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

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

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ABSTRACT

Mixed fermentations with *Starmerella bacillaris* and *Saccharomyces* cerevisiae affects the chemical composition of wines by modulating various metabolites of enological interest. The current study was carried out to elucidate the effect of sequential inoculation of the above mentioned species on the production of white wines, especially on the chemical and aromatic characteristics of Chardonnay, Muscat, Riesling and Sauvignon blanc wines. 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. However, 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 profile 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 (Fleet, 2008). 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; Englezos et al., 2016b; 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 at 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., 2018). 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 & Perez-Coello, 2005) and slightly modified by Rolle et al. (2015, 2018). 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 40 ºC and inserted into the injection port of the GC apparatus for the thermal desorption. Injections were carried out in splitless mode at 250 ºC 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 40 ºC and increased at a rate of 2 ºC/min to 200 ºC for 10 min and 5 ºC/min to 220 ºC. The oven was the held at this temperature for 5 min before returning to the initial temperature. The injection port temperature was 250 ºC, the ion source temperature was 150 ºC and the interface temperature was 280 ºC. 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 VOCs quantification was performed with linear regression using analytical standards (all from Sigma) where available (Supplementary Table 1) (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 data 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 can be seen in Fig. 2 (right panel) and Supplementary Table 2, *Starm. bacillaris* consumed on average more fructose and left glucose mostly untouched during this period. Sequential fermentations started significantly slower as within the first 48 hours only 9.0 g/L of sugars (mainly fructose) were consumed on average, representing 3% of the total sugars. At the same time point, pure fermentations with *S. cerevisiae* consumed on average 81.0 g/L of sugars, representing 34% of total sugars. Sugar consumption rate had a steep increase when *S. cerevisiae* was inoculated in mixed fermentations, and continued until day 7 after which rate of sugar consumption slowed and stopped on day 14 in sequential fermentations. On the other hand, sugar consumption rate decreased on day 4 and stopped on day 9 in pure fermentations. The length of the sequential fermentations is in line with Englezos et al. (2016a) who reported a fermentation time three-days longer when sequential fermentations are compared to pure fermentations with the same *S. cerevisiae* strain Uvaferm BC®.

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

While the ethanol content of the wines was lower in mixed fermentations, the glycerol content was significant higher for all grape variety used in this study, confirming previous

observations (Englezos et al., 2016ab; 2018; Rolle et al., 2018). Glycerol production in the mixed fermented wines ranged from 9.3 to 10.3 g/L compared to pure fermented wines that ranged from 7.8 to 8.4 g/L. This increase in glycerol was also reported in previous studies but in higher levels (more than 4.0 > g/L) (Englezos et al., 2016a). The glycerol yield was between 0.038 - 0.042 for mixed fermented wines and between 0.032 – 0.035 for the control wines.

Titratable acidity (expressed as g/L of tartaric acid) was in average significantly higher in sequential fermented wines (7.1 g/L) compared to pure fermented wines (6.3 g/L). This increase is in line with Sadoudi et al. (2012) and Englezos et al. (2016a) who also reported that 24 and 48 hours inoculation delay resulted in higher titratable acidity (0.16 – 0.50 g/L) compared to pure fermented wines respectively, resulting in a decrease of pH. However, the increase of 0.6 – 0.8 g/L observed in this study could not be explained by the primary organic acids (citric, tartaric, succinic, malic and lactic acid) monitored in study (Supplementary Table 3), suggesting that other acids (such as α-ketoglutaric and pyruvic) are most probably responsible for this increase (van Dijken & Scheffers, 1986). Magyar, Nyitrai-Sárdy, Leskó, Pomázi & Kállay (2014) reported a significantly higher accumulation of pyruvic acid by *Starm. bacillaris* compared to *S. cerevisiae* in pure culture fermentation using synthetic medium. Conversely, pure starter culture fermentations lead to a higher average decrease of malic acid than mixed starter culture fermentations. *S. cerevisiae* in pure fermentations consumed on average 0.7 g/L of malic acid, representing a 36% reduction, while in sequential inoculations the decrease was on average 0.5 g/L representing a 28% reduction. Rantsiou et al. (2017), reported that pure culture fermentations with *Starm. bacillaris* consumed malic acid on a level of 40% in red Barbera cv. musts with differing sugar levels (200-330 g/L), which was in line with earlier research by Tofalo et al (2012), using a red must with 220 g/L of residual sugars.

3.3 Volatile composition

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 (Dzialo et al., 2017). Both pure and mixed fermentations, independent 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

335 threshold (Cullere, Escudero, Cacho & Ferrerira, 2004; Ferreira, Lopez & Cacho, 2000;
336 Guth, 1997; Li, 2006).

337 Isoamylic alcohol (3-methyl-1-butanol), which is produced during fermentation
338 through deamination and decarboxylation reactions from isoleucine (Molina et al., 2009),
339 could negatively contribute to wine quality due to the herbaceous aroma. Chardonnay and
340 Muscat wines produced using pure starter cultures contained significant higher levels of this
341 metabolite, however in concentrations well below its perception threshold. To the contrary,
342 no differences were observed for Riesling and Sauvignon blanc wines. 2-phenylethanol,
343 which is synthesized via Ehrlich pathway through metabolic reactions that involves
344 transamination of the amino acid L-phenylalanine, could contribute to the wine with a
345 pleasant rose-like odour (Swiegers et al., 2005). Riesling and Sauvignon blanc wines
346 produced from mixed starter cultures were distinguished, from the respective wines
347 fermented exclusively with *S. cerevisiae*, by a significant higher amount of this metabolite.
348 Therefore, the increased concentration of 2-phenylethanol would potentially increase the
349 floral aroma in these wines.

350 2-Methyl-1-propanol (also known as isobutanol) is synthesized in the yeast cell
351 through the valine degradation pathway and contributes to herbaceous notes in the wines
352 (Dzialo et al., 2017). Chardonnay and Muscat wines produced from pure *S. cerevisiae*
353 fermentations contained significant higher levels of this metabolite. Conversely, Riesling and
354 Sauvignon blanc wines fermented with pure cultures, contained significantly lower levels of
355 this metabolite, suggesting that valine concentration rather than inoculation strategy affects
356 its production. Moreover, negligible differences were found in wines produced using mixed
357 cultures independently of the grape variety used. Hexanol, usually has a negative influence
358 on wine aroma, by imparting a vegetable and herbaceous odour, when the concentration
359 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 (Dzialo et al., 2017). 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 stain 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 control wine suggesting that grape variety rather than inoculation protocol modulate its production.

The second group of esters, called acetate esters, are those formed from acetic acid and higher alcohols, and are considered to have a greater effect on the perceived aroma than the ethyl esters (Dzialo et al., 2017). In the current study, the acetate esters identified were ethyl acetate, hexyl acetate, octyl acetate, 2-phenyl-ethyl-acetate, and 3-methyl-1-butanol acetate. All wines inoculated with mixed cultures presented significant lower content of this aroma family. Among the quantified acetate esters two compounds (2-phenyl-ethyl-acetate and 3-methyl-1-butanol acetate) associated with the positive attributes, “rose”, “honey” and “banana” presented values above the threshold value in all the wines studied, consequently they are expected to have an influence on the aroma of the wines. Both compounds were found to be significantly higher in pure fermented wines independently of the grape variety used. A significant difference in hexyl acetate, a metabolite with pleasant fruity note was observed. The amount of this metabolite was above the threshold in the control wine however below in mixed starter culture fermented wine, the former having 3-14 times the amount compared to the latter for all the varieties investigated. Similar behaviour was found for 3-methyl-1-butanol acetate. This reduction was more evident in Chardonnay and Muscat wines, suggesting that the grape variety may have an influence on the production of these esters.

Ethyl acetate and 2-phenyl-ethyl-acetate are the most common esters found in wine. Contrary to 2-phenyl-ethyl-acetate, ethyl acetate is known to have an unpleasant nail polish,

vinegar aroma at concentrations above 150 mg/L (Corison, Ough, Berg & Nelson, 1979). At concentrations below this limit, this metabolite contributes positively to white wine quality, with pleasant descriptors such as, pineapple and apple. For both Chardonnay and Muscat wines fermented by pure cultures, the content of ethyl acetate was above the odour threshold, while 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 (Swiegers et al., 2005).

3.3.3 Terpenes and C_{13}-norisoprenoids

Terpenes are a kind of aroma family responsible for the characteristic floral and fruity aroma 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

460 different grape varieties (Roland, Schneider, Razungles & Cavelier, 2011). Among these
461 metabolites, 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH) and
462 its acetate 3-mercaptohexyl acetate (3MHA) contribute positively to the fruity character of
463 young wines with pleasant notes of box tree, grape fruit and exotic fruit aroma, respectively
464 (Rolland et al., 2011; Tominaga, Furrer, Henry & Dubourdieu, 1988). These metabolites are
465 present in grape as non-volatile cysteine or glutathione conjugated precursors and they are
466 released during the fermentation by yeast through their beta-lyase activity (Murat, Masneuf,
467 Darriet, Lavigne, Tominaga & Dubourdieu, 2001). The two inoculation protocols used in this
468 study affected the release of 3MH, while 3MHA was not detected in the samples. Wines
469 fermented using the sequential inoculation protocol showed a significant higher concentration
470 (269 ng/L) of 3MH compared to the control wine (198 ng/L), well above the 60 ng/L
471 perception threshold. This liberation of higher levels of volatile thiols in mixed fermentations
472 could be explained by the beta-lyase activity that favour the cleavage of the conjugated thiols,
473 probably due to involvement of *Starm. bacillaris* in the fermentation process (Swiegers &
474 Pretorius, 2007). Anfang, Brajkovich & Goddard (2009) also reported a significant increase
475 in 3MH in Sauvignon blanc wines, co-fermented with *Starm. bacillaris* and *S. cerevisiae* in a
476 ratio of 9:1, compared to pure fermented wines with *S. cerevisiae*. Conversely, co-inoculation
477 at a ratio 1:9 that favour *S. cerevisiae*, produced wines with similar 3MH content. According
478 to Sadoudi et al. (2012), inoculation of *S. cerevisiae* 24 hours after *Starm. bacillaris*
479 inoculation led to the production of wines, with significant lower levels of this metabolite,
480 compared to the respective control wine. Thus differences in 3MH profile depend on the
481 initial inoculation ratio and the resulting population dynamics, demonstrating that yeast-
482 interactions are strain-dependent.
483
484 **5. Conclusion**
485

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 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 data presented here are obtained from one couple of strains, more investigations are necessary to access the impact of strain selection on wine composition.

Conflict of interest

The authors state no conflict of interest.

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Table 1

Chemical parameters of musts and wines produced by pure and mixed culture fermentations

Grape variety	Inoculation protocol	Residual sugars (g/L)	Malic acid (g/L)	Acetic acid (g/L)	Succinic acid (g/L)	Glycerol (g/L)	Ethanol (% v/v)	Y(gly/sugar) (g/g)	Y(eth/sugar) (g/g)	pH	TA (g/L)
Chardonnay	Prior inoculation	246.0 ± 2.6	2.55 ± 0.03	< 0.1	0.06 ± 0.01	< 0.1	< 0.1	-	-	3.99 ± 0.01	4.33 ± 0.02
	pure	0.4 ± 0.2	1.55 ± 0.03	0.29 ± 0.10	1.27 ± 0.08	8.4 ± 0.1	14.9 ± 0.1	0.034 ± 0.001	0.061 ± 0.001	3.26 ± 0.27	5.84 ± 0.11
	mixed	0.5 ± 0.1	1.88 ± 0.01	0.28 ± 0.01	1.29 ± 0.02	10.3 ± 0.1	14.7 ± 0.1	0.042 ± 0.001	0.06 ± 0.001	3.35 ± 0.06	6.92 ± 0.06
Sign.		NS	***	NS	NS	***	*	***	*	NS	***
Muscat	Prior inoculation	244.0 ± 1.2	1.28 ± 0.03	< 0.1	0.05 ± 0.01	< 0.1	< 0.1	-	-	3.81 ± 0.03	3.15 ± 0.04
	pure	0.5 ± 0.1	0.83 ± 0.01	0.31 ± 0.01	0.94 ± 0.01	7.8 ± 0.1	14.8 ± 0.1	0.032 ± 0.001	0.061 ± 0.001	3.22 ± 0.14	6.69 ± 0.04
	mixed	0.7 ± 0.1	0.94 ± 0.01	0.27 ± 0.01	1.14 ± 0.01	9.3 ± 0.1	14.6 ± 0.1	0.038 ± 0.002	0.06 ± 0.02	3.24 ± 0.11	7.16 ± 0.04
Sign.		NS	***	***	***	***	*	***	*	NS	***
Riesling	Prior inoculation	245.9 ± 1.1	2.26 ± 0.01	< 0.1	0.04 ± 0.01	< 0.1	< 0.1	-	-	3.82 ± 0.01	4.35 ± 0.06
	pure	0.4 ± 0.1	1.44 ± 0.02	0.36 ± 0.03	1.13 ± 0.03	8.6 ± 0.1	14.7 ± 0.1	0.035 ± 0.001	0.06 ± 0.001	3.35 ± 0.08	5.67 ± 0.06
	mixed	0.9 ± 0.1	1.60 ± 0.01	0.32 ± 0.01	1.21 ± 0.01	10.3 ± 0.1	14.6 ± 0.1	0.042 ± 0.001	0.06 ± 0.001	3.34 ± 0.03	6.27 ± 0.05
Sign.		***	***	NS	**	***	*	***	NS	NS	***
Sauvignon blanc	Prior inoculation	245.7 ± 0.6	1.23 ± 0.01	< 0.1	0.03 ± 0.01	< 0.1	< 0.1	-	-	3.56 ± 0.02	6.51 ± 0.04
	pure	0.7 ± 0.1	0.81 ± 0.01	0.40 ± 0.01	0.92 ± 0.01	8.3 ± 0.1	14.9 ± 0.1	0.034 ± 0.001	0.061 ± 0.001	3.09 ± 0.07	7.08 ± 0.01
	mixed	1.1 ± 0.1	0.86 ± 0.01	0.33 ± 0.01	1.02 ± 0.06	9.8 ± 0.1	14.7 ± 0.1	0.04 ± 0.002	0.06 ± 0.002	3.15 ± 0.03	8.11 ± 0.02
Sign.		***	**	***	*	***	***	***	***	NS	***

The values are mean ± standard deviation of three independent experiments. Sign.: *, **, *** and NS indicate significance at $p < 0.05$, $p < 0.01$, $p < 0.001$ and not significant, respectively. TA: titratable acidity expressed as tartaric acid, Y (gly/sugar consumption): glycerol yield and Y (eth/sugar consumption): ethanol yield.

Table 2

Volatile composition of the wines produced by pure and mixed culture fermentations

			Chardonnay		Muscat		Riesling		Sauvignon blanc		Statistical differences						
Metabolites	**Retention index**	**Perception threshold**	**Pure**	**Mixed**	**Pure**	**Mixed**	**Pure**	**Mixed**	**Pure**	**Mixed**	**Variety**	**Yeast**	**Interaction**	**Chard.**	**Mus.**	**Ries.**	**S.b.**
Alcohols																	
2-Methyl-1-propanol	1113	40000a	372 ± 18	271 ± 30	341 ± 58	269 ± 43	153 ± 22	282 ± 66	160 ± 8	264 ± 13	***	NS	***	***	*	**	***
Isoamylic alcohol	1231	30000a	6905 ± 882	4284 ± 436	5163 ± 872	4064 ± 205	4462 ± 882	4196 ± 827	4514 ± 434	4710 ± 494	***	***	***	***	*	NS	NS
Hexanol	1367	8000a	314 ± 51	386 ± 45	72 ± 4	101 ± 9	204 ± 44	287 ± 46	228 ± 21	352 ± 19	***	***	*	*	***	**	***
(R,R)-2,3-Butanediol	1552	120000c	414 ± 102	284 ± 50	619 ± 64	338 ± 51	324 ± 36	240 ± 56	485 ± 202	356 ± 57	***	***	NS	*	***	*	NS
Octanol	1568	900b	7 ± 2	8 ± 4	13 ± 4	12 ± 4	7 ± 2	8 ± 4	7 ± 3	6 ± 5	***	NS	NS	NS	NS	NS	NS
(R,S-meso)-2,3-Butanediol	1587	120000c	96 ± 30	89 ± 19	168 ± 16	108 ± 20	88 ± 20	60 ± 22	122 ± 53	121 ± 14	***	**	NS	NS	***	*	NS
2-phenylethanol	1885	10000a, 14000d	6685 ± 763	8131 ± 1340	7549 ± 1613	8196 ± 1845	4633 ± 955	7369 ± 1999	5176 ± 531	6036 ± 633	***	***	NS	NS	NS	*	*
∑ *Alcohols*			14793 ± 1002	13454 ± 1689	13925 ± 1697	13088 ± 2002	9871 ± 1527	12442 ± 2877	10693 ± 708	11825 ± 477	***	NS	*	NS	NS	NS	**
Esters																	
Ethyl acetate	nd	7500a	7434 ± 850	3909 ± 397	8488 ± 1330	3650 ± 250	3721 ± 569	3688 ± 737	4530 ± 335	4649 ± 315	***	***	***	***	***	NS	NS
Ethyl butanoate	1040	20d	206 ± 26	119 ± 18	248 ± 51	87 ± 28	108 ± 22	80 ± 25	94 ± 28	152 ± 18	***	***	***	***	***	NS	**
3-Methyl-1-butanol acetate	1131	30c	19718 ± 3338	1653 ± 340	19119 ± 3367	1499 ± 232	5844 ± 1174	1097 ± 141	5857 ± 1074	2327 ± 368	***	***	***	***	***	***	***
Ethyl hexanoate	1249	5d,14a	5755 ± 910	3209 ± 832	5711 ± 1079	2456 ± 421	3079 ± 930	2373 ± 431	2770 ± 417	4168 ± 393	***	***	***	***	***	NS	***
Hexyl acetate	1286	670-1500c	5450 ± 1015	572 ± 141	1493 ± 309	113 ± 26	1830 ± 541	227 ± 36	1902 ± 277	648 ± 85	***	***	***	***	***	***	***
Ethyl 2-hexenoate	1355	-	13 ± 3	20 ± 5	0 ± 0	2 ± 1	5 ± 2	13 ± 5	3 ± 2	17 ± 3	***	***	***	*	*	**	***
Methyl octanoate	1398	200f	92 ± 35	51 ± 18	79 ± 9	25 ± 11	57 ± 32	40 ± 10	36 ± 5	60 ± 7	*	***	***	*	***	NS	***
Ethyl octanoate	1445	2a,5d	42583 ± 12382	18266 ± 6110	39625 ± 6078	12680 ± 1891	21525 ± 7409	12036 ± 3738	15044 ± 1802	23904 ± 2822	***	***	***	**	***	*	***
Octyl acetate	1478	50000e	81 ± 27	3 ± 2	92 ± 26	3 ± 2	13 ± 9	1 ± 1	16 ± 6	13 ± 4	***	***	***	***	***	*	NS
Ethyl nonanoate	1543	1300b	15 ± 7	35 ± 18	17 ± 3	20 ± 19	6 ± 4	8 ± 3	20 ± 22	19 ± 19	NS	NS	NS	*	NS	NS	NS
Methyl decanoate	1599	1200e	70 ± 35	33 ± 17	55 ± 8	12 ± 5	32 ± 23	20 ± 8	17 ± 5	47 ± 6	**	**	***	NS	***	NS	***
Ethyl decanoate	1648	200a	34198 ± 10455	15223 ± 3991	27364 ± 5403	11097 ± 1946	14863 ± 5067	10398 ± 2922	12358 ± 1658	22455 ± 2577	***	***	***	*	***	NS	***
3-Methyl-butyl octanoate	1663	-	169 ± 64	84 ± 35	194 ± 36	65 ± 16	51 ± 50	40 ± 15	50 ± 16	131 ± 30	***	**	***	*	***	NS	***
Ethyl 9-decenoate	1697	-	340 ± 151	75 ± 22	241 ± 51	32 ± 9	74 ± 58	51 ± 16	117 ± 18	103 ± 21	***	***	***	**	***	NS	NS

2-Phenyl-ethyl acetate	1815	250a	2585 ± 602	1350 ± 253	3140 ± 640	1209 ± 177	1404 ± 376	772 ± 198	1950 ± 216	1124 ± 117	***	***	***	**	***	**	***
Ethyl dodecanoate	1834	1500-2000b	3008 ± 802	2869 ± 744	3732 ± 813	1901 ± 432	1963 ± 580	1808 ± 594	1682 ± 358	3887 ± 1585	*	NS	***	NS	***	NS	**
3-Methyl-butyl decanoate	1846	-	136 ± 28	112 ± 35	146 ± 38	76 ± 17	55 ± 39	61 ± 18	75 ± 15	163 ± 132	*	NS	**	NS	**	NS	NS
Ethyl tetradecanoate	1974	800b	104 ± 47	196 ± 27	142 ± 44	109 ± 31	59 ± 32	86 ± 20	133 ± 19	211 ± 176	**	NS	NS	**	NS	NS	NS
Ethyl hexadecanoate	2122	1500b	74 ± 28	146 ± 26	61 ± 25	75 ± 24	65 ± 36	50 ± 14	122 ± 14	100 ± 24	***	NS	***	**	NS	NS	NS
∑ *Esters*			122031 ± 19678	47927 ± 10550	109946 ± 13512	35126 ± 4285	45337 ± 20413	32848 ± 8028	46776 ± 5073	64178 ± 5677	***	***	***	***	***	NS	***
Fatty acids																	
Octanoic acid	1986	500a	898 ± 468	329 ± 140	1108 ± 222	319 ± 140	787 ± 174	313 ± 198	900 ± 104	237 ± 186	NS	***	NS	*	***	**	***
Decanoic acid	2138	1000a	389 ± 320	275 ± 62	578 ± 259	70 ± 17	616 ± 144	76 ± 41	587 ± 97	394 ± 36	NS	***	**	NS	***	***	**
∑ *Fatty acids*			1287 ± 648	604 ± 70	1686 ± 463	389 ± 104	1403 ± 316	389 ± 222	1487 ± 197	631 ± 188	NS	***	NS	*	***	***	***
Terpenes																	
D-Limonene	1205	15g, 200f	0 ± 0	0 ± 0	17 ± 4	9 ± 5	0 ± 0	2 ± 5	0 ± 0	0 ± 0	***	NS	**	NS	*	NS	NS
δ-3-Carene	1330	-	0 ± 0	0 ± 0	40 ± 19	24 ± 11	13 ± 7	6 ± 5	0 ± 0	0 ± 0	***	*	NS	NS	NS	NS	NS
t-Furan linalool oxyde	1457	-	0 ± 0	0 ± 0	0 ± 0	0 ± 0	5 ± 8	1 ± 2	0 ± 0	0 ± 0	NS	NS	NS	*	NS	NS	*
Linalool	1556	25.2d	2 ± 1	3 ± 2	647 ± 61	514 ± 68	101 ± 26	80 ± 18	4 ± 2	7 ± 2	***	***	***	NS	**	NS	*
Hotrienol	1617	100g	0 ± 0	0 ± 0	42 ± 10	41 ± 8	35 ± 13	13 ± 8	3 ± 1	1 ± 1	***	**	***	*	NS	**	*
α-Terpineol	1707	250c	0 ± 0	0 ± 0	31 ± 5	25 ± 5	13 ± 8	6 ± 2	0 ± 0	0 ± 0	***	**	*	NS	NS	NS	**
Citronellol	1770	100c	2 ± 0	9 ± 4	4 ± 2	11 ± 5	2 ± 0	11 ± 3	2 ± 1	9 ± 4	NS	***	NS	**	**	***	***
Geraniol	1836	30a	0 ± 0	3 ± 3	4 ± 5	13 ± 3	1 ± 2	4 ± 3	1 ± 2	3 ± 3	***	***	*	*	**	NS	NS
∑ *Terpenes*			5 ± 1	15 ± 8	785 ± 85	639 ± 86	169 ± 51	122 ± 30	12 ± 4	21 ± 6	***	**	***	*	*	NS	**
Other metabolites																	
Methionol	1727	1000d	1 ± 2	3 ± 5	2 ± 2	2 ± 4	3 ± 1	9 ± 3	3 ± 1	6 ± 4	**	**	NS	NS	NS	**	NS
β-Damascenone	1820	0.055a	15 ± 3	11 ± 5	26 ± 7	19 ± 5	35 ± 11	18 ± 5	15 ± 7	8 ± 3	***	***	NS	NS	NS	*	NS
∑ *other metabolites*			17 ± 4	15 ± 5	28 ± 6	22 ± 5	38 ± 12	27 ± 5	19 ± 8	14 ± 5	***	**	NS	NS	NS	NS	NS
Volatile thiols(ng/L)																	
3-mercaptohexanol	-	60h	-	-	-	-	-	-	198 ± 7	269 ± 41	-	-	-	-	-	-	*
3-mercaptohexyl acetate	-	4h	-	-	-	-	-	-	nd	nd	-	-	-	-	-	-	NS

Aroma compounds in wines expressed in μg/L, as mean ± standard deviation of three independent experiments (each replicate was analysed two times (total 6)). Sig: *, **, *** and NS indicate significance at $p < 0.05$, $p < 0.01$, $p < 0.001$ and not significant, respectively. Chard., Chardonnay; Mus., Muscat, Ries., Riesling, S.b., Sauvignon blanc. Perception thresholds (μg/L) were taken from: (a) Guth (1997), (b) Li (2006), (c) Cullere, Escudero, Cacho & Ferreira (2004), (d) Ferreira, Lopez & Cacho (2000), (e) Li, Tao, Wang & Zhang (2008), (f) Cheng, Liu, Yue & Zhang (2015) and (g) Zhang, Petersen, Liu & Toldam-Andersen (2015), (h) Tominaga, Furrer, Henry & Dubourdieu (1998).

Figure captions

Fig.1

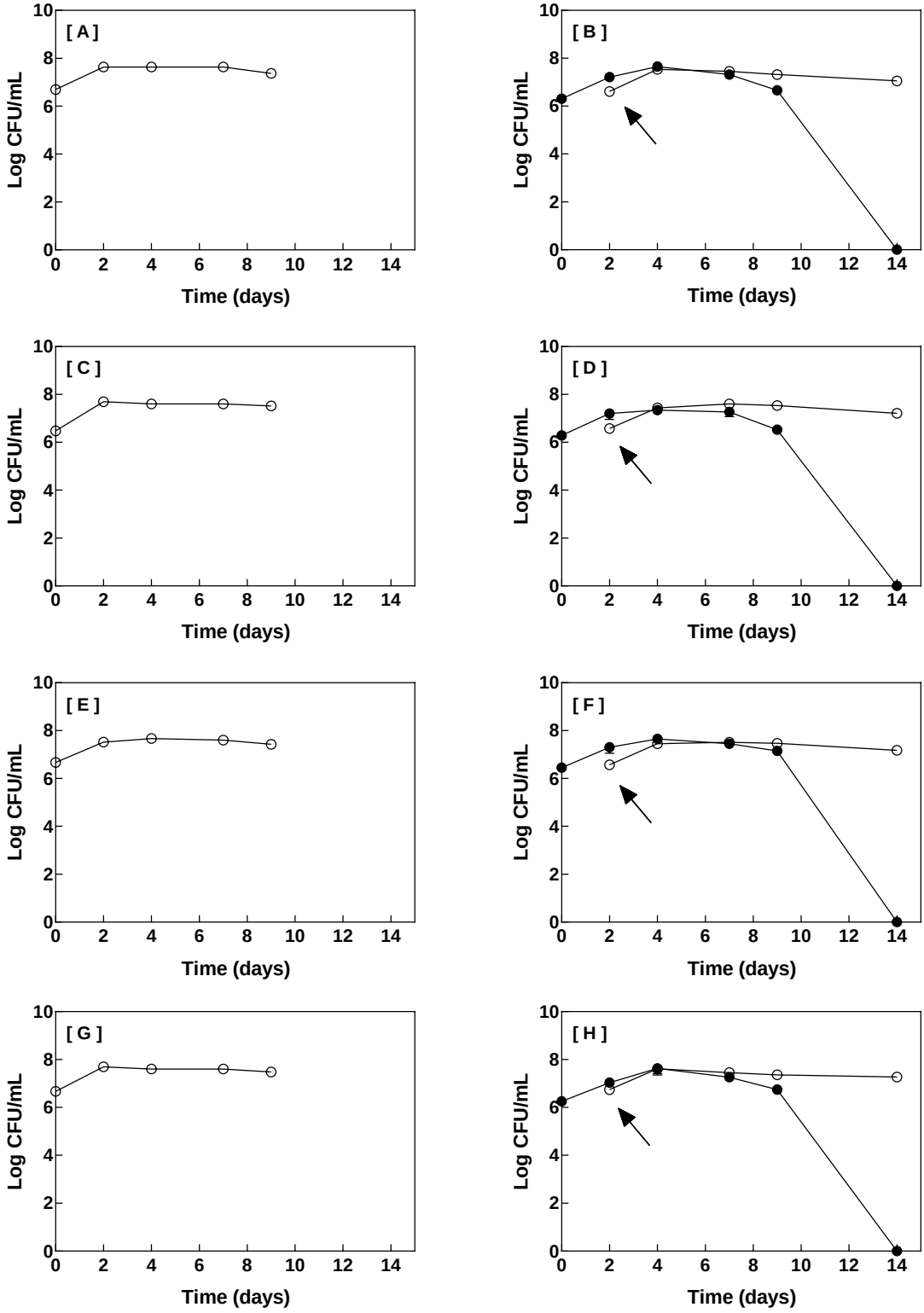
Growth dynamics of yeasts during pure (left panel) and mixed culture fermentations (right panel) using white grape musts: Chardonnay (A, B), Muscat, (C, D), Riesling (E, F) and Sauvignon blanc (G, H). *Starm. bacillaris* strain FC54 (black circle) and *S. cerevisiae* Uvaferm BC® (white circle). The arrow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

Fig.2

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

669 **Figures**

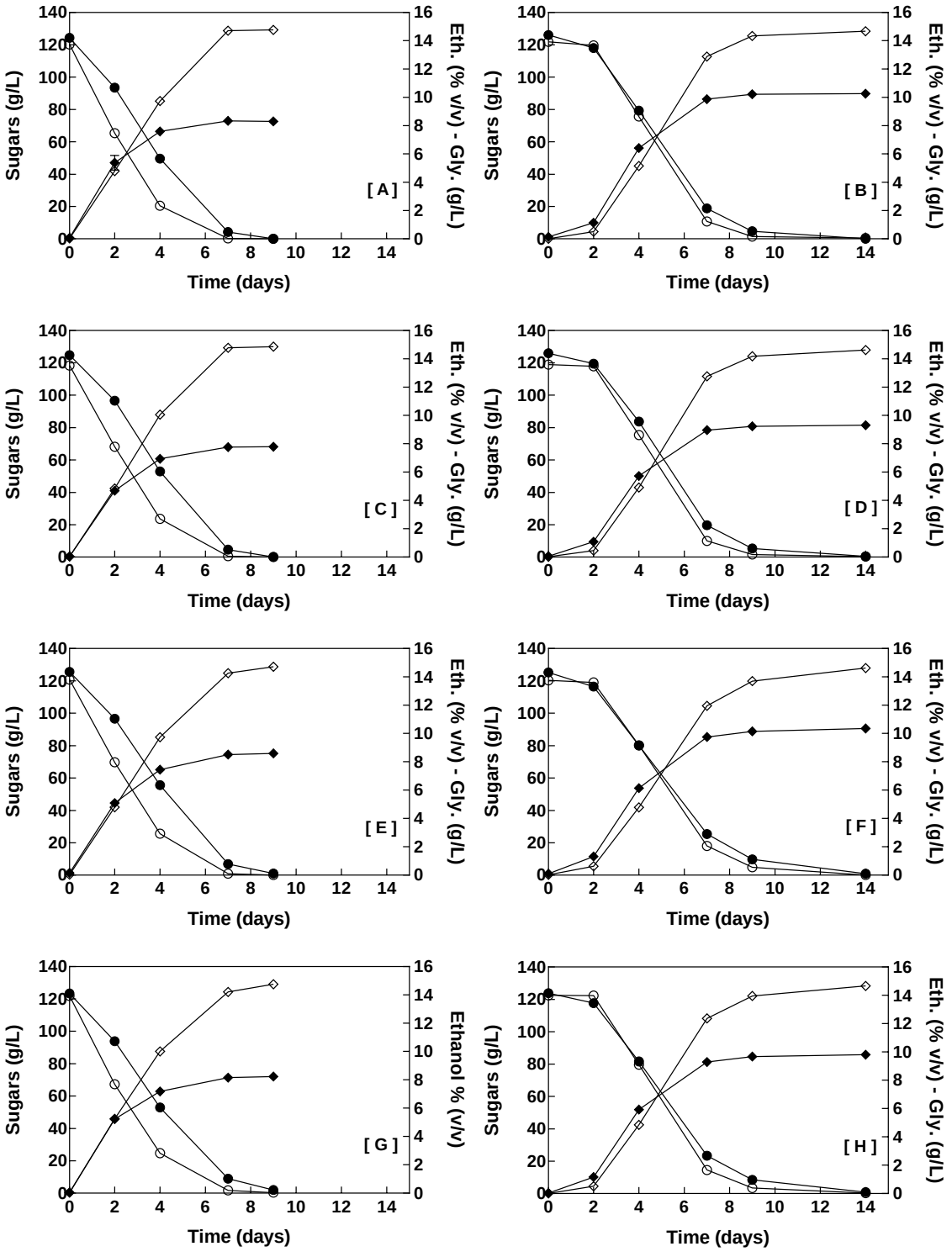
670 **Fig. 1**



671

672

673 **Fig. 2**



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