inoculated tanks and that *Metschnikowia* became predominant only when the pre-fermentative cold maceration was carried out in the presence of MP 346 and MF 98-3 strongly indicate that these strains have the ability to outcompete wild contaminants and their persistence, together with their metabolic repertoire, may contribute to generate specific compounds that can improve wine aroma.

At the end of alcoholic fermentation (End AF) the total yeast population was very similar in all conditions [5.0(±0.2)*10^7 CFU/mL] and was, almost, completely composed (99%) by *S. cerevisiae* cells (supplementary materials, Table 2S). As expected, a clear decline in the total yeast population was observed during the final stages in all fermentations, but, at the end of the MLF phase, clear differences were observed in tanks inoculated and non-inoculated with *Metschnikowia* (Figure 1). Interestingly, viable cell counts for *S. cerevisiae* were 3.3- to 4.5-fold higher in tanks inoculated with MP 346 and MF 98-3 than in the control and the enzyme-treated tanks (supplementary materials, Table 2S).

On analyzing the overall winemaking process, it was observed that non-*Saccharomyces* strains have a significant effect on the initial yeast population dynamics and on the persistence of *S. cerevisiae* at the end of the MLF phase (Figure 1).

Alcoholic and malolactic fermentation

As expected, cold maceration was effective at inhibiting the onset of alcoholic fermentation (Hierro et al. 2006), and sugar consumption only began 48 hours after the inoculum of a commercial *S. cerevisiae* strain (Lalvin RC212®).

The kinetics of sugar consumption in Sangiovese must throughout alcoholic fermentation were comparable during the 2014 vintage (CPM 24h) and 2015 vintage (CPM 72h) (Figure 2) and the experimental data were adequately fitted by a modified Gompertz decay function, as shown by the R^2 values (0.97-0.99) reported in Table 2. It took between 18 and 21 days to finalize alcoholic fermentation at 18 °C, and similar trends were observed between samples (Figure 2) with no significant differences in terms of both kinetic constant (K) and M values, which indicate the time required to consume 50% of the sugars. These data proved that the inoculum of MP 346 and MF 98-3 during the cold pre-fermentative maceration did not significantly affect the fermentation behavior of the *S. cerevisiae* strain under analysis (Lalvin RC212®), which easily governed alcoholic fermentation, thus achieving the completion of the process without delay, as already stated by other authors (Jolly et al. 2003, Belda et al. 2016). Moreover, in a previous study Jolly et al. (2003) demonstrated that the association of *S. cerevisiae* and *M. pulcherrima* in anaerobic conditions did not lead to significant changes in the fermentation rate, when compared with pure cultures of *S. cerevisiae*.

The MLF kinetics, obtained with the *O. oeni* PN4® MBR strain, showed similar trends in 2014 (Figure 1Sa) and 2015 (Figure 1Sb) vintage, despite the different initial amount of malic acid. During the 2014 vintage, approximately 1.5 g/L of malic acid was converted into lactic acid (1.0-1.2 g/L) in all wines. Between 12 and 16 days were required to reach a malic acid content lower than 0.2 g/L. Within a comparable time interval, MLF led to the conversion of approximately 0.9 g/L malic acid into 0.5-0.6 g/L of lactic acid in all samples during 2015 vintage. Figure 1S illustrates that no significant differences were observed during MLF and in the evolution of the

malic and lactic acid content, when non-*Saccharomyces* were inoculated during the cold pre-fermentative maceration.

Volatiles profile

A total of 78 volatile compounds (i.e. terpenes and norisoprenoids, aldehydes and ketones, esters, alcohols, acids, phenols and lactones) were identified and quantified by means of GC-MS analysis in all wine samples (Table 3S).

Terpenes and norisoprenoids

Terpenes and C13-norisoprenoids contribute to the varietal character of many wines, especially aromatic varieties (Marais 2017; Ristic et al. 2010). Both groups of odorants are present in grapes in glycoside forms and they can be released by glycosidase enzymes during winemaking.

On comparing the total amount of terpene and C13-norisoprenoid, non-*Saccharomyces* yeasts did not induced a clear defined effect in both studied vintages (2014 and 2015). However, in the 2015 vintage, when a longer cold-maceration was performed (CPM 72h), wines inoculated with *Metschnikowia* strains (MP 346 and MF 98-3), similarly to the enzyme treated wine, revealed a greater amount of total terpens respect to the control wine. Moreover, the presence of *Metschnikowia* strains had a discriminating effect on some individual terpene and norisoprenoid compounds. In 2015 vintage, nerol and geraniol levels, although lower than their odor thresholds, were higher when MP 346 and MF 98-3 strains were used (Table 3S), compared to the other wine samples (enzyme preparation and control) as further proved by the OAV indicated in Table 3. Some slight but significant differences were found also in 8- hydroxy-linalool (*cis*) levels, when MP 346 and MF 98-3 strains were used compared to the control wine. No significant differences were observed for these compounds compared to the enzyme treated wine. With regard to the C13-norisoprenoids, in the same vintage (2015), higher levels of 3-oxo-α-ionol were found in MP 346 and MF 98-3 wines (similarly to enzyme treated wine) than in control wine.

Aldehydes and Ketones

In both vintages, more aldehydes and ketones were detected in the wine in which cold pre-fermentative maceration occurred using enzyme preparation compared to the other wine samples (MP 346, MF 98-3 and control). Moreover, the MF 98-3 sample showed the lowest level of benzaldehyde compared with control wine in both vintages, irrespective of CPM duration. However, in all wines, the concentration of the latter did not exceed the olfactory threshold (2000 µg/l, Cai et al. 2014).

Esters

Esters, including acetate esters and fatty acid ethyl esters, are the main source of fruity aromas in wine. Most of them are secondary metabolites produced by yeast during alcoholic fermentation.

In both vintages, non-*Saccharomyces* yeasts (MP 346 and MF 98-3) did not have a defined effect on the total concentration of esters in Sangiovese wines. Nevertheless, the final levels of some specific esters, such as isoamyl lactate and ethyl isoamyl succinate, were higher in MP 346 and MF 98-3 (similarly to enzyme treated wine), than in control wine, in both 2014 and 2015 vintages. Previous research has shown that non-*Saccharomyces* species, in particular *M. pulcherrima*, are able to produce a relatively high amount of several esters (Whitener et al. 2017).

However, by extending the maceration time to 72 hours (2015 vintage), the pre-fermentative and fermentative metabolism, in presence of non-*Saccharomyces* (MP 346 and MF 98-3), resulted in a higher level of isoamyl acetate (acetate ester), ethyl butanoate, ethyl hexanoate (ethyl esters), than the control wine. Among these ester compounds, only isoamyl acetate and ethyl hexanoate (which have a banana aroma) exceeded the corresponding thresholds (30 and 14 µg/L respectively, Ferreira et al. 2000). Data reported in Table 3 showed that *Metschnikowia* strains lead to an increase (about 21-24 % in OAV) for both compounds compared to the control wine. However, as recently demonstrated (Pineau et al. 2009), these esters could have an indirect impact on fruity wine aroma, due to the additive effect of these compounds in red wines. In particular, it has recently been suggested (Ferreira et al. 2009), that ethyl esters of branched or cyclic fatty acids could act additively with other wine ethyl esters, thus contributing to the fruity notes of red wines. It is

important to note that for both vintages, the sum of ethyl 2-hydroxy-4-methylpentanoate (or ethyl leucate, a compound directly associated with a ‘‘fresh blackberry’’ aroma, Falcao et al. 2011) and ethyl 3-methylbutyl succinate was higher in presence of MF 98-3 than in the control wine. Concerning ethyl acetate, it may add aroma complexity at low levels (concentrations below 80 mg/L), whereas it is associated with negative sensory descriptors (solvent odour) at concentration above 150 mg/L. In both vintages, MP 346 and MF 98-3 samples showed significantly lower concentration of ethyl acetate than the control (Table 3 and 3S). Wines produced with *Metschnikowia* strains had an appreciable decrease in OAV (about 24% in 2014 and 61% in 2015 vintage) compared to the control wine (Table 3). Benito et al. (2015) reported a similar result, proving that Riesling wine produced with *M. pulcherrima* followed by *S. cerevisiae* inoculation showed less ethyl acetate than wine produced by *S. cerevisiae* alone. Irrespective of CPM duration, wines treated with enzyme preparation showed the lowest level of ethyl acetate. Finally, for both vintages, less ethyl 4-hydroxybutanoate was found in the MP 346 and MF 98-3 samples compared to enzyme treated wine and control irrespective of CPM duration.

Alcohols

Four of the C_6 alcohols, which generally have negative vegetal and herbaceous characters, were identified in this study. Despite the total alcohol level was similar between treatments, in both vintages, MP 346 and MF 98-3 samples resulted in lower concentration of 1-hexanol respect to control wine, despite it did not exceed the olfactory threshold (8000 μg/L, Ferreira et al. 2000). The higher alcohols (fusel alcohols) can positively contribute to the complexity of wine aroma, while they may have negative effects at very high concentrations. Moreover data showed that, among all samples, MF 98-3 wines had lower concentration of 3-(methylthio)-propanol (methionol) in both vintages, as further revealed by the corresponding OAV calculated (Table 3).

Acids

This group of volatile compounds is produced by yeast during fatty acid metabolism and are characterized by rancid, fruit or cheesy odours. Nevertheless, volatile fatty acids can improve the

complexity of wine bouquet. In this study six volatile acids were detected and of these, decanoic acid did not exceed the olfactory threshold (1000 μg/l, Ferreira et al. 2000). In particular, Table 3S shows that the MF 98-3 inoculum increased, compared to the control and enzyme treated wine, the concentration of isovaleric and homovanillic acid in both the 2014 (CPM 24h) and the 2015 vintages (CPM 72h), irrespective of CPM duration.

Phenols and Lactones

Volatile phenols are generally present in wine at concentrations ranging from a few dozen to several hundred micrograms per liter. These compounds are likely to give sensory characteristics generally classified among the "off-flavours". The results obtained showed some slight but significant differences in the total phenol concentration between treatment. Wines inoculated with *Metschnikowia* strains (MP 346 and MF 98-3), similarly to enzyme treated wine, showed a lower amount of total phenols in both vintages (2014 and 2015), irrespective of CPM duration. In all cases, 4-ethylphenol and 4-ethylguaiacol compounds were far below their perception threshold [440 μg/L (Lopez et al. 2002) and 33 μg/L (Ferreira et al. 2000) respectively] in both vintages, as indicated in Table 3.

The odor of lactones is usually described as "buttery, fruity and coconut-like"; three lactones were also identified in this study; however, their levels did not provide evidence on the impact of both non-*Saccharomyces* strains.

Conclusions

Overall, both non-*Saccharomyces* strains (MP 346 and MF 98-3), inoculated during cold pre-fermentative maceration, had a significant effect on the initial yeast population dynamics and on the persistence of *S. cerevisiae* at the end of the malolactic fermentation phase. Irrespective of CPM duration, the inoculum of *Metschnikowia* strains did not significantly affect or interfere with the rate of sugar consumption of the *S. cerevisiae* strain under analysis, or the kinetics of malolactic fermentation induced at the end of alcoholic fermentation. Considering the volatile composition, in both vintages, non-*Saccharomyces* yeasts (MP 346 and MF 98-3) did not have a clear defined effect

on the total concentration of the main classes of aroma. Nevertheless, the final levels of some specific terpenes and C13-norisoprenoids (such as nerol, geraniol, 8- hydroxy-linalool (*cis*) and 3-oxo-α-ionol) were higher in tanks inoculated with *Metschnikowia* strains than in the control and enzyme treated wine, when a longer cold-maceration was performed (72h, 2015 vintage). Moreover, higher amount of some specific esters (isoamyl lactate and ethyl isoamyl succinate in both vintages), was revealed in presence of *Metschnikowia* strains respect to the control and enzyme treated wine, thus confirming that non-*Saccharomyces* yeasts certainly affected aroma formation. The influence of *Metschnikowia* strains in the production of some esters (isoamyl acetate, ethyl butanoate, ethyl hexanoate) was more evident extending the cold-maceration time.

Moreover, some other specific molecules such as isovaleric and homovanillic acids, the sum of ethyl 2-hydroxy-4-methylpentanoate and ethyl 3-methylbutyl succinate (higher in MF 98-3 inoculated wines) and 3-(methylthio)-propanol (lower in MF 98-3 inoculated wines) were differently affected by the two non-*Saccharomyces* strains. This evidence suggests that a species and strain effect is also present within the yeast genus *Metschnikowia* and that further research is required to determine whether it is possible to fine-tune wine aroma profiles with non-*Saccharomyces* strain specificities.

As regards the winemaking practice, this study shows for the first time that inoculum of non-*Saccharomyces* yeasts (MP 346 and MF 98-3) during cold pre-fermentative maceration is effective, since impacted both *Metschnikowia* population dynamics during the maceration time and wine volatile composition. Further studies could be carried out to assess the effectiveness of non-*Saccharomyces* yeasts in improving wine color stability and phenolic composition.

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Table 1: *S. cerevisiae* population in different tanks at the end of AF and MLF phase for 2015 vintage.

Phase	*S. cerevisiae* (CFU/mL)*			
	MP 346 (Tank 1-2)	**MF 98-3 (Tank 3-4)**	**Enzyme preparation (Tank 5-6)**	**control (Tank 7-8)**
End AF	$3.94\pm0.48*10^{7\ a}$	$4.95\pm0.45*10^{7\ a}$	$5.53\pm1.45*10^{7\ a}$	$4.27\pm2.69*10^{7\ a}$
End MLF	$2.12\pm0.93*10^{5\ a}$	$2.01\pm1.49*10^{5\ a}$	$4.68\pm2.48*10^{4\ b}$	$6.35\pm1.10*10^{4\ b}$

*Data are mean values of two tanks ± standard deviation. Values with different letters are significantly different according to the Tukey test (95%)

Table 2: Parameters obtained by fitting the altered Gompertz equation to the experimental data of sugar consumption in Sangiovese must during alcoholic fermentation, carried out during 2014 vintage (CPM 24h) and 2015 vintage (CPM 72h).[a]

Thesis	MP 346	MF 98-3	Enzyme preparation	control	Sign.
Vintage 2014 (CPM 24h)					
K (g/ L day)	0.122 (± 0.028)	0.093 (± 0.024)	0.070 (± 0.024)	0.076 (± 0.020)	ns
M (1/day)	6 (± 1)	7 (± 2)	9 (± 2)	9 (± 2)	ns
R^2	0.97	0.98	0.98	0.98	
Vintage 2015 (CPM 72h)					
K (g/L day)	0.102 (± 0.012)	0.104 (± 0.013)	0.084 (± 0.011)	0.087 (± 0.014)	ns
M (1/day)	7 (± 1)	7 (± 1)	8 (± 1)	8 (± 1)	ns
R^2	0.99	0.99	0.99	0.99	

Abbreviations: K, fermentation rate; M, half-time of sugar consumption; ns, not significant

Table 3: Odor activity value (OAV) and odor descriptor of key odorants (positive or negative) in Sangiovese wines produced by adding *Metschnikowia* strains (MP 346 or MF 98-3) or a commercial pectinolytic enzyme (Enzyme preparation) in pre-fermentative cold maceration followed by sequential inoculation with *S. cerevisiae*, compared with the same wine produced by the pure culture of *S. cerevisiae* (control).

Compounds	Odor descriptor	Odor threshold (µg/L)	OAV Vintage 2014				OAV Vintage 2015			
			MP 346	MF 98-3	Enzyme preparation	control	MP 346	MF 98-3	Enzyme preparation	control
Terpenes										
Nerol	Violets, floreal	500[a]	0.0066	0.0072	0.0062	0.0074	0.0068	0.0056	0.0046	0.0040
Geraniol	Citric, geranium	20 [a]	0.010	0.012	0.013	0.015	0.012	0.013	0.011	0.010
cis-8- hydroxy –linalool		Nf[e]	-	-	-	-	-	-	-	-
C_{13}- Norisoprenoids										
3-oxo-α-ionol	Nf	Nf	-	-	-	-	-	-	-	-
Aldehydes and ketones										
Benzaldehyde	Roasted, almond	2000[a]	0.005	0.004	0.004	0.006	0.004	0.003	0.003	0.004
Esters										
Ethyl acetate	Fruity, solvent,	7500[b]	9.6	9.2	6.1	12.4	11.4	13.9	8.5	32.6
Isoamyl acetate	Fruity, Banana	30[c]	17.6	12.5	20.5	21.4	11.3	9.9	9.4	8.7
Ethyl butanoate	Banana, pineapple strawberry	400[a]	0.4	0.3	0.4	0.4	0.3	0.3	0.3	0.2
Ethyl hexanoate	Banana, green apple	14[c]	14.2	12.0	15.7	18.7	10.8	9.0	8.7	8.4
Ethyl 3-methylbutyl succinate	Nf	Nf	-	-	-	-	-	-	-	-
Isoamyl lactate	Cream, nut	200[d]	0.95	0.94	0.84	0.92	0.46	0.47	0.44	0.37
Alcohols										
1-Hexanol	Herbaceous, woody	8000[c]	0.10	0.10	0.10	0.17	0.06	0.06	0.09	0.09
Methionol	Cooked vegetable	1000[c]	0.86	0.68	0.76	0.71	0.67	0.58	0.64	0.69
Acids										
Isovaleric acid	Acid, rancid	3000 [a]	0.15	0.18	0.14	0.17	0.17	0.17	0.15	0.15
Homovanillic acid	Nf	Nf	-	-	-	-	-	-	-	-
Phenols										
4-ethylphenol	Phenolic	440[d]	0.0043	0.0021	0.0016	0.0016	0.0047	0.0053	0.0072	0.0043
4-ethylguaiacol	Phenolic	33[c]	0.030	0.036	0.030	0.033	0.130	0.139	0.100	0.097

Odor descriptor and odor threshold of the main aroma compounds, were indicated accordingly to previous references: [a] Cai et al. 2014; [b] Peinado et al. 2006; [c] Ferreira et al. 2000; [d] Zhang et al. 2016; [e] Lopez et al. 2002.

[e] Nf (Not found): odor descriptor or odor threshold is not available in the literature.

Figure captions

Figure 1. Evolution of total yeast population during the different phases of winemaking process for 2015 vintage.

Figure 2. Kinetic of sugar consumption in Sangiovese must throughout alcoholic fermentation carried out during 2014 vintage (CPM 24h) (a) and 2015 vintage (CPM 72h) (b).

Sangiovese wines were produced by adding *Metschnikowia* strains (● MP 346; ▲ MF 98-3) or a commercial pectinolytic enzyme (■ Enzyme preparation) in cold pre-fermentative maceration followed by sequential inoculation with *S. cerevisiae*. The same wine was produced by the pure culture of *S. cerevisiae* (□ control).

Figure 1.

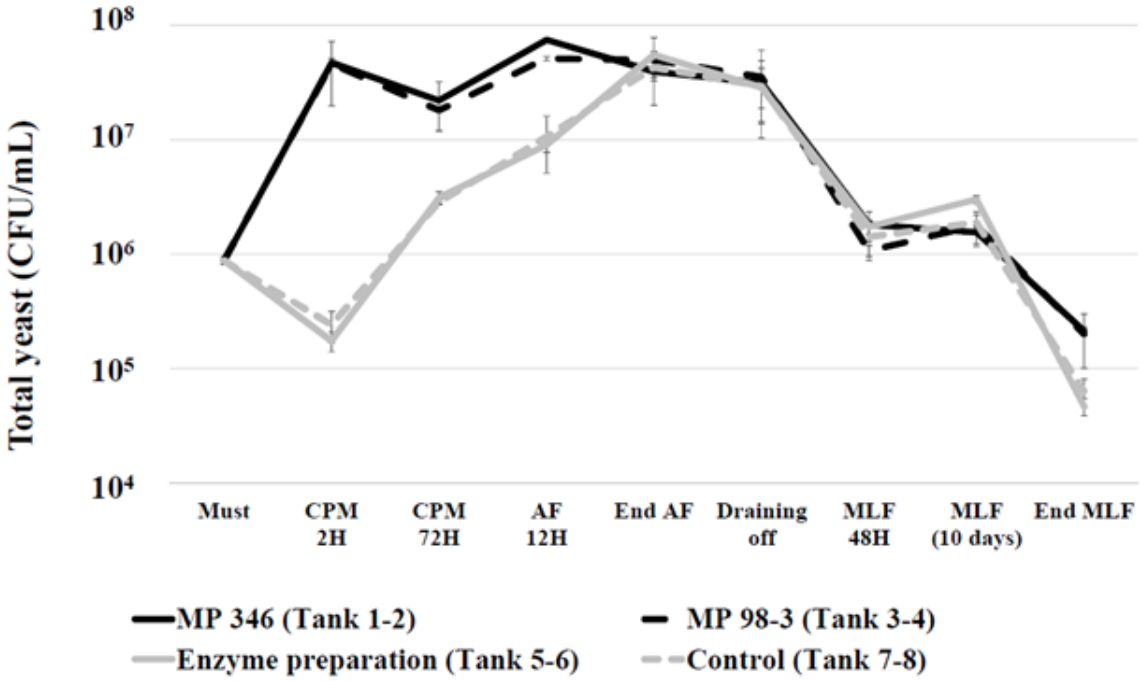

Total yeast (CFU/mL)
10⁸
10⁷
10⁶
10⁵
10⁴
Must
CPM 2H
CPM 72H
AF 12H
End AF
Draining off
MLF 48H
MLF (10 days)
End MLF
MP 346 (Tank 1-2)
MP 98-3 (Tank 3-4)
Enzyme preparation (Tank 5-6)
Control (Tank 7-8)

Figure 2.

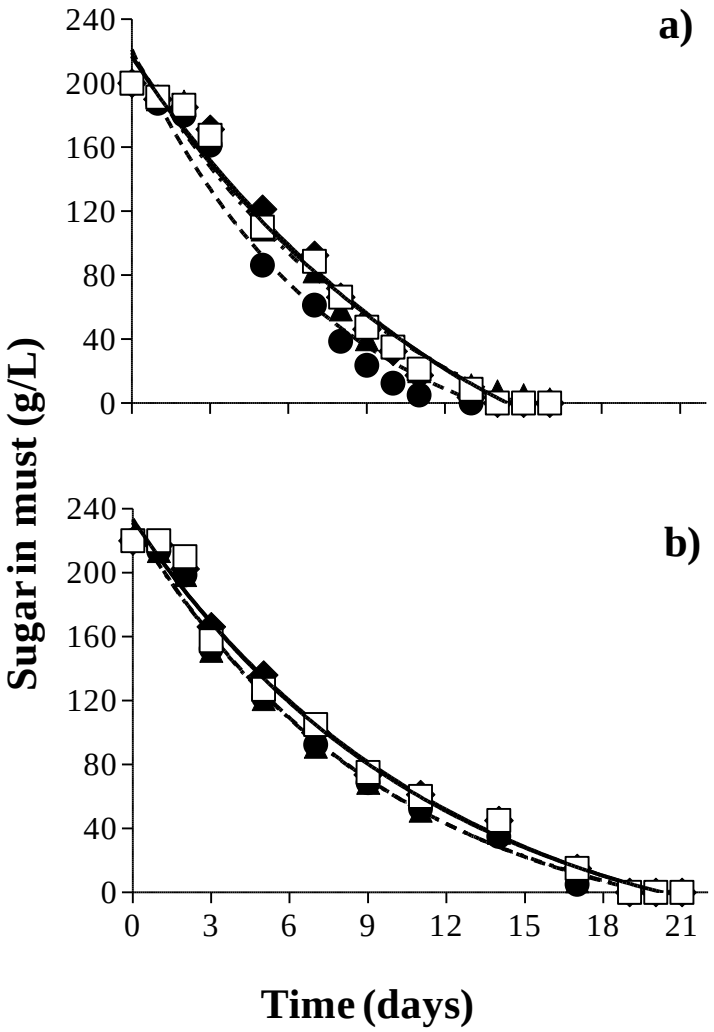

a)
b)
240
200
160
120
80
40
0
0
3
6
9
12
15
18
21
Sugar in must (g/L)
Time (days)

Table 1S: Ratio between *Metschnikowia* and total yeast population in tanks inoculated with strain MP 346 and MF 98-3 for 2015 vintage.

Phase	*Metschnikowia* / Total yeasts* (%)	
	MP 346 (Tank 1-2)	**MF 98-3 (Tank 3-4)**
CPM 2h	99.8±0.02	99.9±0.08
CPM 72h	99.1±0.05	96.3±0.5
AF12h	96.4±2.9	99.0±0.7

*The mean numbers of total yeasts were: $4.7\pm0.6*10^7$ CPM2H; $2.0\pm0.2*10^7$ CPM72H; $7.5\pm0.4*10^7$ AF 12H.

Table 2S: Composition of the Sangiovese wines obtained.

	Vintage 2014 (CPM 24h)				Vintage 2015 (CPM 72h)			
	MP 346	**MF 98-3**	**Enzyme preparation**	**control**	**MP 346**	**MF 98-3**	**Enzyme preparation**	**control**
pH	3.43	3.39	3.42	3.47	3.64	3.66	3.57	3.63
Alcohol concentration (% v/v)	13.26	13.55	13.66	13.37	14.12	14.14	14.27	14.39
Total acidity (g/L tartaric acid)	4.90	5.27	5.10	5.16	5.70	5.81	6.00	5.82
Volatile acidity (g/L acetic acid)	0.42	0.45	0.41	0.42	0.66	0.62	0.93	0.92
Total sulfur dioxide (mg/L)	74	71	71	64	67	67	59	71
Free sulfur dioxide (mg/L)	17	11	22	17	16	17	16	20

Table 3S: Concentration of volatile compounds in Sangiovese wines.

Compounds	Vintage 2014 (CPM 24h)				Vintage 2015 (CPM 72h)			
	MP 346	MF 98-3	Enzyme preparation	control	MP 346	MF 98-3	Enzyme preparation	control
Terpenes								
Linalool	9.1±1.3^{a}	10±1^{a}	9.7±0.8^{a}	10.6±2.2^{a}	6.3±0.7^{a}	6.7±0.3^{a}	7.7±0.6^{a}	6.8±0.8^{a}
α-terpineol	6.1±0.7^{a}	6.3±0.7^{a}	6.4±0.6^{a}	6.9±1.2^{a}	3.8±0.3^{a}	4.1±0.2^{a}	4.3±0.3^{a}	4±0.3^{a}
Citronellol	4.9±0.5^{a}	5.3±0.7^{a}	5.4±0.6^{a}	6.7±0.8^{a}	4.6±0.4^{a}	5±0.4^{a}	5.1±0.5^{a}	4.8±0.2^{a}
Nerol	3.3±0.3^{a}	3.6±0.5^{a}	3.1±0.3^{a}	3.7±0.3^{a}	3.4±0.1^{a}	2.8±0.2^{b}	2.3±0.3^{c}	2±0.1^{c}
Geraniol	4.8±0.5^{b}	5.9±0.8^{ab}	6.6±0.5^{ab}	7.5±1^{a}	6.1±0.4^{a}	6.6±0.2^{a}	5.4±0.3^{b}	5.2±0.2^{b}
trans-furan linalool oxide	5.2±0.4^{a}	4±0.4^{bc}	3.8±0.3^{c}	4.9±0.5^{ab}	1.2±0.2^{ab}	1±0.1^{b}	1.4±0.1^{a}	1±0.1^{b}
trans-pyran linalool oxide	9.9±1.1^{b}	11.8±1.2^{ab}	11.2±0.8^{ab}	14.1±1.9^{a}	6.7±0.3^{a}	7.1±0.3^{a}	7±0.5^{a}	6.5±0.2^{a}
cis- pyran linalool oxide	3.5±0.2^{b}	3.9±0.4^{b}	3.4±0.4^{b}	4.7±0.3^{a}	7.5±0.5^{ab}	7.6±0.7^{a}	6.1±0.3^{c}	6.4±0.4^{bc}
Diendiol 1	5.7±0.3^{b}	6±1^{b}	7.5±0.6^{ab}	9.1±1.2^{a}	2.1±0.1^{a}	2.4±0.2^{a}	2.2±0.2^{a}	2.4±0.3^{a}
Diendiol 2	4.5±0.3^{a}	3.7±0.3^{bc}	3.3±0.1^{c}	3.8±0.2^{b}	5.7±0.3^{a}	5.6±0.6^{a}	4.6±0.2^{b}	5.1±0.3^{ab}
Endiol	11.8±0.9^{b}	16.5±1.6^{a}	14.2±1.2^{ab}	14.2±0.9^{ab}	15±0.8^{a}	14.4±0.7^{a}	16.6±0.6^{a}	16±1.6^{a}
trans-8- hydroxy –linalool	6.6±0.7^{b}	14.4±2.5^{a}	9±0.3^{b}	13.8±1.8^{a}	6.3±0.3^{b}	12.1±1.2^{a}	13.9±1.5^{a}	7±0.6^{b}
cis-8- hydroxy –linalool	11.9±1.4^{ab}	12.6±1.2^{a}	9±0.6^{b}	14.6±1.4^{a}	12.4±1.2^{a}	10.9±1^{ab}	12.3±0.5^{a}	9.6±0.6^{b}
Terpinen-4-ol	1.7±0.1^{a}	1.6±0.2^{a}	1.8±0.3^{a}	1.7±0.2^{a}	7.8±0.8^{a}	8.3±0.6^{a}	8.3±0.4^{a}	7.6±0.7^{a}
Total	89±1.8 ^{c}	106±5.7 ^{b}	94±1 ^{c}	116±4.5 ^{a}	89±1 ^{b}	95±2.9 ^{a}	97±1.9 ^{a}	84±0.2 ^{c}
C_{13}- Norisoprenoids								
β-damascenone	1.5±0.2^{a}	1.7±0.2^{a}	1.6±9.9^{a}	1.6±0.1^{a}	1.4±0.2^{a}	1.5±0.1^{a}	1.3±0.3^{a}	1.2±0.2^{a}
Actinidol 1 (*cis*)	3.8±0.4^{a}	3±0.4^{ab}	2.1±0.3^{c}	2.4±0.2^{bc}	1.8±0.3^{a}	1.9±0.1^{a}	2.1±0.2^{a}	2±0.2^{a}
Actinidol 2 (trans)	2.2±0.2^{b}	3±0.3^{a}	2.9±0.2^{a}	3.1±0.2^{a}	3.7±0.3^{a}	3.7±0.3^{a}	4.3±0.3^{a}	3.9±0.3^{a}
3-oxo-α-ionol	32.1±4^{b}	41±3.5^{a}	44.1±2.5^{a}	44.9±2.5^{a}	60.4±1.5^{a}	60.5±2^{a}	58.6±1^{ab}	55.9±1^{b}
Total	40±3.7 ^{b}	49±4.3 ^{a}	51±2.7 ^{a}	52±2.4 ^{a}	67±1.9 ^{a}	68±2.5 ^{a}	66±1.2 ^{a}	63±1.3 ^{a}
Aldehydes and ketones								
Phenylacetaldehyde	1.8±0.1^{c}	1.9±0.1^{c}	48.4±1.5^{a}	29.1±1^{b}	7.1±0.9^{c}	14.2±1^{b}	32.7±2^{a}	5.3±0.4^{c}
Benzaldehyde	10±0.8^{ab}	7.4±0.5^{b}	8.3±0.6^{b}	11.8±1.8^{a}	8.6±0.5^{a}	5.6±0.4^{b}	5±0.5^{b}	8.2±0.3^{a}
Vanillin	20.1±2.3^{b}	8.8±1^{c}	18.3±0.7^{b}	24.9±2.1^{a}	22.8±1.1^{b}	26.9±1.7^{a}	22.4±0.6^{b}	17±0.9^{c}
Norfuraneol	8.1±0.4^{a}	6.5±0.3^{b}	8.6±0.7^{a}	7.6±0.6^{ab}	12.1±0.5^{b}	10.3±0.8^{b}	10.7±0.5^{b}	14.2±1.1^{a}
Syringaldehyde	17.9±1^{ab}	9.3±0.7^{c}	18.8±1.2^{a}	15.5±0.9^{b}	4.2±0.4^{a}	3.8±0.3^{a}	4.6±0.5^{a}	3.9±0.1^{a}
Furfural	21±1^{b}	11.3±0.5^{c}	29.4±1.5^{a}	21.4±1.4^{b}	21.1±1^{ab}	19.8±0.8^{ab}	23.4±2.5^{a}	19.1±1.3^{b}
5-Methylfurfural	4.3±0.2^{b}	1.8±0.03^{c}	21.5±1.2^{a}	3.6±0.1^{b}	1±0.1^{a}	1±0.1^{a}	1±0.1^{a}	1±0.2^{a}
Furaneol	8.1±0.7^{a}	6.5±0.3^{b}	8.6±0.6^{a}	7.6±0.2^{ab}	12.1±1.6^{ab}	10.3±0.4^{b}	10.7±1.3^{b}	14.2±1.1^{a}
Total	91±3.7 ^{c}	54±1.3 ^{d}	162±5.3 ^{a}	122±1.6 ^{b}	89±0.9 ^{b}	92±4.5 ^{b}	110±2.8 ^{a}	83±4.4 ^{b}
Esters								
Ethyl acetate*	71.8±1.1^{b}	68.7±1.1^{b}	45.5±1.1^{c}	93.1±1.1^{a}	85.8±3.4^{bc}	104.2±19.8^{b}	63.9±7.1^{c}	244.2±4.7^{a}
Hexyl acetate	13.8±1.1^{b}	5.5±0.5^{c}	13.5±1.2^{b}	18.2±0.8^{a}	5.1±0.6^{ab}	4.5±0.5^{b}	6.3±0.7^{a}	4±0.3^{b}
Isoamyl acetate	528.8±21^{b}	376±26^{c}	616±40^{a}	642±22^{a}	340±11^{a}	296±7^{b}	283±3^{b}	261±7^{c}

2-phenylethyl acetate	57.2±2.5^{b}	55.4±3.5^{b}	60±2.5^{b}	69.7±4.3^{a}	48.4±3^{b}	50±3.7^{b}	61.7±2.5^{a}	53.1±2.9^{b}
Ethylphenyl acetate	2.1±0.4^{a}	2.2±0.3^{a}	1.9±0.2^{a}	2.4±0.2^{a}	2.3±0.3^{a}	2.5±0.2^{a}	2.4±0.3^{a}	2.2±0.1^{a}
Ethyl butanoate	147±7^{b}	124±5^{c}	146±4^{b}	172±6.5^{a}	127±3.6^{a}	109±3 ^{b}	107±2.2^{b}	99±3.2^{c}
Ethyl hexanoate	199±8^{c}	168±8.5^{d}	220±4.5^{b}	261.5±6.5^{a}	151.6±6.1^{a}	125.6±3^{b}	121.6±4.5^{bc}	117.6±2.6^{c}
Ethyl octanoate	169±4^{c}	176±4.5^{c}	195±5.5^{b}	220±8.5^{a}	93±5^{a}	79±3^{b}	82±3^{b}	82±3^{b}
Ethyl decanoate	23.5±3.5^{c}	25.8±0.7^{bc}	39.8±2.6^{a}	30.2±2.5^{b}	29.3±2.4^{a}	24.7±1.5^{a}	27.3±2.3^{a}	28.4±2.1^{a}
Ethyl 9-decenoate	1.3±0.2^{c}	3.1±0.2^{b}	4.4±0.4^{a}	3.6±0.2^{b}	1±0.1^{a}	1±0.2^{a}	1±0.2^{a}	1±0.3^{a}
Ethyl 3-hydroxybutanoate	519±305^{a}	418±19^{b}	486±15^{a}	533±0.7^{a}	265±16^{a}	275±21 ^{a}	268±9.1^{a}	255.8±9.8^{a}
Ethyl 4-hydroxybutanoate	4687±236^{c}	5073±184.5^{c}	6215±215^{b}	8643±254^{a}	7005±280^{bc}	6389±389^{c}	7230±230^{b}	8523±262^{a}
Ethyl 2-hydroxyvalerate	13.2±1.2^{a}	12.4±0.9^{a}	12.4±0.6 ^{a}	14.7±1.3 ^{a}	8±1^{a}	7.9±1.9^{a}	8.6±0.5^{a}	8±1.2^{a}
Ethyl 2-hydroxy-4-Methylpentanoate	76.7±3.6^{ab}	84.1±4^{a}	74.1±1.8 ^{a}	82.4±2.5^{a}	65.3±4^{ab}	64.8±2.7^{b}	72.2±2.7^{a}	65.9±1^{ab}
Ethyl 3-methylbutyl succinate (Ethyl isoamyl succinate)	7±0.4^{b}	8.8±0.5^{a}	6 ±0.4^{c}	6.8±0.3 ^{c}	12.6±0.4^{ab}	14.4±1.5^{a}	10.2±1^{bc}	10±1^{c}
Diethyl succinate	395±16^{ab}	460±36^{a}	380±26^{b}	446±22^{ab}	369±19^{ab}	347±8^{b}	410±10^{a}	359±30^{b}
Ethyl lactate	24910±1595^{a}	21879±2025^{a}	17667±866^{b}	24279±1689^{a}	9434±665^{a}	7394±158^{b}	7650±150^{b}	7581±220^{b}
Isoamyl lactate	190±4^{a}	187±25^{ab}	168±4^{c}	184±3^{b}	92±3^{a}	94±2^{a}	87±2^{a}	74±5^{b}
Diethyl malate	115±4^{b}	126±4^{a}	127±5^{a}	116±4^{b}	93±4^{b}	91±2^{b}	117.2±2.5^{a}	97±3^{b}
Diethyl 2-hydroxyglutarate	161±3^{a}	156±6^{a}	154±6^{a}	151±3^{a}	132±7^{a}	127.5±5^{a}	138.9±6.5^{a}	128±9^{a}
Methyl vanillate	26.4±0.7^{b}	25.6±1.3^{b}	29.3±1.5 ^{a}	29.2±0.8 ^{a}	6.5±0.6^{a}	6.9±0.7^{a}	7.3±0.8^{a}	6.5±0.5 ^{a}
Methyl salicylate	1±0.1^{a}	1.1±0.1^{a}	1.1±0.2 ^{a}	1.2±0.2^{a}	1±0.2^{a}	1±0.1^{a}	1±0.1^{a}	1±0.1^{a}
Ethyl vanillate	67.6±2.6^{c}	88.3±3^{b}	80.1±3.7^{b}	103.5±3.5^{a}	131.1±1.8^{b}	137.7±2.5^{a}	127.4±1.2^{b}	108.3±3^{b}
Ethyl pyroglutamate	92±4^{d}	138±5^{b}	200±11^{a}	111±4^{c}	258±8^{c}	261±3^{c}	308±7^{a}	283±44^{b}
Total	32402±1871^{ab}	29593±1835^{bc}	26894±1051^{c}	36119±1949^{a}	18669±340^{a}	15904±206^{c}	17128±344^{b}	18149±56^{a}
Alcohols								
1-Hexanol	789±11^{b}	784±13^{b}	809±9^{b}	1341±45^{a}	468±19^{b}	461±10^{b}	749±18.5^{a}	757±10^{a}
trans-3-Hexen-1-ol	17.4±1.3^{ab}	15.3±0.7^{bc}	14±1.1^{c}	19.6±1.5^{a}	16.2±0.9^{a}	17.2±1.3^{a}	17.4±0.6^{a}	17.1±1^{a}
cis-3-Hexen-1-ol	53.2±3^{b}	53.9±1.6^{b}	54.6±0.5^{b}	82.7±6.5^{a}	9.7±0.8^{a}	11.4±0.5^{a}	11.8±0.8^{a}	10.6±1.2^{a}
2-Hexen-1-ol	5.5±0.3^{b}	8.2±0.8^{a}	5.5±0.5^{b}	8.4±1.4^{a}	5.5±0.3^{a}	5.4±0.2^{a}	4±0.3^{b}	3.6±0.5^{b}
Benzyl alcohol	270±10^{b}	264±9^{b}	269±12^{b}	323±250^{a}	219±18^{a}	227±7^{a}	212±9^{a}	217±6^{a}
2-phenylethanol	34700±1200^{a}	36200±800^{a}	33800±2150^{a}	35900±1350^{a}	40200±800^{a}	40100±1295^{a}	41600±503^{a}	40400±491^{a}
3-(methylthio)-propanol (Methionol)	862±22^{a}	676±6^{d}	759±15^{b}	713±6^{c}	668±18^{a}	576±25^{b}	643±20^{a}	685±26^{a}
Furfuryl alcohol	1124±94^{a}	685±15^{c}	946±17^{b}	1023±75^{ab}	248±9^{a}	232±12^{b}	168±14^{c}	286±18^{a}
Homovanillyl alcohol (Vanillic alcohol)	79.4±6^{a}	77.9±7^{a}	87.7±2.5^{a}	81.4±4.5^{a}	52.7±2.5^{a}	53.8±4^{a}	48.5±4.1^{a}	50.3±3.2^{a}
1-Octen-3-ol	3.9±0.4 ^{a}	2.8±0.2^{b}	2±0.1^{c}	4.5±0.3^{a}	3.1±0.3^{b}	4±0.2^{a}	3.5±0.4^{ab}	2.9±0.3^{b}
Total	37905±1159^{a}	38768±826^{a}	36746±2159 ^{a}	39496±1453 ^{a}	41890±55^{a}	41687±1299^{a}	43457±560^{a}	42428±488^{a}
Acids								
Butyric acid	508±19^{ab}	471±12^{b}	475±13^{b}	542.5±15^{a}	558±33^{a}	581±18.5^{a}	468±12^{b}	442±11^{b}
3-Methylbutanoic acid (Isovaleric acid)	447±14^{c}	545±11^{a}	434±15^{c}	501±14^{b}	497±13^{ab}	519±11^{a}	447±32^{c}	451±14^{bc}
Hexanoic acid	1018±37^{b}	996±28^{b}	1138±38^{b}	1410±177^{a}	1074±75^{a}	1129±130^{a}	1022±106^{a}	932±44^{a}
Octanoic acid	1275±75^{c}	1215±38^{c}	1535±66^{b}	1701±57^{a}	1162±63^{a}	1204±167^{a}	1167±68^{a}	1041±65^{a}

Decanoic acid	171±6^{c}	199±13^{bc}	267±17^{a}	226±14^{b}	301±13^{a}	306±8^{a}	314±15^{a}	285±5^{a}
Homovanillic acid	58.9±0.9^{a}	61.8±1.1^{a}	57.3±1.3^{b}	44.8±4.5^{b}	24.8±1.3^{a}	25.5±0.5^{a}	18.9±1^{b}	20.3±0.8^{b}
Total	3479±71^{c}	3489±50^{c}	3906±74^{b}	4425.2±85^{a}	3617±72^{a}	3764±85^{a}	3436±259^{ab}	3171±125^{b}
Phenols								
Phenol	3.7±0.2^{b}	3.5±0.3^{bc}	3±0.2^{c}	4.6±0.3^{a}	4.1±0.5^{a}	4.6±0.7^{a}	4.2±0.5^{a}	4.1±0.4^{a}
4-ethylphenol	1.9±0.1^{b}	2.3±0.1^{a}	1.8±0.2^{b}	1.8±0.1^{b}	5.2±0.5^{b}	5.8±0.9^{b}	7.9±0.9^{a}	4.7±0.3^{b}
4-ethylguaiacol	1±0.1^{b}	1.2±0.1^{a}	1±0.1^{ab}	1.1±0.0^{ab}	4.3±0.3^{a}	4.6±0.2^{a}	3.3±0.3^{b}	3.2±0.2^{b}
Eugenol	2.6±0.1^{a}	2.8±0.1^{a}	2±0.1^{b}	2.7±0.2^{a}	2.3±0.2^{ab}	2.4±0.2^{ab}	2.5±0.1^{a}	2±0.2^{b}
Guaiacol	2.6±0.3^{bc}	3.1±0.2^{ab}	2.5±0.2^{c}	3.2±0.2^{a}	3.5±0.2^{a}	3.5±0.1^{a}	3.3±0.1^{a}	3.6±0.3^{a}
o-cresol	1.1±0.1^{ab}	1.3±0.1^{a}	1±0.1^{b}	1±0.1^{b}	1±0.1^{a}	1.2±0.2^{a}	1±0.1^{a}	1±0.1^{a}
p-cresol	2.3±0.2^{a}	1.6±0.1^{b}	1.2±0.1^{c}	1.5±0.1^{bc}	1.1±0.1^{a}	1±0.2^{a}	1±0.1^{a}	1±0.1^{a}
Vanillin fenol	20.1±0.8^{b}	8.8±0.9^{c}	18.3±1^{b}	24.9±1.7^{a}	22.8±1.3^{b}	26.9±1^{a}	22.4±0.8^{b}	17±1.5^{c}
Acetovanillone	146±7^{b}	169±9^{a}	169±5^{a}	175±5^{a}	83±5^{a}	86±4^{a}	82±2^{a}	76±6^{a}
Total	181±7.5^{c}	193±9^{bc}	199±6.3^{ab}	216±3.2^{a}	127±7^{a}	136±4.8^{a}	127±1.5^{a}	112±4.3^{b}
Lactones								
γ-nonalactone	5.6±0.5^{c}	6.4±0.4^{bc}	7.1±0.3^{b}	9.2±0.4^{a}	15.2±1.3^{a}	13.9±0.8^{ab}	11±1.5^{bc}	10.6±0.5^{c}
γ-Butyrolactone	1578±111^{b}	1486±54^{b}	1475±74^{b}	1859±80^{a}	1471±120^{a}	1573±87^{a}	1585±108^{a}	1460±116^{a}
4-carboxyethoxy-butyrolactone	549±32^{a}	531±21^{a}	515±15^{a}	515±5^{a}	354±14^{a}	349±15^{a}	399.5±28^{a}	358±44^{a}
Total	2132±79^{b}	2024±75^{b}	1997±89^{b}	2383±85^{a}	1840±135^{a}	1935±103^{a}	1995±137^{a}	1829±160^{a}
Others								
N-(3-Methylbutyl)acetamide	276±27^{a}	172±13^{bc}	211±9^{a}	152±20^{c}	121±4^{a}	127±7^{a}	128±5^{a}	97±14^{b}

For each treatment within the same year, values with different letters in the same row are significantly different according to the Tukey test (95%).

*Ethyl acetate is expressed as mg/L, however it is not included in ester total amount, due to its higher concentration compared with the other esters.

Figure 1S Kinetics of malolactic fermentation carried out, by the strain *O. oeni* PN4® MBR, during 2014 vintage (CPM 24h) (a) and 2015 vintage (CPM 72h) (b). Solid lines represent the evolution of malic acid and dashed lines indicate the lactic acid increase.

Sangiovese wines were produced by adding *Metschnikowia* strains (● MP 346; ▲ MF 98-3) or a commercial pectinolytic enzyme (■ Enzyme preparation) in cold pre-fermentative maceration followed by sequential inoculation with *S. cerevisiae*. The same wine was produced by the pure culture of *S. cerevisiae* (□ control).

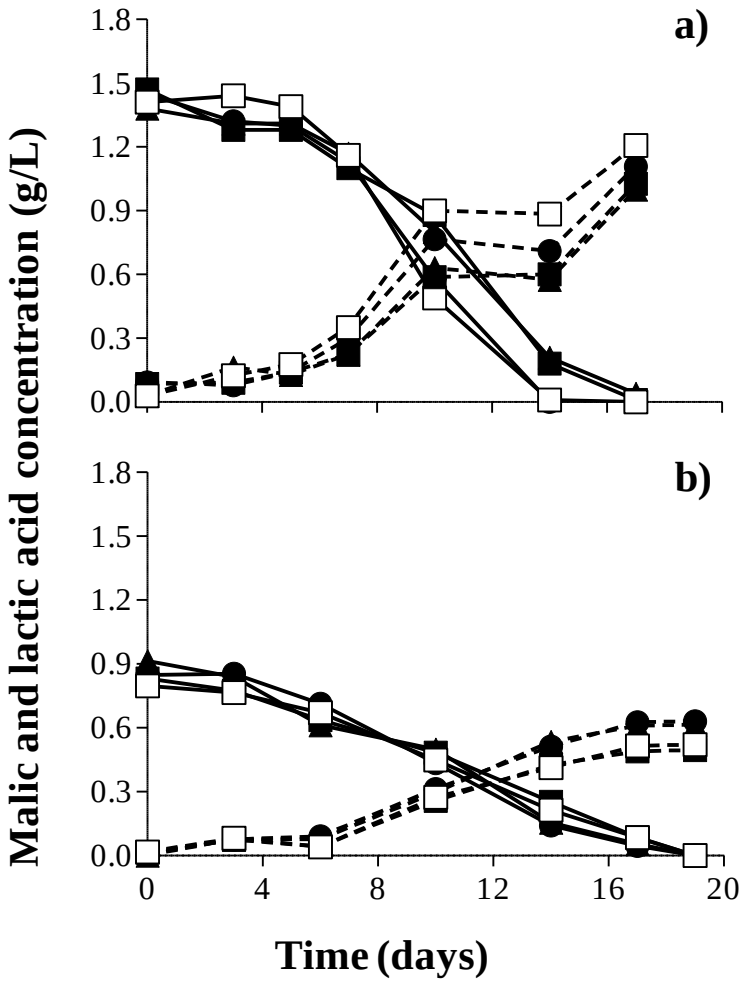
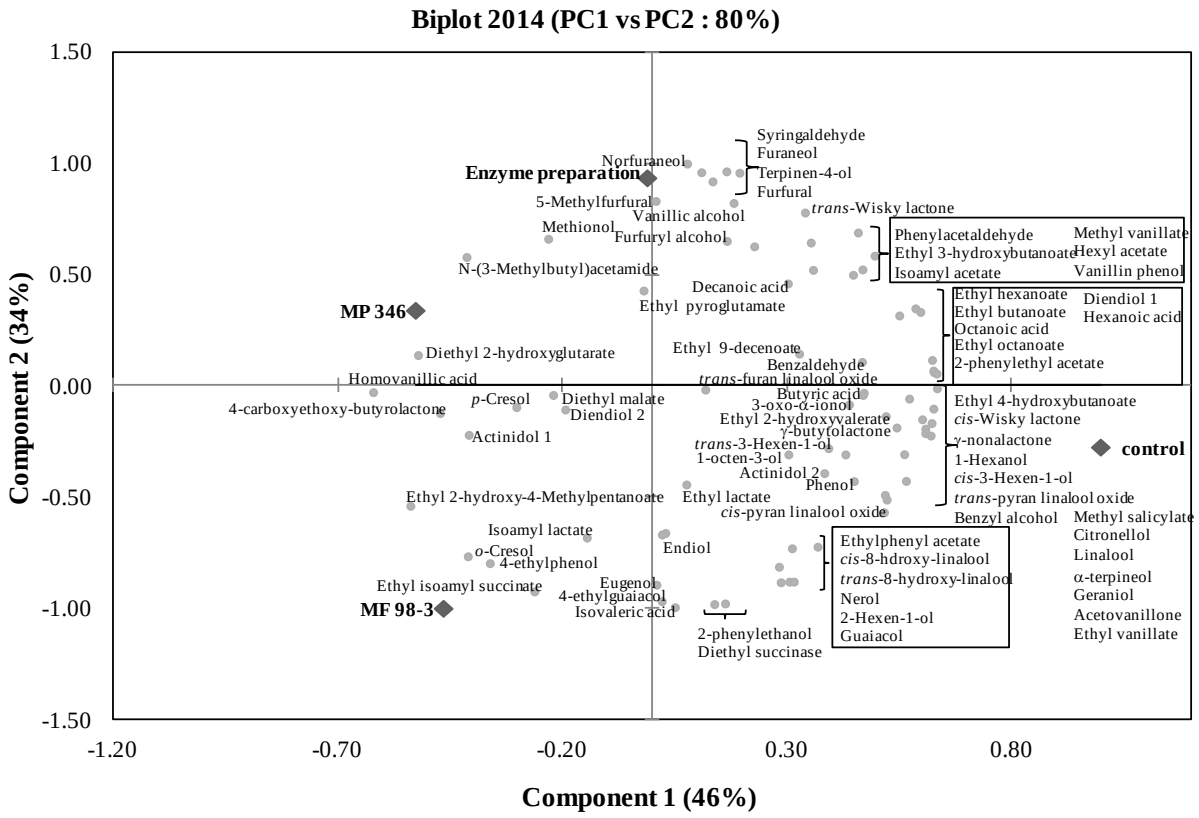
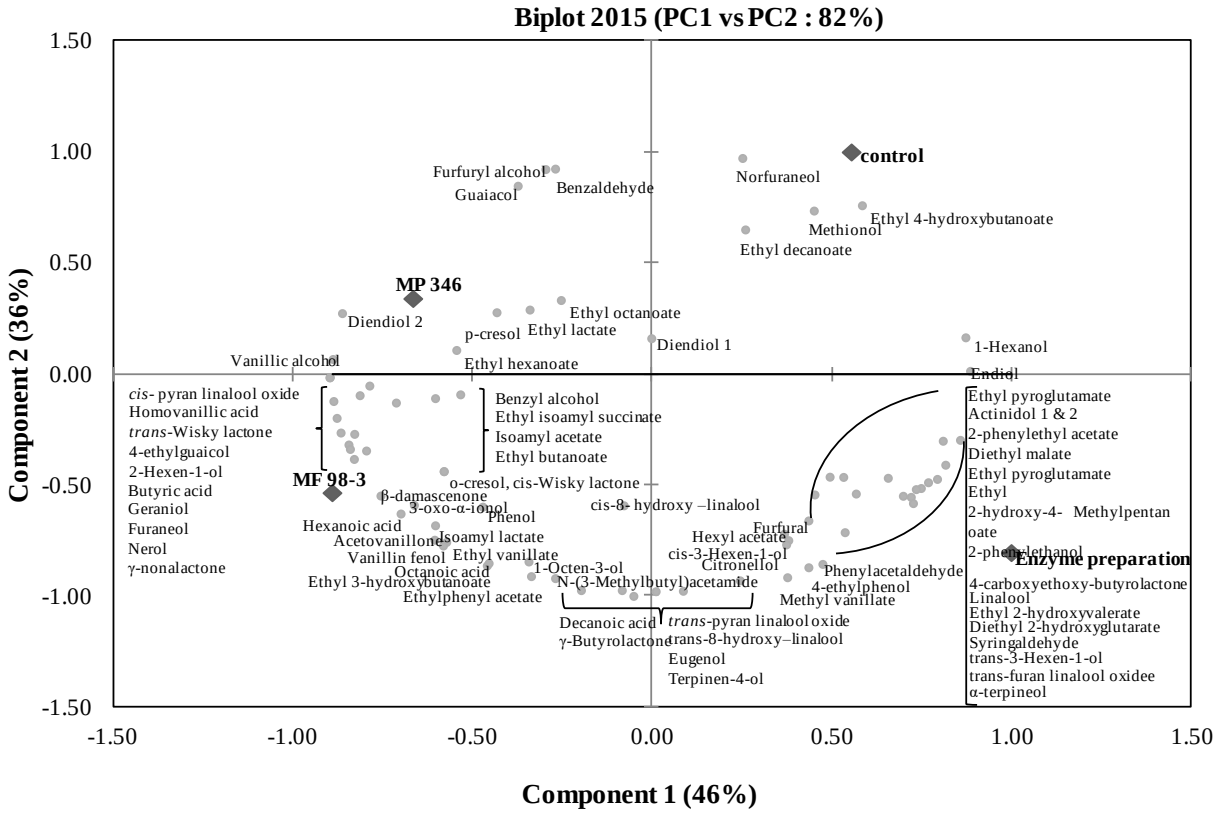


Figure 2S Biplot of the principal components analysis (PC 1 vs. PC 2) of volatile compounds in Sangiovese wines produced by adding *Metschnikowia* strains (MP 346 or MF 98-3) or a commercial pectinolytic enzyme (Enzyme preparation) in cold pre-fermentative maceration followed by sequential inoculation with *S. cerevisiae*, compared with the same wine produced by the pure culture of *S. cerevisiae* (control).




Biplot 2015 (PC1 vs PC2 : 82%)
Component 2 (36%)
Component 1 (46%)
1.50
1.00
0.50
0.00
-0.50
-1.00
-1.50
-1.50
-1.00
-0.50
0.00
0.50
1.00
1.50
control
MP 346
MF 98-3
Enzyme preparation
Furfuryl alcohol
Guaiacol
Benzaldehyde
Norfuraneol
Ethyl 4-hydroxybutanoate
Methionol
Ethyl decanoate
Diendiol 2
p-cresol
Ethyl lactate
Ethyl octanoate
Diendiol 1
1-Hexanol
Vanillic alcohol
Ethyl hexanoate
Endiol
cis- pyran linalool oxide
Homovanillic acid
trans-Wisky lactone
4-ethylguaicol
2-Hexen-1-ol
Butyric acid
Geraniol
Furaneol
Nerol
γ-nonalactone
Benzyl alcohol
Ethyl isoamyl succinate
Isoamyl acetate
Ethyl butanoate
o-cresol, cis-Wisky lactone
β-damascenone
3-oxo-α-ionol
Phenol
cis-8- hydroxy –linalool
Hexanoic acid
Acetovanillone
Isoamyl lactate
Vanillin fenol
Ethyl vanillate
Octanoic acid
1-Octen-3-ol
Ethyl 3-hydroxybutanoate
N-(3-Methylbutyl) acetamide
Ethylphenyl acetate
Decanoic acid
γ-Butyrolactone
trans-pyran linalool oxide
trans-8-hydroxy–linalool
Eugenol
Terpinen-4-ol
Furfural
Hexyl acetate
cis-3-Hexen-1-ol
Citronellol
Phenylacetaldehyde
4-ethylphenol
Methyl vanillate
Ethyl pyroglutamate
Actinidol 1 & 2
2-phenylethyl acetate
Diethyl malate
Ethyl pyroglutamate
Ethyl
2-hydroxy-4- Methylpentan
oate
2-phenylethanol
4-carboxyethoxy-butyrolactone
Linalool
Ethyl 2-hydroxyvalerate
Diethyl 2-hydroxyglutarate
Syringaldehyde
trans-3-Hexen-1-ol
trans-furan linalool oxidee
α-terpineol