Pre-fermentative cold maceration in the presence of non-*Saccharomyces* strains: effect on fermentation behaviour and volatile composition of a red wine

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Abstract

Background and Aims: This study evaluated the impact of pre-fermentative cold maceration (PCM), in the presence of two non-*Saccharomyces* yeasts, *Metschnikowia pulcherrima* MP 346 and *Metschnikowia fructicola* MF 98-3, and of a commercial pectic enzyme, on fermentation kinetics and on the 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 a pectic enzyme preparation during PCM, at 5°C for 24 or 72 h. A Control wine was produced by a pure culture of *Saccharomyces cerevisiae*. Both non-*Saccharomyces* strains affected the initial yeast population dynamics and the persistence of *S. cerevisiae* at the end of malolactic fermentation. Irrespective of the duration of PCM, the inoculum of *Metschnikowia* strains did not influence the rate of sugar consumption or the kinetics of malolactic fermentation. The volatile composition of the final wines was evaluated with solid-phase extraction, followed by GC/MS. The concentration of some terpenes and C13-norisoprenoids, nerol, geraniol, 8-hydroxy-linalool (*cis*) and 3-oxo-α-ionol, and of some esters, isoamyl lactate and ethyl isoamyl succinate, was higher in wines inoculated with *Metschnikowia* strains than in the Control and wine treated with pectic enzyme.

Conclusions: *Metschnikowia* yeast strains MP 346 and MF 98-3 affect wine volatile composition.

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

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

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 be 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 (Jolly et al. 2014, Azzolini et al. 2015, Belda et al. 2015, Benito et al. 2015, 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 colour (Capozzi et al. 2015, Padilla et al. 2016). 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. 1988) and exo-glucanases (Gil et al. 2005).

Since it was demonstrated that the aromatic components of certain grape cultivars 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 pectic enzyme preparations, which are mainly used in red wine production for breaking down the cell walls of red grape skins, thus improving overall colour intensity and colour stability (Gunata et al. 1988, Gil and Vallés 2001, Fia et al. 2005, 2016). All of these winemaking practices have enhanced interest in pre-fermentative maceration stages and have recently attracted considerable attention from researchers (Gil-Muñoz et al. 2009, González-Neves et al. 2015, Mihnea et al. 2015, Baiano et al. 2016).

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

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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 pre-fermentative cold maceration (PCM), carried out in the presence of two *Metschnikowia* strains (*Metschnikowia pulcherrima* MP 346 or *Metschnikowia fructicola* MF 98-3) or of a commercial pectic 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 *Saccharomyces cerevisiae* in order to evaluate the sole temperature impact during PCM.

Materials and methods

Experimental design

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

Microorganisms and media

Metschnikowia pulcherrima MP 346 and *M. fructicola* MF 98-3 strains (in ADY preparation), the *S. cerevisiae* Lalvin RC212 strain (in ADY preparation), the *Oenococcus oeni* PN4 strain (in MBR lyophilised preparation) and the pectic enzyme LALLZYME Cuvée Rouge (containing pectinases with glucosidases side activities) used for this study were kindly provided by Lallemand (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, Milan, Italy). *Oenococcus oeni* PN4 MBR strain was maintained frozen at −20°C in Man Rogosa Sharpe (MRS) broth (BD Difco) containing 20% glycerol (v/v).

In order to evaluate the total yeast population, must and wine samples were serially diluted in 1% (m/v) peptone solution (pH 7.0) and spread in duplicate onto Wallerstein Laboratory (WL) Nutrient Agar (Thermo Scientific Oxoid, Milan, 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 colour, 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) 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, Italy). For both vintages, four vinifications (each in duplicate) were carried out using the same experimental procedure, at the Azienda Agricola Ciucci, Orte, Italy, 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, TA 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/m, was bled off (saignée). Before alcoholic fermentation (AF), PCM was performed at 5°C for 24 h (vintage 2014) or 72 h (vintage 2015). The set of experiments consisted of: two trials subjected to PCM in the presence of 0.25 g/L *M. pulcherrima* MP 346 (tanks 1–2) or 0.5 g/L *M. fructicola* MF 98-3 (tanks 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); one trial subjected to cold maceration with 3 g/hL of the pectic enzyme LALLZYME Cuvée Rouge (tanks 5–6); a Control trial (tanks 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). The amount of yeast assimilable nitrogen (YAN) added after pre-fermentative maceration during the 2014 and 2015 vintages was 145 and 110 mg N/L, respectively. The YAN was supplemented by the addition of 30 g/hL of FERMAID E (Lallemand) at 1/3 of AF in the 2014 samples. Otherwise, during the 2015 vintage, 20 g/hL of FERMAID E was added 12 h after the inoculum of *S. cerevisiae* and at one-third of the AF.

Once the fermentation of sugars was complete, 3 days of post-fermentation extended maceration was carried out. After devatting, all wines were inoculated with 1 g/hL *O. oeni* PN4 MBR (Lallemand), which was rehydrated according to the manufacturer's instructions. Malolactic fermentation (MLF) was carried out at 20°C; at the end of MLF the wines were transferred into stainless steel tanks and sodium metabisulfite was added in order to obtain a similar free SO_2 concentration (20 mg/L) in all tanks. Following the post-fermentation stabilisation process, 60 L of each wine sample were bottled and the volatile compounds were analysed after 4 months.

Estimation of the parameters of AF

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 Equation 1:

$$Y = A + C \times e^{-e[K,x(t-M)]} \quad (1)$$

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. Equation 1 was fitted to the experimental data by a non-linear regression procedure (GraphPad Prism 5.0; GraphPad software, La Jolla, CA, USA) and the quality of the regression was evaluated by the coefficient of determination (R^2).

Analytical procedures

The Sangiovese grapes at harvest were analysed for pH, TA and TSS (°Brix). pH was measured potentiometrically with a

Mettler Toledo pH meter (Steroglass, Perugia, Italy); TA was determined as g tartaric acid/L of juice sample by titrating 10 mL of juice with 0.1 mol NaOH reaching pH 7; and TSS was measured at 20°C with a digital refractometer HI 96801 (Hanna Instruments, Milan, Italy). During AF, sugar consumption was measured by the decrease in the medium density with a standard wine densimeter. The concentration of L-lactic acid and malic acid was determined with K-LATE and K-LMALR kits (Megazyme International Ireland, Wicklow, Ireland).

Volatile compounds were analysed by GC/MS after solid-phase extraction (SPE), carried out by ENV+ cartridge (IST, Ystrad Mynach, Wales). The process was performed by an Aspec XL Sample Processor for SPE (Gilson, 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 dichloromethane. The solution was dried with Na_2SO_4 and concentrated to 0.4 mL by nitrogen flow stream.

The volatile compounds were analysed by GC/MS with a 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 (60 m × 320 µm i.d. × 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 a splitless mode. The analyses were done in SCAN mode. Compounds were identified by the NIST data bank and co-injection of pure reference standards. All compounds were quantified using 1-heptanol as internal standard with Response factor (RF) = 1.

Odour activity value

The odour activity value (OAV), a parameter used to evaluate the contribution of the volatiles to wine aroma, was calculated as the ratio between the concentration of an individual volatile and the corresponding odour threshold found in the literature (Ferreira et al. 2000, Cai et al. 2014).

Statistical analysis

Data of wine composition and flavour compounds were analysed for statistical significance by one-way ANOVA in order to test for significant differences between treatments. When significance was reached, a Tukey's (honest significant difference) post-hoc test (confidence interval: 95%) was performed using EXCEL (Microsoft, Redmond, WA, USA) Add-in macro DSAASTAT program (Onofri 2006).

Results and discussion

Yeast population dynamics

One grape cultivar (Sangiovese) and two vintages (2014 and 2015) were analysed to evaluate the combined effect of PCM and the addition of selected *Metschnikowia* strains (*M. pulcherrima* MP 346 and *M. fructicola* MF 98-3) or an enzyme preparation containing pectic enzyme activity.

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. Figure 1 shows the typical evolution of the total yeast population observed throughout AF during the 2015 vintage. Comparing tanks inoculated (tanks 1–4) and non-inoculated with *Metschnikowia* (tanks 7–8), a significant difference was observed in total yeast population both in PCM and AF phases. A similar difference was not observed when comparing the yeast population of the Control (tanks 7–8) and tanks treated with the enzyme preparation (tanks 5–6) (Figure 1).

During the first 2 h of PCM (PCM 2 h; Figure 1), the total yeast population increased 50-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 fourfold [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 12 h, it was mainly composed (96–99%) by *Metschnikowia* cells (Table S1). The observations that an increase in the yeast cell count occurred only in inoculated tanks and that *Metschnikowia* became predominant only when the PCM 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 similar under all conditions [5.0 (±0.2) × 10^7 CFU/mL] and was, almost, completely composed (99%) of *S. cerevisiae* cells (Table S1). 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 3.3–4.5-fold higher in tanks inoculated with MP 346 and MF 98-3 than in the Control and the enzyme-treated tanks (Table 1).

Analysis of the overall winemaking process indicated 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 MLF

As expected, cold maceration was effective at inhibiting the onset of AF (Hierro et al. 2006), and sugar consumption

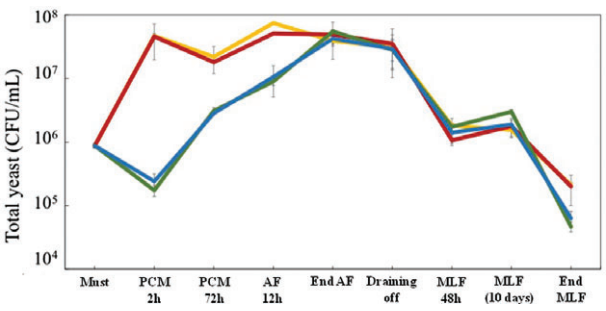

Figure 1. Evolution of total yeast population during the different phases of the winemaking process for the 2015 vintage for *Metschnikowia pulcherrima* MP 346 (tanks 1–2) (——), *Metschnikowia fructicola* MF 98-3 (tanks 3–4) (——), pectic enzyme preparation (——) and Control (——).

began 48 h only after the inoculum of a commercial *S. cerevisiae* strain.

The kinetics of sugar consumption in Sangiovese must throughout AF was comparable during the 2014 vintage (PCM 24 h) and 2015 vintage (PCM 72 h) (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 finalise AF at 18°C, and similar trends were observed between samples (Figure 2) with no significant difference 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 of MF 98-3 during the PCM did not significantly affect the fermentation behaviour of the *S. cerevisiae* strain under analysis, which easily governed AF, thus achieving the completion of the process without delay, as already indicated 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 change in the fermentation rate, when compared with that of pure cultures of *S. cerevisiae*.

The MLF kinetics, obtained with the *O. oeni* PN4 MBR strain, showed a similar trend in 2014 (Figure S1a) and 2015 (Figure S1b) 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 concentration 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 the 2015 vintage. When non-*Saccharomyces* was inoculated during the PCM, no significant difference was observed during MLF and in the evolution of the malic and lactic acid concentration (Figure S1). Moreover, no differences were evident in the final composition of the four Sangiovese wines (Table S2).

Volatiles profile

Seventy-eight volatile compounds, 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 S3).

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

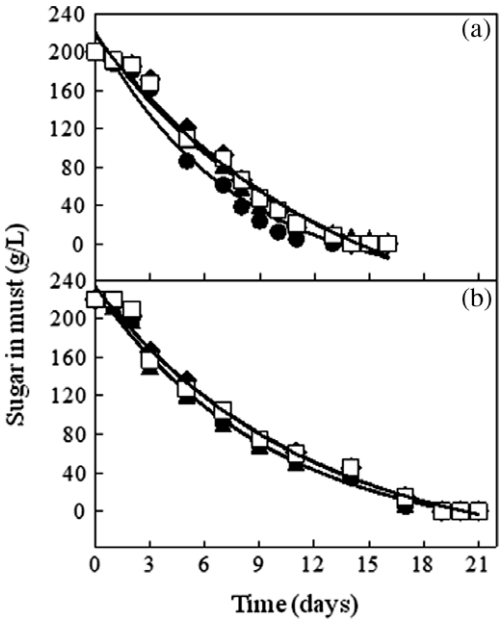

Figure 2. Kinetics of sugar consumption in Sangiovese must throughout alcoholic fermentation during (a) 2014 vintage [pre-fermentative cold maceration (PCM) 24 h] and (b) 2015 vintage (PCM 72 h). Sangiovese wines were produced by adding *Metschnikowia* strains MP 346 (●) and MF 98-3 (▲) or by adding a commercial pectic enzyme preparation (■) in PCM followed by sequential inoculation with *Saccharomyces cerevisiae*. The same wine was produced by the pure culture of *S. cerevisiae* (□).

The non-*Saccharomyces* yeasts did not clearly affect the total concentration of terpenes and C13-norisoprenoids in 2014 and 2015 vintages (Table S3). In the 2015 vintage, however, when a longer cold-maceration was performed (PCM 72 h), wines inoculated with the two *Metschnikowia* strains, similarly to the enzyme-treated wine, contained a greater concentration of terpenes compared to that of the Control wine. Moreover, the presence of the *Metschnikowia* strains had a discriminating effect on some individual terpenes and norisoprenoids. In the 2015 vintage, the concentration of nerol and geraniol, although lower than their odour threshold, was higher when the two *Metschnikowia* strains were used (Table S3), compared to that in the other wine samples (enzyme-treated and Control) as further proved by the OAV (Table 3). Some slight but significant differences were found also in the 8-hydroxy-linalool (*cis*) concentration, when the two *Metschnikowia* strains were compared to the Control wine. No significant difference was observed for these compounds compared to the enzyme-treated wine. In the same vintage (2015), the concentration of the C13-norisoprenoid 3-oxo-α-ionol was higher in the MP 346 and MF 98-3 wines (similarly to enzyme-treated wine) than in the Control wine.

Table 1. Effect of pre-fermentative cold maceration with *Metschnikowia* strains and with a pectic enzyme preparation on the population of *Saccharomyces cerevisiae* at the end of alcoholic fermentation and of malolactic fermentation for the 2015 vintage.

	Saccharomyces cerevisiae (CFU/mL)			
Phase	**MP 346 (tanks 1–2)**	**MF 98-3 (tanks 3–4)**	**Enzyme preparation (tanks 5–6)**	**Control (tanks 7–8)**
End AF	$3.94 \pm 0.48 \times 10^7$ a	$4.95 \pm 0.45 \times 10^7$ a	$5.53 \pm 1.45 \times 10^7$ a	$4.27 \pm 2.69 \times 10^7$ a
End MLF	$2.12 \pm 0.93 \times 10^5$ a	$2.01 \pm 1.49 \times 10^5$ a	$4.68 \pm 2.48 \times 10^4$ b	$6.35 \pm 1.10 \times 10^4$ b

Data are mean values of two tanks ± SD. Values with different letters are significantly different according to the Tukey's test (95%). AF, alcoholic fermentation; MLF, malolactic fermentation; MF, *Metschnikowia fructicola*; MP, *Metschnikowia pulcherrima*.

Table 2. Parameters obtained by fitting the altered Gompertz equation to the experimental data of sugar consumption in Sangiovese must during alcoholic fermentation, during the 2014 and 2015 vintages.

Thesis	MP 346	MF 98-3	Enzyme preparation	Control	Significance
Vintage 2014 (PCM 24 h)					
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 (PCM 72 h)					
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	

K, fermentation rate; M, half-time of sugar consumption; MP, *Metschnikowia pulcherrima*; MF, *Metschnikowia fructicola*; ns, not significant; PCM, pre-fermentative cold maceration.

Aldehydes and ketones. In both vintages, more aldehydes and ketones were detected in the wine in which PCM occurred using the enzyme preparation compared to the other wine samples (MP 346, MF 98-3 and Control). Moreover, the MF 98-3 sample showed the lowest concentration of benzaldehyde compared with that of the Control wine in both vintages, irrespective of PCM duration. In all wines, however, 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 AF.

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 the Sangiovese wines (Figures S2,S3). Nevertheless, the final concentration of some specific esters, such as isoamyl lactate and ethyl isoamyl succinate, was higher in MP 346 and MF 98-3 (similarly to enzyme-treated wine) than in the Control wine, in both 2014 and 2015 vintage. Previous research has shown that non-*Saccharomyces* species, in particular *M. pulcherrima,* are able to produce a relatively high concentration of several esters (Whitener et al. 2017).

By extending the maceration time to 72 h (2015 vintage), however, the pre-fermentative and fermentative metabolism, in the presence of non-*Saccharomyces* (MP 346 and MF 98-3), resulted in a higher concentration of isoamyl acetate (acetate ester), ethyl butanoate, ethyl hexanoate (ethyl esters) than in the Control wine. Among these ester compounds, only isoamyl acetate and ethyl hexanoate (which have a banana aroma) exceeded the corresponding threshold [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. As recently demonstrated (Pineau et al. 2009), however, 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. 2012)] and ethyl 3-methylbutyl succinate was higher in the presence of MF 98-3 than of the Control wine. Ethyl acetate may add aroma complexity at low concentration (below 80 mg/L); however, it is associated with negative sensory descriptors (solvent odour) at a concentration above 150 mg/L. In both vintages, MP 346 and MF 98-3 samples showed significantly lower concentration of ethyl acetate than that of the Control (Tables 3,S3). Wines produced with *Metschnikowia* strains had an appreciable decrease in OAV (about 24% in 2014 and 61% in 2015 vintage) compared to that of 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 PCM duration, wines treated with the pectic enzyme preparation showed the lowest concentration of ethyl acetate. Finally, for both vintages, less ethyl 4-hydroxybutanoate was found in the MP 346 and MF 98-3 samples compared to the pectic enzyme-treated wine and Control irrespective of the duration of PCM.

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 being similar between treatments, in both vintages, MP 346 and MF 98-3 samples resulted in lower concentration of 1-hexanol compared to that of the Control wine, although not exceeding the olfactory threshold [8000 μg/L (Ferreira et al. 2000)]. The higher alcohols (fusel alcohols) can contribute positively to the complexity of wine aroma, while they may have a negative effect at high concentration. Moreover, data showed that, among all samples, MF 98-3 wines had a 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 via fatty acid metabolism and is characterised 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 S3 shows that the MF 98-3 inoculum, compared to the Control and enzyme-treated wine, increased the concentration of isovaleric and homovanillic acid in both the 2014 (PCM 24 h) and the 2015 vintages (PCM 72 h), irrespective of the duration of PCM.

Phenols and lactones. Volatile phenols are generally present in wine at a concentration ranging from a few dozen to several hundred micrograms per litre. These

Table 3. Effect of pre-fermentative cold maceration with added *Metschnikowia* strains or a commercial pectic enzyme followed by sequential inoculation with *Saccharomyces cerevisiae* on the odour activity value and odour descriptor of key odorants (positive or negative) in Sangiovese wines.

			OAV Vintage 2014				OAV Vintage 2015			
Compounds	**Odour descriptor**	**Odour threshold (μg/L)**	**MP 346**	**MF 98-3**	**Enzyme preparation**	**Control**	**MP 346**	**MF 98-3**	**Enzyme preparation**	**Control**
Terpenes										
Nerol	Violets, floral	500†	0.0066	0.0072	0.0062	0.0074	0.0068	0.0056	0.0046	0.0040
Geraniol	Citric, geranium	20†	0.010	0.012	0.013	0.015	0.012	0.013	0.011	0.010
cis-8-hydroxy-linalool		Nf	—	—	—	—	—	—	—	—
C_{13}-Norisoprenoids										
3-Oxo-α-ionol	Nf	Nf	—	—	—	—	—	—	—	—
Aldehydes and ketones										
Benzaldehyde	Roasted, almond	2000†	0.005	0.004	0.004	0.006	0.004	0.003	0.003	0.004
Esters										
Ethyl acetate	Fruity, solvent	7500‡	9.6	9.2	6.1	12.4	11.4	13.9	8.5	32.6
Isoamyl acetate	Fruity, banana	30¶	17.6	12.5	20.5	21.4	11.3	9.9	9.4	8.7
Ethyl butanoate	Banana, pineapple strawberry	400†	0.4	0.3	0.4	0.4	0.3	0.3	0.3	0.2
Ethyl hexanoate	Banana, green apple	14¶	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††	0.95	0.94	0.84	0.92	0.46	0.47	0.44	0.37
Alcohols										
1-Hexanol	Herbaceous, woody	8000¶	0.10	0.10	0.10	0.17	0.06	0.06	0.09	0.09
Methionol	Cooked vegetable	1000¶	0.86	0.68	0.76	0.71	0.67	0.58	0.64	0.69
Acids										
Isovaleric acid	Acid, rancid	3000†	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§	0.0043	0.0021	0.0016	0.0016	0.0047	0.0053	0.0072	0.0043
4-Ethylguaiacol	Phenolic	33¶	0.030	0.036	0.030	0.033	0.130	0.139	0.100	0.097

Odour descriptor and odour threshold of the main aroma compounds, were indicated according to the following references: †Cai et al. (2014); ‡Peinado et al. (2006); §Lopez et al. (2002); ¶Ferreira et al. (2000); ††Zhang et al. (2016). MF, *Metschnikowia fructicola*; MP, *Metschnikowia pulcherrima*; Nf, not found: odour descriptor or odour threshold is not available in the literature; OAV, odour activity value; –, odour threshold not available in the literature and therefore OAV could not be calculated.

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 treatments. Wines inoculated with the two *Metschnikowia* strains, similarly to enzyme-treated wine, showed a lower concentration of total phenols in both vintages, irrespective of the duration of PCM. In all cases, the concentration of 4-ethylphenol and 4-ethylguaiacol was well below their perception threshold [440 μg/L (Lopez et al. 2002) and 33 μg/L (Ferreira et al. 2000), respectively] in both vintages (Table 3).

The odour of lactones is usually described as buttery, fruity and coconut-like; three lactones were also identified in this study; their concentration, however, provided no evidence of the impact of either non-*Saccharomyces* strain.

Conclusions

Overall, both non-*Saccharomyces* strains (MP 346 and MF 98-3), inoculated during PCM, had a significant effect on the initial yeast population dynamics and on the persistence of *S. cerevisiae* at the end of the MLF. Irrespective of the duration of PCM, the inoculum of *Metschnikowia* strains did not significantly affect or interfere with the rate of sugar consumption by the *S. cerevisiae* strain used, or the kinetics of MLF induced at the End AF. The non-*Saccharomyces* yeasts (MP 346 and MF 98-3), in both vintages, did not have a clear, defined effect on the total concentration of the main classes of aroma compounds. Nevertheless, the final concentration of some specific terpenes and C13-norisoprenoids [such as nerol, geraniol, 8-hydroxy-linalool (*cis*) and 3-oxo-α-ionol] was higher in tanks inoculated with the two *Metschnikowia* strains than in the Control and enzyme-treated wine, when a longer cold-maceration was performed (72 h, 2015 vintage). Moreover, a higher concentration of some specific esters (isoamyl lactate and ethyl isoamyl succinate in both vintages) was revealed in the presence of *Metschnikowia* strains compared to that of 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 as the cold-maceration time was extended.

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 affected differently by the two non-*Saccharomyces* strains. Such 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 specific non-*Saccharomyces* strain.

For the first time, this study shows that an inoculum of non-*Saccharomyces* yeasts (MP 346 and MF 98-3) during PCM is an effective winemaking practice, since it impacted both *Metschnikowia* population dynamics during the maceration time and the volatile composition of the wine. Further studies should be carried out to assess the effectiveness of non-*Saccharomyces* yeasts in improving wine colour stability and phenolic composition.

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Supporting information

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Figure S1. Effect of malolactic fermentation by the strain *Oenococcus oeni* PN4 MBR during the (a) 2014 vintage [24 h pre-fermentative cold maceration (PCM)] and during the (b) 2015 vintage (PCM 72 h) on the concentration of malic (●, ▲, ■, □) and lactic acid (●, ▲, ■, □) in Sangiovese wines. The wines were produced by adding *Metschnikowia* strains MP 346 (●, ●) and MF 98-3 (▲, ▲) or a commercial pectic enzyme (■, ■) during PCM followed by sequential inoculation with *Saccharomyces cerevisiae*. The Control wine was produced with the pure culture of *S. cerevisiae* (□, □).

Figure S2. Biplot of the principal components analysis (PC 1 vs PC 2), for 2014 vintage, of volatile compounds in Sangiovese wines produced by adding *Metschnikowia* strains (MP 346 or MF 98-3) or a commercial pectic enzyme preparation during pre-fermentative cold maceration followed by sequential inoculation with *Saccharomyces cerevisiae*, compared with the same wine produced by the pure culture of *S. cerevisiae* (Control).

Figure S3. Biplot of the principal components analysis (PC 1 vs PC 2), for 2015 vintage, of volatile compounds in Sangiovese wines produced by adding *Metschnikowia* strains (MP 346 or MF 98-3) or a commercial pectic enzyme preparation during pre-fermentative cold maceration followed by sequential inoculation with *Saccharomyces cerevisiae*, compared with the same wine produced by the pure culture of *S. cerevisiae* (Control).

Table S1. Ratio between *Metschnikowia* and total yeast population in tanks inoculated with two *Metschnikowia* strains during the 2015 vintage.

Table S2. Effect of pre-fermentative cold maceration with added *Metschnikowia* strains or a commercial pectic enzyme followed by sequential inoculation with *Saccharomyces cerevisiae* on the composition of Sangiovese wines.

Table S3. Effect of pre-fermentative cold maceration with added *Metschnikowia* strains or a commercial pectic enzyme preparation followed by sequential inoculation with *Saccharomyces cerevisiae* on the concentration of volatile compounds in Sangiovese wines.