Volatile profile of white wines fermented with sequential inoculation of *Starmerella bacillaris* and *Saccharomyces cerevisiae*

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ABSTRACT

The current study was carried out to elucidate the effect of sequential inoculation of *Starmerella bacillaris* and *Saccharomyces cerevisiae* on the production of white wines, in terms of both chemical and aromatic characteristics. Chardonnay, Muscat, Riesling and Sauvignon blanc wines were produced by sequential inoculation of the two yeasts and the single inoculation of *S. cerevisiae* was carried out as control. Analysis form chemical composition showed that titratable acidity and glycerol content exhibited evident differences among the wines after fermentation. For volatile compounds, mixed fermentations led to a reduction of the total ester, including ethyl acetate, which is a compound responsible for wine deterioration. Moreover, Sauvignon blanc wines fermented by mixed cultures contained significantly higher levels of esters and thiols, both associated with positive sensory attributes. These findings suggest that sequential inoculations posed a great potential in affecting and modulating the chemical and aromatic profiles of white wines, especially those produced from Sauvignon blanc grapes.

Keywords: non-*Saccharomyces*, *Starmerella bacillaris*, sequential inoculation, white grape varieties, aroma profile

1. Introduction

Aroma is an important aspect of grape and wine quality, since it has a substantial influence on consumer acceptance (Sáenz-Navajas, Ballester, Fernández-Zurbano, Ferreira, Peyron & Valentin, 2016). Several aroma families construct the volatile composition of wines, among them alcohols are known to contribute to herbaceous characters, esters and terpenes to fruity and floral characters, C_{13}-norisoprenoids to balsamic and violet aromas (Dzialo, Park, Steensels, Lievens & Verstrepen, 2017; Swiegers, Bartowsky, Henschke & Pretorius, 2005). Meanwhile, thiols generally contribute to blackcurrant, passion fruit and citrus zest descriptors (Francis & Newton, 2005). Many of these metabolic compounds are produced from non-volatile precursors through complex metabolic reactions, which begin during grape ripening and continue throughout fermentation, ageing and bottling (Swiegers et al., 2005).

During fermentation the yeasts, through their central glycolytic pathway, transform the sweet and low aroma must into an alcoholic, high aroma beverage. In this process, each glucose and fructose molecule is split and converted to ethanol, carbon dioxide and plenty of volatile metabolites that contribute individually or synergistically to wine composition and sensory profile, in order to provide energy necessary for cell growth maintenance and reproduction (Belda et al., 2017; Fleet, 2008; Molina, Guadalupe, Varela, Swiegers, Pretorius & Agostin, 2009). In addition to this, many volatile metabolites are also released from non-volatile grape derived precursors by yeast enzymes (Swiegers et al., 2005). Examples are monoterpenes and C_{13}-norisoprenoids, which are released from glycosidic precursors, and long-chain polyfunctional thiols, which are derived from S-cysteinylated conjugates. The production of these metabolites is strictly correlated with the fermentation conditions which the yeasts strain(s) is subjected to, that is: strain compatibility, physicochemical and nutrition parameters (Belda et al., 2017).

Grapes and winery equipment contain a large variety of indigenous yeasts, that are involved in spontaneously fermented wines (Bokulich, Ohta, Richardson & Mills, 2013). Allowing the must to ferment with indigenous yeasts can potentially increase the complexity of wine aromas due to the diversity of yeast species and strains, which are present (Belda et al., 2017). However, the lack of reproducibility and predictability on these fermentations has favoured the use of yeast starters, generally strains of *Saccharomyces cerevisiae*, with several phenotypes (Fleet, 2008). In addition to the choice of *S. cerevisiae* strain, the use of mixed starter cultures with selected non-*Saccharomyces* and *S. cerevisiae* yeasts can result in greater complexity and diversity of volatile metabolites in ways not reachable with pure starter cultures of *S. cerevisiae*, by simulating a spontaneous fermentation (Belda et al., 2017; Sadoudi et al., 2012).

Among non-*Saccharomyces* yeasts, *Starmerella bacillaris* (synonym *Candida zemplinina*) can tolerate relatively high concentrations of ethanol and persist until the middle-end stages of fermentation, making them more suitable for mixed fermentations (Englezos, Giacosa, Rantsiou, Rolle & Cocolin, 2017). Recent studies have revealed several potentially useful winemaking attributes, including high glycerol and low ethanol production, preference towards fructose rather than glucose, ability to tolerate relative high concentrations of ethanol, while acetic acid and acetaldehyde production is highly variable among strains (Englezos et al., 2018, Rantsiou et al., 2017). These phenotypic characteristics make this non-*Saccharomyces* species an optimum candidate to accompany *S. cerevisiae* in mixed fermentations (Mestre, Maturano, Combina, Mercado, Toro & Vasquez, 2017). In the last decade, many studies have focused on mixed fermentations with *Starm. bacillaris* and *S. cerevisiae* to ferment grape must and have made noticeable progress in many aspects, including the importance of strain selection, inoculation density and delay on the chemical profile of the wines (Englezos et al., 2017). However, several efforts must be performed in order to establish

a link between an inoculation protocol and chemical composition of wines using the same couple of strains and fermentation conditions.

Hence, the present study sought to investigate the effect of mixed fermentations with *Starm. bacillaris* and *S. cerevisiae* on the aroma profile of some monovarietal white wines. To this end, four of the world's most planted white wine grape varieties, namely Chardonnay, Muscat, Riesling and Sauvignon blanc, were fermented with *Starm. bacillaris* FC54 and *S. cerevisiae* Uvaferm BC® using an inoculation delay of 48 hours. Control fermentations with *S. cerevisiae* Uvaferm BC® were performed in parallel. The aroma profile of the resultant wines was determined by Head Space-Solid Phase Micro Extraction (HS-SPME) combined with Gas Chromatography-Mass Spectrometry (GC-MS).

2. Materials and methods

2.1. Strains

The yeast strains for this experiment were the commercial *S. cerevisiae* Uvaferm BC® and *Starm. bacillaris* FC54 obtained from Lallemand Inc. (Montreal, Canada) and the yeast culture collection of DISAFA (Department of Agricultural, Forest and Food Sciences, University of Turin, Italy), respectively. These strains were selected for their enological attributes in mixed fermentations in grape must at the laboratory and pilot scale (Englezos et al., 2016a).

2.2. Must preparation

Four white wine grape varieties (*Vitis vinifera* L.) cultivars, namely Chardonnay, Muscat, Riesling and Sauvignon blanc were harvested at technological ripening from the experimental vineyard of the University of Turin at Grinzane Cavour (Cuneo, Piedmont, NW Italy). After harvesting, the grapes were destemmed, crushed and the juice obtained without the skins was sterilized by adding 200 mg/L dimethyl dicarbonate from Sigma (Milan, Italy) as previously described by Delfini, Gaia, Schellino, Strano, Pagliara & Ambrò (2002). The absence of culturable yeast population in the musts prior to inoculation was checked by plating an aliquot of the must on Wallerstein laboratory nutrient (WLN) medium (Biogenetics, Milan, Italy). The sanitization protocol was deemed successful, since no colonies were formed on the medium after 3-5 days of incubation at 28 °C. Grape musts were standardized for providing a unified starting point of sugars and YAN (Yeast Assimilable Nitrogen) for the fermentations. To this end the musts were standardized to 245 ± 5 g/L of sugar and 180 ± 5 mg/L of YAN using the commercial product Fermaid O® from Lallemand Inc., in order to ensure complete sugar fermentation. The chemical composition of the musts is reported in Table 1.

2.3. Fermentation trials

The four musts were each divided into six samples comprising three replicates of each of two types of inoculation protocols, a. inoculation with *S. cerevisiae* Uvaferm BC® (pure culture fermentation), b. initial inoculation with *Starm. bacillaris* FC54 followed by *S. cerevisiae* Uvaferm BC® after 48 hours of fermentation (mixed, sequential inoculation). Twenty-four fermentations (4 grape varieties x 2 inoculation protocols x 3 replicates = 24) in total were performed under semi-anaerobic conditions in 1 L sterile glass bottles containing 800 mL of must. Each yeast strain was inoculated with 5.0 x 10^6 cells/mL, which corresponds to a dose of 25 g/hL of ADY (Active Dry Yeast) (Lallemand SAS, Toulouse, France),

previously activated in a sterile glucose solution (5 %), incubated at 37 °C. Fermentors were fitted with air-lock to ensure semi-anaerobic conditions, after all the oxygen in the headspace is consumed and kept at 20 °C without shaking. Fermentations were considered finished when the sum of glucose and fructose was less than 2 g/L. At the end of fermentation, samples were taken from each fermentor for analysis of the volatile fermentation compounds.

2.4. Microbiological analysis

The growth dynamics of the two yeasts during fermentation were monitored by plate counts. Aliquots of 1 mL were taken from each must at days 0, 2, 4, 7, 9 and 14 (only for the mixed culture fermentation), diluted in sterile Ringer's solution (Oxoid, Milan, Italy) and plated on WLN medium. Enumeration of the yeast colonies was performed after 3-5 days of incubation at 28 °C and the differentiation of the two species was carried out visually as previously described by Englezos et al. (2018) and subsequently counted. In this medium, *Starm. bacillaris* forms light to intense green with white border, whereas *S. cerevisiae* forms creamy white to light green colonies enabling the concurrent enumeration of both species.

2.5. Chemical analysis

Extracellular metabolites concentration such as sugars (glucose and fructose), glycerol, organic acids (citric, tartaric, succinic, malic, lactic and acetic acid) (g/L) and ethanol (% v/v) were quantified during (0, 2, 4, 7, 9 and 14 days) and at the end of fermentation were quantified by an Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA, USA) using a UV detector (UV100) at 210 nm and a refractive index detector (RI-150). Analyses were performed isocratically at 0.8 mL min^{-1} flow-rate and at 65 ^{o}C column temperature with a 300 mm x 7.8

mm i.d cation exchange column (Aminex HPX-87H) and a Cation H^+ Microguard cartridge (Bio-Rad Laboratories, Hercules, CA, USA). The mobile phase was 0.0065 mol L^{-1} H_2SO_4 (Rolle et al., 2017). At the end of fermentation, total acidity (expressed as g/L of tartaric acid) was determined according to the official method proposed by the International Organization of Vine and Wine (OIV, 2008), while pH was registered using an InoLab 730 pH meter (WTW, Weilheim, DE). Total YAN concentration was determined spectrophotometrically by using two enzymatic kits (catalog codes: K-Large and K-PANOPA, Megazyme International, Wicklow, Ireland).

2.6. Volatile profile

Volatile compounds formed through yeast metabolism in pure and mixed culture fermentations were extracted and determined by Head Space – Solid Phase Micro Extraction (HS-SPME) coupled by Gas Chromatograpghy – Mass Spectroscopy (GC-MS). The chromatographic and MS conditions were previously described by Sánchez-Palomo, Diaz-Maroto and Perez-Coello (2005) and slightly modified by Rolle et al. (2015, 2017). For each sample, a 5 mL aliquot was transferred to a 20 mL glass headspace vial with a headspace screw cap, containing 5 mL of water, 2 g of sodium chloride and 1-heptanol solution (200 µL of 15.52 mg/L solution in 10 % v/v ethanol) as internal standard (IS). The vials were sealed with 18 mm diameter silicon septa caps (Supelco, Bellefonte, PA, USA) and carefully shaken to dissolve sodium chloride before the analysis. A 50/30 µm DVB/CAR/PDMS fibre from Supelco was used to extract the volatile compounds, using a Gerstel MPS2 XL auto sampler (Gerstel, Baltimore, MD, USA). The fibre was exposed to the headspace of each vial for 20 min at 40ºC and inserted into the injection port of the GC apparatus for the thermal desorption. Injections were carried out in splitless mode at 250ºC for 5 min, during which the desorption of analytes

from the fibre was occurred.

Analyses were carried out using an Agilent 7890C gas chromatograph (Little Falls, DE, USA) associated with an Agilent 5975 mass selective detector and DB-WAXETR capillary column (30 m x 0.25 mm, 0.25 µm, J&W Scientific Inc., Folsom, CA, USA). Helium was used as a carrier gas with a flow rate of 1 mL/min. the software used was Agilent G1701-90057 MSD ChemStation. Chromatographic conditions are as follows: 5 min at 40ºC and increased at a rate of 2ºC/min to 200ºC for 10 min and 5ºC/min to 200ºC. The oven was the held at this temperature for 5 min before returning to the initial temperature.. The injection port temperature was 250ºC, the ion source temperature was 150ºC and the interface temperature was 280ºC. The detection was carried out by electron impact mass spectroscopy in total ion current (TIC) mode, using an ionisation energy of 70 eV. The mass acquisition range was between m/z 30-330. Volatile compounds were identified according to retention indices and mass spectra of pure standards and the NIST database (http://webbook.nist.gov/chemistry/). The pure standard used are reported by Englezos et al. (2016b). Quantitative determination was performed using 1-heptanol as internal standard and calibration with pure standard previously reported and data expressed as µg/L. The thiols analysis in the wines produced from Sauvignon blanc grapes was performed using the method reported by Piano et al. (2015) and Fracassetti et al. (2018). Data are the average of the duplicate sample preparation for each fermentor (n=6) and are expressed as ng/L.

2.7. Statistical analyses

The data obtained were subjected to statistical analysis using IBM SPSS Statistics software package (version 19.0, IBM Corp., Armonk, NY, USA). Significant differences between samples were established using one-way Analysis of Variance (ANOVA). When

statistical differences were found, a Tukey-b post hoc test comparison was performed using $p<0.05$ as the threshold significance.

3. Results and discussion

3.1. Yeast growth during fermentation

The yeast growth dynamics during pure and mixed fermentations were followed by plate counts and the results are illustrated in Fig. 1. In pure culture fermentations, *S. cerevisiae* Uvaferm BC® reached the maximum population (about 5.0-8.0 x 10^7 colony forming units [cfu]/mL) in two days. The viable population then remained stable until the end of the fermentation (9 days). In sequential fermentations, *Starm. bacillaris* FC54 reached the highest cell population on day 4 (5.0-7.0 x 10^7 cfu/mL). Its population became undetectable in sequential inoculations on day 14, while *S. cerevisiae* population remained at levels from 10^6 cfu/mL in Sauvignon blanc to 10^7 cfu/mL in Muscat wines. *Starm. bacillaris* impacted *S. cerevisiae* population in sequential inoculations. More specifically, *S. cerevisiae* was slightly lower (range 0.1 to 0.2 Log cfu/mL, data not shown) in comparison to pure culture *S. cerevisiae* fermentations, after similar periods of post-inoculation.

3.2. Chemical parameters

The extracellular metabolites concentrations, for the fermented wines from each grape variety and inoculation protocol, are shown in Table 1. While both glucose and fructose were almost consumed (< 2.0 g/L) at the end of fermentation, the strong fructophilic character of *Starm. bacillaris* compared to *S. cerevisiae* was confirmed on the first 48 hours of fermentation, in agreement with previous studies (Englezos et al., 2017, 2018; Rantsiou et al., 2017). As it

252 can be seen in Fig. 2 (right panel) and Supplementary Table 1, *Starm. bacillaris* consumed on
253 average more fructose and left glucose mostly untouched during this period. Sequential
254 fermentations started significantly slower as within the first 48 hours only 9.0 g/L of sugars
255 (mainly fructose) were consumed on average, representing 3% of the total sugars. At the same
256 time point, pure fermentations with *S. cerevisiae* consumed on average 81.0 g/L of sugars,
257 representing 34% of total sugars. Sugar consumption rate had a steep increase when *S.*
258 *cerevisiae* was inoculated in mixed fermentations, and continued until day 7 after which rate
259 of sugar consumption slowed and stopped on day 14 in sequential fermentations. On the other
260 hand, sugar consumption rate decreased on day 4 and stopped on day 9 in pure fermentations.
261 The length of the sequential fermentations is in line with Englezos et al. (2016a) who reported
262 a fermentation time three-days longer when sequential fermentations are compared to pure
263 fermentations with the same *S. cerevisiae* strain Uvaferm BC®.

264 Ethanol production in the sequential fermented wines was slightly lower (0.1 to 0.2 %
265 v/v) compared to pure fermented wines, independently of the grape variety used (Table 1).
266 These differences are lower than observed in a previous work (0.5 % v/v) with the same couple
267 of strains and inoculation delay using red Barbera grape must, compared to pure fermented
268 wines with *S. cerevisiae* (Englezos et al. 2016a). The lower fermentation temperature compared
269 to the previous study (20 °C vs. 25 °C), could explain the low sugar consumption by *Starm.*
270 *bacillaris* in the first 48 hours of fermentation and as a consequence the low ethanol reduction
271 in this work.

272 While the ethanol content of the wines was lower in mixed fermentations, the glycerol
273 content was significant higher for all grape variety used in this study, confirming previous
274 observations (Englezos et al., 2016ab; Englezos et al., 2018; Rolle et al., 2017). Glycerol
275 production in the mixed fermented wines ranged from 9.3 to 10.3 g/L compared to pure
276 fermented wines that ranged from 7.8 to 8.4 g/L. This increase in glycerol was also reported in

277 previous studies but in higher levels (more than 4.0 > g/L) (Englezos et al., 2016a). The
278 glycerol yield was between 0.038 - 0.042 for mixed fermented wines and between 0.032 –
279 0.035 for the control wines.

280 Titratable acidity (expressed as g/L of tartaric acid) was in average significantly higher
281 in sequential fermented wines (7.1 g/L) compared to pure fermented wines (6.3 g/L). This
282 increase is in line with Sadoudi et al. (2012) and Englezos et al. (2016a) who also reported that
283 24 and 48 hours inoculation delay resulted in higher titratable acidity (0.16 – 0.50 g/L)
284 compared to pure fermented wines respectively, resulting in a decrease of pH. However, the
285 increase of 0.6 – 0.8 g/L observed in this study could not be explained by the primary organic
286 acids (citric, tartaric, succinic, malic and lactic acid) monitored in study (Supplementary Table
287 2), suggesting that other acids (such as α-ketoglutaric and pyruvic) are most probably
288 responsible for this increase (van Dijken & Scheffers, 1986). Magyar, Nyitrai-Sárdy, Leskó,
289 Pomázi & Kállay (2014) reported a significantly higher accumulation of pyruvic acid by *Starm.*
290 *bacillaris* compared to *S. cerevisiae* in pure culture fermentation using synthetic medium.
291 Conversely, pure starter culture fermentations lead to a higher average decrease of malic acid
292 than mixed starter culture fermentations. *S. cerevisiae* in pure fermentations consumed on
293 average 0.7 g/L of malic acid, representing a 36% reduction, while in sequential inoculations
294 the decrease was on average 0.5 g/L representing a 28% reduction. Rantsiou et al. (2017),
295 reported that pure culture fermentations with *Starm. bacillaris* consumed malic acid on a level
296 of 40% in red Barbera cv. musts with differing sugar levels (200-330 g/L), which was in line
297 with earlier research by Tofalo et al (2012), using a red must with 220 g/L of residual sugars.
298
299 *3.3 Volatile composition*
300

Identification and quantification of the volatile metabolites was carried out in order to determine the effect of the inoculation protocol on white wines aroma. As shown in Table 2, a total of 38 volatile compounds were identified and subsequently divided into 4 volatile families, including 7 alcohols, 19 esters, 2 fatty acids, 8 terpenes and C_{13}-norisoprenoids. The total aroma volatile composition exhibited significant differences between pure and mixed culture fermentations, highlighting a metabolic interaction between the two species. In particular, significant lower levels of volatile compounds were registered for the mixed compared to pure fermented wines.

3.3.1 Higher alcohols

Higher alcohols, known as fusel alcohols, constitute the largest group of volatile metabolites, synthesized by yeast during alcoholic fermentation (Ugliano & Henschke, 2009). Both pure and mixed fermentations, independently of the grape variety used, produced the same levels of alcohols, at concentrations ranging from 9.9 mg/L to 14.8 mg/L, well below the level of 300 mg/L which enhance the complexity in the wines (Rapp & Versini, 1991). The only exception was Sauvignon blanc wines, in which the involvement of *Starm. bacillaris* in the fermentation process increased significantly the levels of this group of metabolites (11.8 μg/L vs 10.7 μg/L). The total concentration of the alcohols in the wines was strongly associated with the concentration of isoamylic alcohol and 2-phenyl ethanol, which constituted up to 91% of total alcohols. However, none of them surpassed their perception threshold (Cullere, Escudero, Cacho & Ferrerira, 2004; Ferreira, Lopez & Cacho, 2000; Guth, 1997; Li, 2006).

Isoamylic alcohol (3-methyl-1-butanol), which is produced during fermentation through deamination and decarboxylation reactions from isoleucine (Molina et al., 2009), could negatively contribute to wine quality due to the herbaceous aroma. Chardonnay and Muscat

wines produced using pure starter cultures contained significant higher levels of this metabolite, however in concentrations well below its perception threshold. To the contrary, no differences were observed for Riesling and Sauvignon blanc wines. 2-phenylethanol, which is synthesized via Ehrlich pathway through metabolic reactions that involves transamination of the amino acid L-phenylalanine, could contribute to the wine with a pleasant rose-like odour (Swiegers et al., 2005). Riesling and Sauvignon blanc wines produced from mixed starter cultures were distinguished, from the respective wines fermented exclusively with *S. cerevisiae*, by a significant higher amount of this metabolite. Therefore, the increased concentration of 2-phenylethanol would potentially increase the floral aroma in these wines.

2-Methyl-1-propanol (also known as isobutanol) is synthesized in the yeast cell through the valine degradation pathway and contributes to herbaceous notes in the wines (Ugliano & Henschke, 2009). Chardonnay and Muscat wines produced from pure *S. cerevisiae* fermentations contained significant higher levels of this metabolite. Conversely, Riesling and Sauvignon blanc wines fermented with pure cultures, contained significantly lower levels of this metabolite, suggesting that valine concentration rather than inoculation strategy affects its production. Moreover, negligible differences were found in wines produced using mixed cultures independently of the grape variety used. Hexanol, usually has a negative influence on wine aroma, by imparting a vegetable and herbaceous odour, when the concentration exceeds 100 mg/L (Satora & Tuszynski, 2010). This metabolite, was present in significant higher levels in mixed starter culture fermented wines, independently of the grape variety used, but still significantly lower than its olfactory detection threshold.

3.3.2 Esters

Fermentation derived esters are responsible for the fruity character of the wines (Ugliano & Henschke, 2009). In general, mixed fermentations produced Chardonnay and

Muscat wines with significant lower levels of esters, compared to pure fermented wines. To the contrary, a completely different picture was captured in Sauvignon blanc wines, in fact mixed starter cultures produced higher levels of this aroma family. No significant differences were found for Riesling wines, in the amount of total esters produced, between the pure and mixed fermented wines. Among the identified esters, ethyl esters deriving from medium chain fatty acids and responsible for the fruity character of the wines were the most representative aroma family in all the wines produced, accounting for 72 % and 85 % of total esters in the pure and mixed fermentations, respectively. Ethyl octanoate and ethyl decanoate associated with pleasant notes “pineapple”, “pear”, and “floral” were the most abundant ethyl esters and significant differences were registered between pure and mixed fermented wines, independently of the grape variety used. Significant lower levels were found in mixed fermented wines. To the contrary, Sauvignon blanc wines fermented by mixed cultures were characterized by significant higher content of these two compounds. The higher level of ethyl decanoate in this wine is in line with previous findings (Sadoudi et al., 2012) in sequential inoculated Sauvignon blanc with 24 h inoculation delay, while ethyl hexanoate was not affected by the inoculation protocol used in both studies. Concerning the level of ethyl octanoate in the wines, the results of the present study are in agreement with those of Sadoudi et al. (2012) who observed a lower level of this compound in pure fermented Sauvignon blanc wines with *S. cerevisiae*. Conversely, Chardonnay, Muscat and Riesling wines fermented with pure *S. cerevisiae* cultures contained significant higher levels of this metabolite, indicating that strain selection and grape variety can modulate its production. Ethyl dodecanoate (pear, fruity, floral) was found in significant higher levels in pure culture fermented wines compared to mixed culture fermented wines. On the other hand, Sauvignon blanc wines fermented with mixed cultures contained significant higher levels of this metabolite compared to the respective

375 control wine suggesting that grape variety rather than inoculation protocol modulate its
376 production.

377 The second group of esters, called acetate esters, are those formed from acetic acid and
378 higher alcohols, and are considered to have a greater effect on the perceived aroma than the
379 ethyl esters (Ugliano & Henschke, 2009). In the current study, the acetate esters identified were
380 ethyl acetate, hexyl acetate, octyl acetate, 2-phenyl-ethyl-acetate, and 3-methyl-1-butanol
381 acetate. All wines inoculated with mixed cultures presented significant lower content of this
382 aroma family. Among the quantified acetate esters two compounds (2-phenyl-ethyl-acetate and
383 3-methyl-1-butanol acetate) associated with the positive attributes, "rose", "honey" and
384 "banana" presented values above the threshold value in all the wines studied, consequently
385 they are expected to have an influence on the aroma of the wines. Both compounds were found
386 to be significantly higher in pure fermented wines independently of the grape variety used. A
387 significant difference in hexyl acetate, a metabolite with pleasant fruity note was observed. The
388 amount of this metabolite was above the threshold in the control wine however below in mixed
389 starter culture fermented wine, the former having 3-14 times the amount compared to the latter
390 for all the varieties investigated. Similar behaviour was found for 3-methyl-1-butanol acetate.
391 This reduction was more evident in Chardonnay and Muscat wines, suggesting that the grape
392 variety may have an influence on the production of these esters.

393 Ethyl acetate and 2-phenyl-ethyl-acetate are the most common esters found in wine.
394 Contrary to 2-phenyl-ethyl-acetate, ethyl acetate is known to have an unpleasant nail polish,
395 vinegar aroma at concentrations above 150 mg/L (Corison, Ough, Berg & Nelson, 1979). At
396 concentrations below this limit, this metabolite contributes positively to white wine quality,
397 with pleasant descriptors such as, pineapple and apple. For both Chardonnay and Muscat wines
398 fermented by pure cultures, the content of ethyl acetate was above the odour threshold, while
399 it was lower than the perception threshold in sequential inoculation wines. The difference

between pure and mixed fermentation was statistically significant for these varieties. Ethyl acetate was not above the threshold in any of the fermentations of Riesling and Sauvignon blanc. Generally, wines produced with *Starm. bacillaris*, showed a reduction in ethyl acetate, hexyl acetate and 2-phenyl-ethyl acetate when compared with pure culture fermented wines.

3.3.4 Fatty acids

Two fatty acids, decanoic and octanoic acid were identified across the pure and mixed fermented wines (Table 2). Both are medium-chain fatty acids (C_6 – C_{10}), which can impart a butter-like, cheesy aroma (Francis & Newton, 2005), however, they impact negatively wine quality only when their concentration exceeds 20 mg/L (Ribéreau-Gayon, Dubourdieu, Donèche & Lonvaud, 2006). Wines produced from pure *S. cerevisiae* culture contained significant higher levels of these metabolites independently of the grape variety used. In these wines, the decanoic acid ranged from 578 to 616 μg/L and the octanoic acid concentration from 787 to 1108 μg/L. Even though these volatile fatty acids are well below the concentration at which become unpleasant, octanoic acid concentration in pure starter fermentations was present at levels above its perception threshold, which is 500 μg/L. In small quantities, volatile fatty acids contribute to the aromatic equilibrium of wine, since they counteract the hydrolysis of their esters (Ugliano & Henschke, 2009).

3.3.3 Terpenes and C_{13}-norisoprenoids

Terpenes are a kind of aroma family responsible for the characteristic floral and fruity aromas of Muscat and Riesling wines. Generally, they are present in grape berries in free or bound form and synthesized from glucose via the isoprenoid pathway (Mateo & Jimenez,

2000). The terpenes compounds with high odour activity are linalool, geraniol and nerol. Geraniol has aromas described as rose-like and linalool aromas described as floral-like (Swiegers et al., 2005), whereas oxidized geraniol and linalool are described as vegetative and camphorous, respectively. The concentration of monoterpene linalool in mixed fermented Muscat wines, was almost 21 times above the odour threshold, however significantly lower (514 μg/L) than in the control wine (647 μg/L). This result suggests that the interaction between the two yeast species has a negative influence in the expression of the varietal character of the wines. This result is in line with those reported by Sadoudi and and co-workers (2012), where a negative interaction was registered between *Starm. bacillaris* and *S. cerevisiae* resulting in a decrease in terpenes content compared to pure fermentations with *S. cerevisiae*. Similarly, linalool and terpenes concentration in Riesling was above the odour threshold in both inoculation protocols investigated, however no significant differences were found between the two protocols.

3.3.4 Thiols

Volatile thiols, such as hydrogen sulphide (H_2S), ethanenthiol and methanenthiol are responsible for wine defects, however, certain volatile thiols are considered important aroma constituents of Sauvignon blanc wines and other white, rosé and red wines elaborated with different grape varieties (Roland, Schneider, Razungles & Cavelier, 2011). Among these metabolites, 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH) and its acetate 3-mercaptohexyl acetate (3MHA) contribute positively to the fruity character of young wines with pleasant notes of box tree, grape fruit and exotic fruit aroma, respectively (Rolland et al., 2011; Tominaga, Furrer, Henry & Dubourdieu, 1988). These metabolites are present in grape as non-volatile cysteine or glutathione conjugated precursors and they are released during

the fermentation by yeast through their beta-lyase activity (Murat, Masneuf, Darriet, Lavigne, Tominaga & Dubourdieu, 2001). The two inoculation protocols used in this study affected the release of 3MH, while 3MHA was not detected in the samples. Wines fermented using the sequential inoculation protocol showed a significant higher concentration (269 ng/L) of 3MH compared to the control wine (198 ng/L), well above the 60 ng/L perception threshold. This liberation of higher levels of volatile thiols in mixed fermentations could be explained by the beta-lyase activity that favour the cleavage of the conjugated thiols, probably due to involvement of *Starm. bacillaris* in the fermentation process (Swiegers & Pretorius, 2007). Anfang, Brajkovich & Goddard (2009) also reported a significant increase in 3MH in Sauvignon blanc wines, co-fermented with *Starm. bacillaris* and *S. cerevisiae* in a ratio of 9:1, compared to pure fermented wines with *S. cerevisiae*. Conversely, co-inoculation at a ratio 1:9 that favour *S. cerevisiae*, produced wines with similar 3MH content. According to Sadoudi et al. (2012), inoculation of *S. cerevisiae* 24 hours after *Starm. bacillaris* inoculation led to the production of wines, with significant lower levels of this metabolite, compared to the respective control wine. Thus differences in 3MH profile depend on the initial inoculation ratio and the resulting population dynamics, demonstrating that yeast-interactions are strain-dependent.

5. Conclusion

The current study examined the effect of mixed fermentations with *Starm. bacillaris* and *S. cerevisiae* on the production of white wines using four different white grape varieties. Results obtained from chemical composition showed that the level of glycerol and titratable acidity varied significantly among wines after fermentation. For the volatile components determined, inoculation protocol influenced the aroma profile of the wines in a variety-dependent manner, since only the wines produced from Sauvignon blanc grapes contained significant higher levels of esters and alcohols compared to pure fermented wines. Since all the

476 data presented here are obtained from one couple of strains, more investigations are necessary
477 to access the impact of strain selection on wine composition.
478
479 **Conflict of interest**
480 The authors state no conflict of interest.
481
482

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610 **Figure captions**

611

612 **Fig.1**

613 Growth dynamics of yeasts during pure (left panel) and mixed culture fermentations (right
614 panel) using white grape musts: Chardonnay (A, B), Muscat, (C, D), Riesling (E, F) and
615 Sauvignon blanc (G, H). *Starm. bacillaris* strain FC54 (black circle) and *S. cerevisiae* Uvaferm
616 BC® (white circle). The arrow indicates the *S. cerevisiae* inoculation. Counts are the mean
617 CFU/mL values ± standard deviations of three independent experiments.

618

619 **Fig.2**

620 Evolution of metabolites during pure (left panel) and mixed culture fermentations (right panel)
621 using white grape musts: Chardonnay (A, B), Muscat, (C, D), Riesling (E, F) and Sauvignon
622 blanc (G, H). Glucose (white circle) fructose (black circle), ethanol (white diamond) and
623 glycerol (black diamond). Data are the mean ± standard deviation of three independent
624 experiments

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