Saccharomyces cerevisiae-Starmerella bacillaris strains interaction modulates chemical and volatile profile in red wine mixed fermentations

Saccharomyces cerevisiae–Starmerella bacillaris strains interaction modulates chemical and volatile profile in red wine mixed fermentations

Vasileios Englezos[1#], Matteo Pollon[1#] Kalliopi Rantsiou[1], Anne Ortiz-Julien[2], Riccardo Botto[1], Susana Río Segade[1], Simone Giacosa[1], Luca Rolle[1], Luca Cocolin[1*].

[1]Università degli Studi di Torino, Dipartimento di Scienze Agrarie, Forestali e Alimentari, Largo Braccini 2, 10095 Grugliasco, Italy.

[2]Lallemand SAS, Blagnac, France.

[*]Corresponding author: Luca Cocolin, Fax: +39-011-6708553, email: lucasimone.cocolin@unito.it.

[#] Vasileios Englezos and Matteo Pollon contributed equally to this article.

ABSTRACT

The use of *Starmerella bacillaris* in combination with *Saccharomyces cerevisiae* is considered as a state-of-the-art biological application to modulate wine composition. This application implies a detailed understanding of yeast-yeast interactions during mixed fermentations and their effect on the composition of the resulting wines. In this context, ten commercial *S. cerevisiae* strains were used as partners of an indigenous, previously characterized *Starm. bacillaris* strain in order to get a better insight into the impact of *S. cerevisiae* strain employed. The different combinations of strains tested influenced the growth dynamics, the fermentation behavior and, as a consequence, wine composition in a couple-dependent manner. In addition, wines produced from mixed fermentations had significantly lower levels of ethanol, acetic acid and ethyl acetate, and showed higher amounts of glycerol, higher alcohols and esters compared to pure *S. cerevisiae* control fermentations. This study reveals the importance of *S. cerevisiae* strain choice on the chemical composition of the wines produced from mixed culture fermentations with *Starm. bacillaris*.

Keywords: non-*Saccharomyces; Starmerella bacillaris*; mixed fermentation; yeast interactions; extracellular metabolites

1. Introduction

The presence of increased percentages of non-*Saccharomyces* yeasts appears to be one of the reasons for the higher complexity found in spontaneously fermented wines (Fleet, 2003). In an effort to replicate the aroma complexity, derived from indigenous strains in spontaneous fermentations, mixed fermentations using selected non-*Saccharomyces* and *S. cerevisiae* strains have been proposed (Ciani, Comitini, Mannazzu, & Domizio, 2010; Fleet, 2008). A large body of research has focused on incorporating non-*Saccharomyces* yeasts to improve the aroma complexity and quality of wines (Mate & Maicas, 2016; Padilla, Gil, & Manzanares, 2016; Varela, 2016; Whitener et al., 2016). This approach encompasses the employment of non-*Saccharomyces* yeasts together with *S. cerevisiae*, either in co-inoculation or in sequential inoculation. The success of this mixed fermentation protocol depends greatly on the metabolic activity and contribution of non-*Saccharomyces* yeasts on wine composition (Ciani & Comitini, 2015).

Another path of research into the application of non-*Saccharomyces* yeasts aims at providing enological advantages, which are not possible with *S. cerevisiae* strains. In particular, increased enzymatic activity, decreased volatile acidity, glycerol and mannoproteins enhancement, and reduced ethanol yield are some attributes reported for non-*Saccharomyces* yeasts (Domizio, Liu, Bisson, & Barile, 2014; Gobbi et al., 2013; Jolly, Varela, & Pretorius, 2014; Nisiotou et al., 2018; Suzzi et al., 2012). In addition, certain non-*Saccharomyces* species are able to perform well in musts with high sugar concentration. Therefore, mixed fermentation using selected non-*Saccharomyces* and *S. cerevisiae* yeasts has been proposed to enhance or reduce wine target metabolites and response to current challenges faced by the winemaking industry, such us reducing the ethanol content in wines produced using grapes with high sugar amounts without the risks associated with spontaneous fermentations (Belda et al. 2017; Ciani et al., 2010; Jolly et al., 2014).

Starmerella bacillaris is a non-*Saccharomyces* yeast with interesting enological features, such as growth at high concentration of sugars, ability to tolerate relatively high

ethanol levels, production of significant amounts of glycerol and low levels of ethanol from consumed sugars (Englezos, Giacosa, Rantsiou, Rolle, & Cocolin, 2017; Rantsiou et al., 2017). Thus, the reduction in ethanol yield can be partially explained by the production of secondary metabolites alternative to ethanol, mainly glycerol and pyruvic acid (Englezos et al., 2018a). Taking into consideration these phenotypic characteristics, coupling of *Starm. bacillaris* with selected *S. cerevisiae* strains has been proposed in order to improve wine quality by enhancing or reducing the production of target metabolites (Englezos et al., 2017). In particular, the impact of *Starm. bacillaris* in mixed fermentations with *S. cerevisiae* can be more effective when specific metabolites are targeted, exploiting reduced ethanol content, increased glycerol amount and total acidity, and improved chromatic characteristics in the wines (Englezos et al., 2017). Therefore, the inoculation timing of *S. cerevisiae* in association with fermentations conditions (inoculum density, temperature, sulphur dioxide, nitrogen and ethanol concentrations) could be used to promote the growth of *Starm. bacillaris* and consequently their impact on wine composition (Englezos et al., 2017).

An important question still open is how strain–strain interaction in wine mixed fermentations with *Starm. bacillaris* and *S. cerevisiae* affects the microbial growth and, as a consequence, metabolites production. As a proof of the concept, the goal of this study was to acquire further knowledge about the impact of *S. cerevisiae* strain choice on mixed fermentation performance with *Starm. bacillaris*. With this aim, ten commercial *S. cerevisiae* strains were tested as partners of a well-characterized *Starm. bacillaris* strain in mixed fermentations using an inoculation delay of 48 hours. Metabolic profiles of wines produced were compared in order to understand the impact of the specific couple on the finished wine composition.

2. Materials and methods

2.1. Strains

One indigenous strain of *Starm. bacillaris* (FC54) and ten commercial *S. cerevisiae* strains (D80, QA23, RBS133, D254, Opale 2.0, VRB, Rhone 2056, Charme Fruity, Exence,

Okay, all from Lallemand Inc. [Montreal, Canada]) were used in this study. *Starm. bacillaris* FC54 was selected for its enological attributes in pure and mixed fermentations (Englezos et al., 2016) and came from the yeast culture collection of the Department of Agricultural, Forest and Food Sciences (DISAFA) of the University of Turin, Italy.

2.2. Must preparation

Vitis vinifera L. cv. Barbera red grapes were harvested manually in good phytosanitary conditions. Once in the laboratory, they were destemmed, crushed, and the grape mash obtained (liquid, skins, and seeds) was heated to 60 °C for 1 hour to promote the diffusion of phenolic compounds from the skins and to deactivate the indigenous yeast populations (Boulton, Singleton, Bisson, & Kunkee, 1996). After cooling, the grape juice was separated from the skins and seeds using a stainless-steel sieve. Afterwards, 800 mL of grape juice were transferred to 1000 mL sterile glass bottle and the absence of viable yeast and bacteria populations was evaluated by plating an aliquot of the pasteurised must on WLN (Biogenetics, Milan, Italy) and MRS agar medium (Biogenetics), respectively. The grape must contained 234 g/L of sugars (118 g/L of glucose and 116 g/L of fructose) and had a pH value of 3.29, a total acidity of 6.40 g/L as tartaric acid, while the yeast assimilable nitrogen (YAN) was composed of 110 mg/L of amino acids and 40 mg/L of ammonium. The YAN concentration was adjusted to 180 mg/L using the commercial product Fermaid O® (Lallemand Inc.).

2.3. Fermentation trials

Two inoculation protocols were carried out: a) pure culture fermentations with each *S. cerevisiae* strain; and b) mixed culture fermentations by inoculating *S. cerevisiae* 48 hours after the *Starm. bacillaris* inoculation. In total sixty fermentations (2 inoculation protocols × 10 *S. cerevisiae* strains × 3 independent replicates) were performed in 1000 mL sterile glass bottles containing 800 mL of grape must, closed with fermentation locks to maintain semi-anaerobic condition during fermentation. In pure and mixed fermentations, each yeast species

was inoculated at 1×10^6 cells/mL as determined by methylene blue staining as marker of cell viability and direct microscope count. *S. cerevisiae* strains were inoculated as active dry yeast (ADY), previously rehydrated in sterile glucose solution (5 %) and then incubated at 37 °C for 20 minutes before inoculation. *Starm. bacillaris* was prepared by pre-adaptation in the must described above for 48 hours at 25 °C. Fermentations were performed at 25 °C under static conditions. At the end of the fermentation (< 2.0 g/L of residual sugars), wines were analysed for standard chemical parameters and volatile organic compounds.

2.4. Microbiological analysis

Growth kinetics were monitored in three replicates by plating performed at day 0, 2, 4, 7, 10 and 14. Briefly, samples were serially diluted with sterile Ringer's solution (Biogenetics) and the number of colony forming units per millilitre (CFU/mL) was determined by plating 100 µL of three appropriately chosen dilutions on WLN medium. The plates were incubated at 28°C for 5 days. The two types of colonies were differentiated visually and subsequently counted. In this medium *Starm. bacillaris* forms flat green colonies with white border and *S. cerevisiae* forms creamy white colonies (Englezos et al., 2018b).

2.5. Chemical analysis

Ethanol, glycerol, and organic acids production, as well as the glucose and fructose consumption were determined during and at the end of the fermentation by high-performance liquid chromatography (HPLC) using an Agilent 1260 Infinity (Aglient Technologies, Santa Clara, CA, USA) apparatus as described by Rolle et al. (2018). Briefly, the analyses were performed isocratically at 0.7 mL/min flow-rate and 65°C temperature with a 300 × 7.8 mm i.d. cation exchange column (Aminex HPX-87H) and a Cation H^+ Microguard cartridge (Bio-Rad Laboratories, Hercules, CA, USA), using 0.0065 N sulphuric acid (H_2SO_4) as the mobile phase. The OIV-MA-AS313-01:R2015 official method (OIV, 2015) was applied to determine titratable acidity, as g/L of tartaric acid. The pH was determined by using an InoLab 730 pH meter (WTW, Weilheim, DE). The yeast assimilable nitrogen (YAN) was determined

enzymatically with the Megazyme kit following the manufacturer instructions (Megazyme International Ireland).

2.6. Volatile organic compounds (VOCs) profile

Volatile organic compounds (VOCs) were determined using the protocol described by Andujar-Ortiz, Moreno-Arribas, Martín-Álvarez, & Pozo-Bayón (2009) with some modifications as follows: 50 mL of wines were supplemented with 0.5 mL of an internal standard (80.344 µg/mL of 1-heptanol in a 10% (v/v) ethanol solution) and extracted twice with 5 mL of dichloromethane (Sigma-Aldrich, Milan, Italy). The dichloromethane extract was dried with Na_2SO_4 anhydrous (Sigma-Aldrich) and concentrated using N_2 stream. Then, 1 µL of the extract was injected into a 7890A gas chromatograph (Agilent Technologies) equipped with a split/splitless injector set to split mode (1:20 ratio). The column used was a DB-WAX capillary column (30 m × 0.25 mm × 0.50 µm; J&W Scientific Inc., Folsom, CA, USA), the carrier gas was He with a flow-rate of 1 mL/min. The oven initial temperature was 40 °C, maintained for 1 min, increased at a rate of 3 °C/min until 190 °C and newly increased at a rate of 4 °C/min until 230 °C and maintained for 20 min. The detection and semi-quantitative determination of VOCs were performed using a 5975C mass spectrometer (Agilent Technologies). The energy of ionization was 70 eV and the *m/z* acquisition range was 35−350. The identification of the analytes was performed through comparison of the mass spectra obtained with the libraries NIST 08 and Wiley 05. The VOCs were semi-quantified in relation to the area of the 1-heptanol internal standard.

2.7. Statistical analyses

Data were analysed using the software R Studio (R Foundation for Statistical Computing, Vienna, Austria). The following packages were used: stats, car, agricolae, FactoMineR, factoextra. The data were subjected to analysis of variance, in the case of null hypothesis rejection (F test, p-value < 0.05) the differences among more than two groups were assessed through Tukey's HSD *post hoc* test. Concerning multivariate analysis, both standard chemical parameters and VOCs data were elaborated through cluster analysis and

principal component analysis (PCA). In cluster analysis, the Euclidean's distance and the Ward's method were used. The number of clusters was determined through gap statistics method. The PCA was performed scaling the data to unit standard deviation. The first two components were used for the graphical representations. The PCA individuals were distinguished through different colours based on cluster analysis results.

3. Results

3.1. Population dynamics during fermentation

The yeast population dynamics in both pure and mixed fermentations are presented in Fig. 1. Yeast cell viability of *S. cerevisiae* strains in pure culture fermentations, independently of the strain used, achieved an average maximum population of 1.3 x 10^8 CFU/mL and remained at these levels throughout the fermentation process. In mixed culture fermentations, *Starm. bacillaris* achieved populations from 1.2 x 10^8 to 3.4 x 10^8 CFU/mL after 2 days, while *S. cerevisiae* populations reached an average maximum population of 3.4 x 10^7 CFU/mL. In all the mixed fermentations, the *Starm. bacillaris* population was higher than that of *S. cerevisiae* and it persisted to the middle of the fermentation (7th day). In mixed fermentations with strains VRB, Rhone 2056, Charme Fruity and Exence, the population of *Starm. bacillaris* was even greater than that using the other six strains (more than 3.0 x 10^8 CFU/mL at the beginning of the stationary phase), and it persisted beyond the middle of the alcoholic fermentation (10th day). Additionally, in these four fermentations, on day 10, populations of *S. cerevisiae* were lower compared to other couples, ranging from 1.0 x 10^7 to 2.0 x 10^7 CFU/mL.

3.2. Standards chemical parameters of wines

The evolution of the metabolites during pure and mixed culture fermentations is presented in Fig. 2. Stuck or sluggish fermentations were not observed for any of the pure or mixed fermentations, however the fermentation length differed between the *S. cerevisiae* strains and inoculation protocols tested. Pure fermentations were completed in 7 days, except

for strains D80 and RBS133 that needed 10 days. On the other hand, mixed fermentations were slower and finished in 14 days, independently of the couple. *Starm. bacillaris* FC54 exhibited a strong fructophilic character, as evidenced by the almost exclusive fructose consumption during the first 2 days of fermentation (average sugar consumption: 24.8 g/L of fructose versus 1.4 g/L of glucose; Fig. 2). In the same time frame, in pure fermentations with *S. cerevisiae,* glucose consumption ranged from 5 to 81 g/L and fructose consumption ranged from 2 to 44 g/L. Sugar consumption rate, especially glucose, had a steep increase when *S. cerevisiae* was inoculated in mixed culture fermentations. Furthermore, couples could be distinguished based on fructose consumption pattern. The fermentations with the *S. cerevisiae* strains VRB, Rhone 2056, Charme Fruity and Exence consumed completely the fructose by day 7 and tended to have higher total production of glycerol in the final wines, compared to the other couples (Table 1).

As can be seen in Table 1 and Tables S1 and S2, *S. cerevisiae* strain choice influenced greatly the chemical composition of wines. Pure fermented wines with *S. cerevisiae* D80, D254 and QA23 contained significantly higher ethanol amounts (13.96% v/v for D80, and 13.94% v/v for both D254 and QA23; Table S1) than the pure fermented wines with the strain VRB having the lowest ethanol concentration registered in pure fermented wines (13.66 % v/v). Compared to wines produced by *S. cerevisiae* in pure culture, a significant average reduction of 0.55% v/v in the ethanol levels was observed in mixed fermented wines, corresponding the largest reduction (−0.81 g/L, 6% reduction) to Charme Fruity strain. When comparing the pure fermentations with their respective mixed culture fermentations, significant differences were also observed in ethanol yield in 9 out of 10 fermentations (except for the couple FC54 and VRB, Table 1). The ethanol yield ranged from 0.59 to 0.62 (mL/g) for pure fermented wines and from 0.56 to 0.58 (mL/g) for the mixed fermented wines (Table 1).

Among pure fermented wines, *S. cerevisiae* strains OKAY and Opale 2.0 produced significantly more glycerol than other strains (9.91 g/L for OKAY and 9.85 g/L for Opale 2.0; Table S1), while the D80 strain produced the lowest level of this metabolite (7.48 g/L). Wines fermented with mixed cultures presented significantly higher levels of glycerol compared to their respective pure fermentations (Table 1). The smallest difference in glycerol production compared to the control ferment was registered for the couple with *S. cerevisiae* OKAY that

increased glycerol by 3.06 g/L. The most notable increases in glycerol production were observed for couples with Rhone 2056 (6.00 g/L) and Charme Fruity (6.33 g/L) resulting in wines with 14.49 and 14.54 g/L, respectively. The glycerol yield ranged from 0.030 to 0.040 (g/g) for pure fermented wines and 0.050 to 0.060 for mixed fermented wines (Table 1).

Concerning acetic acid, the pure culture fermented wines with *S. cerevisiae* D80 and Rhone 2056 accounted for significantly higher concentration (0.34 g/L both), while the strain OKAY showed the lowest value (0.16 g/L), compared to the other pure fermented wines (Table S1). On average, pure fermentations produced 0.25 g/L of acetic acid and the mixed ferments produced 0.13 g/L. Mixed culture fermentations showed significantly lower levels of this metabolite compared to the respective pure culture ferments in 8 of the 10 comparisons (Table 1). The mixed fermentation performed with couple FC54 and Rhone 2056 produced the largest reduction of acetic acid (−0.24 g/L, 70% reduction) compared to the pure culture ferment (Table 1).

Pure fermented wines with *S. cerevisiae* strains D80 and QA23 resulted in the highest values of pH (both 3.24), while Okay showed the lowest pH (3.19) (Table 1). Strains VRB and D254 produced the highest levels of total acidity (7.11 g/L both) for pure fermentations, while using D80 and QA23 (6.68 and 6.71 g/L, respectively) the lowest values were found. A significant decrease in pH values with a parallel increase in total acidity was seen for the wines produced using mixed cultures, independently of the *S. cerevisiae* strain used (except the couple FC54 and D254), compared to the respective pure culture fermentation (Table 1). On average, mixed fermented wines had a pH of 3.16 and total acidity of 7.60 g/L, while pure fermented wines had a pH of 3.20 and total acidity of 6.91 g/L. The strain-strain couple that provided the greatest difference of pH compared to the pure culture control was FC54 and D80. In this case the pH value was significantly reduced from 3.24 to 3.15. The mixed fermentation of *Starm. bacillaris* with *S. cerevisiae* RBS133 resulted in the largest increase in total acidity from 7.01 to 8.26 g/L.

The standard chemical parameters dendrogram of similarity formed two different clusters; one grouped the pure fermentations and the other the mixed fermentations (Fig. 3A). The same data were subjected to a principal component analysis (PCA) and the observations were plotted in a bi-dimensional space formed by the first two principal components (PCs) in different colours based on cluster analysis results. Fig. 4 shows the distribution of each pure

and mixed fermented wine (Fig. 4A) in the plane defined by the first two PCs and the loadings of each variable in the first and second PC (Fig. 4B and 4C, respectively). The first principal component (PC1) accounted for 72.11% of the total variance, and it was strongly positively correlated (coefficient ≥ 0.8) with ethanol, pH, and acetic acid but negatively associated (coefficient ≤ -0.8) with total acidity and glycerol. This component permitted a good separation of the wines according to the fermentation protocol, in particular pure fermented wines were located in the right side, while mixed fermented wines were in the left side. The second PC (PC2) explained 15.42% of the total variance and was mainly related to residual sugars, with coefficient ≥ 0.8. Mixed fermented wines were differentiated from pure fermented wines due to the relatively high levels of titratable acidity and glycerol and low levels of ethanol, acetic acid and pH. The significant variability at level of residual sugars (Table S2) allowed to differentiate relatively the mixed fermented wines according to the strain-strain couple used, as observed in Fig. 4A.

3.3. Volatile composition of wines

A total of 37 VOCs belonging to seven chemical families were identified in the wine samples, including 12 higher alcohols, 13 esters, 6 fatty acids, 2 diols, 1 lactone, and 2 terpenes (Table S3). From the univariate point of view, in pure fermentations with *S. cerevisiae* strains four wines showed different volatile profile (Table S3). Pure fermented wine with D254 contained the highest total concentration of fatty acids, particularly octanoic acid and decanoic acid, as well as of some esters such as hexyl acetate, ethyl octanoate, ethyl-3-hydroxybutanote, and ethyl decanoate. Pure fermented wine with Okay was the second richest in fatty acids having the highest amounts of hexanoic acid and octanoic acid, and in higher alcohols mainly due to 1-butanol, 2-phenylethanol, S-(+)-3-methylpentanol, 4-vinylguaiacol, and tyrosol, and it was characterized by the highest concentration in certain esters such as ethyl hexanoate, ethyl acetate, and ethyl succinate. Opale 2.0 wine was distinct by the highest total concentration of higher alcohols, particularly isoamyl alcohol, 4-methylpentanol, and methionol, as well as in other compounds such as isovaleric acid, 2-phenylethyl acetate, isopentyl isobutanoate, and citronellol. Lastly, pure fermented wine by

RBS133 was characterized by high concentration in dodecanoic acid, ethyl lactate, diethyl succinate, ethyl-4-hydroxybutanoate, isoamyl acetate, γ-butyrolactone, and (*Z*)-3-hexenol.

Comparing pure with mixed fermented wines, it was possible to note some key differences, independently of the *S. cerevisiae* strain used (Table S4). Mixed fermented wines were characterized by significantly higher concentrations of isobutyric acid, isovaleric acid, hexanoic acid, ethyl hexanoate, ethyl lactate, benzyl alcohol, and citronellol, and significantly lower amounts of ethyl acetate, ethyl octanoate, and octanoic acid.

Volatile compounds were subjected to hierarchical clustering and the dendrogram of similarity is shown in Fig. 3B. As can be observed, wines were clustered in two groups, the first cluster grouped together 8 of the 10 pure fermented wines, while the second cluster grouped all the mixed fermented wines and wines produced by Okay and Opale 2.0 in pure culture. The same data were used to perform a PCA by plotting the observations with different colours based on cluster analysis (Fig. 5A). The first two principal components explained 66.09% of the total variance and allowed the differentiation of the pure and mixed culture fermented wines. PC1 (52.62% of the variance) was highly positively correlated with hexanoic acid, benzyl alcohol, isovaleric acid, ethyl hexanoate, isoamyl alcohol, citronellol, methionol, ethyl lactate, γ-butyrolactone, 2-phenylethyl acetate, isobutyric acid, tyrosol, isopentyl isobutanoate, S-(+)-3-methylpentanol, and 4-methylpentanol (coefficients ≥ 0.70) and strong negatively correlated to terpin, decanoic acid, ethyl decanoate, ethyl octanoate, and octanoic acid (coefficients ≤ -0.70) (Fig. 5B). The PC2 (13.47% of variance) was mostly correlated to 2-phenylethanol and 4-vinylguaiacol (coefficients ≥ 0.70) and negatively correlated to (*Z*)-3-hexenol and diethyl succinate (coefficients ≤ -0.57) (Fig. 5C). Figure 5A showed a lesser variability in the VOCs composition of the wines from mixed fermentation compared to those derived from pure fermentation. For the mixed trials, the extreme values on the first component corresponded to FC54 plus OKAY and FC54 plus RBS133, which are located at 1.77 points of distance from each other; instead for pure fermentations, the most extreme points on the first component are Charme Fruity and OPALE 2.0 with a distance of 4.73 points from each other. The same evaluation was done also for the second component, in which it is possible to see the biggest differences between pure and mixed wines. FC54 plus QA23 and FC54 plus Excence are distant 2.69 points on PC2, while OKAY and D254 are well far away each other (10.30 points on PC2). Therefore, the mixed fermentations were less

variable on PC1 and PC2 compared to pure fermentations, advancing the hypothesis that FC54 is able not only to change the VOCs profile of wines giving them some specific features, but it is able to reduce also the variability in VOCs composition induced by the inoculation of different *S. cerevisiae* strains in pure fermentations (Table S5).

4. Discussion

Recent studies focused on the potential application of *Starm. bacillaris* jointly with *S. cerevisiae* have highlighted the ability of mixed fermentations to modulate metabolites of enological interest, like glycerol, ethanol and titratable acidity (Englezos et al., 2017; Nisiotou et al., 2018; Tofalo et al., 2012; Zara et al., 2014). In mixed fermentations, important parameters such as strain choice and fermentation conditions (SO_2, temperature, nutrient concentration and composition, and oxygen availability) should be taken into consideration. We have previously shown, using two *S. cerevisiae* strains as partners of *Starm. bacillaris* in mixed fermentations, that the strain used has a great impact on wine quality, in particular on residual sugar content (Englezos et al., 2016). However, for any practical application, better knowledge about the physiological and metabolic interactions between *Starm. bacillaris* and *S. cerevisiae* is required. For this reason, in the present study we have investigated the effect of ten commercial *S. cerevisiae* strains on the overall performance of mixed fermentations when coupled with an indigenous *Starm. bacillaris* strain FC54.

The high fructophilic character of *Starm. bacillaris* and its ability to persist into the mid to late stages of the mixed culture fermentations (population become undetectable after 10 or 14 days of fermentation) observed in this study are in line with Englezos et al. (2018). However, it is worth to mention that metabolically active populations of *Starm. bacillaris* at low cell densities in mixed culture fermentations may be present in the last stages of fermentation (day 10 and 14), but they cannot be easily determined by plate counting on WLN medium when coexisting with high populations of *S. cerevisiae*. *Starm. bacillaris* also showed ability to dominate and reduce the growth and population size of *S. cerevisiae* regardless of the strain used, in accordance with the findings of Englezos et al. (2016). In the present study, using a 48-hour sequential inoculation protocol, *Starm. bacillaris* strain FC54 had a limiting effect on the growth of *S. cerevisiae*, which was couple-dependent. On the

other hand, *Starm. bacillaris* established a high population and persisted up to the 10th day, particularly for VRB, Rhone 2056, Charme Fruity, and Exence. The exact mechanism of the observed inhibition is not known but it could be explained by the early consumption of nutrients, mainly ammonium prior to the *S. cerevisiae* inoculation, as observed for other non-*Saccharomyces* yeasts (Ciani & Comitini, 2015; Englezos et al., 2018).

Mixed culture fermentations when compared to their pure culture controls showed reduced ethanol levels in 9 out of 10 couples. This finding is in agreement with previous observations in which the involvement of *Starm. bacillaris* in mixed fermentations greatly influences the ethanol production (Di Maio et al., 2016; Englezos et al., 2017; Giaramida et al., 2016). The lowest reduction (0.37% v/v) was achieved using the couple FC54 and RBS133 and the highest decrease (0.80% v/v) was with the couple FC54 and Charme Fruity, this last one being higher than that found by Englezos et al. (2016) of 0.70% v/v. Therefore, the results of this study indicate that the magnitude of ethanol reduction is *Starm. bacillaris* and *S. cerevisiae* couple-dependent. The use of the most suitable *S. cerevisiae* strain with *Starm. bacillaris* FC54 was shown to be important as it resulted in a difference of more than 0.30% v/v in ethanol levels in the resulting wine. This reduction of ethanol production contributes to reduce the risks associated to excessive alcohol consumption and exposure to volumetric taxation (Room, Babor, & Rehm, 2015).

The couples able to reduce the ethanol levels were the same producing higher glycerol concentrations. In addition, all mixed fermented wines contained significantly more glycerol than their respective pure fermentations, in accordance with previous studies (Englezos et al., 2016; Giaramida et al., 2016; Rolle et al., 2018; Zara et al., 2014). However, the magnitude of glycerol production is greatly influenced by the *S. cerevisiae* strain selection. In the present study, coupling *Starm. bacillaris* FC54 with *S. cerevisiae* OKAY increased up to 3.06 g/L of glycerol, compared to the respective control wine. Glycerol is a non-aromatic compound but it can significantly contribute to wine quality. In wines, glycerol concentrations ranging 1–15 g/L are usually present and the higher levels are thought to contribute to the viscosity (Swiegers et al., 2005). Therefore, the increase found in wine fermented with *Starm. bacillaris* FC54 and *S. cerevisiae* OKAY could have a favourable impact on wine quality and in particular on sensory traits. The association of *Starm. bacillaris* and *S. cerevisiae* strains also influenced pH and titratable acidity, in agreement with previous reports (Englezos et al.,

2018b; Mangani, Buscioni, Collina, Bocci, & Vincenzini, 2011). This observation could be explained by the strong tendency of *Starm. bacillaris* to produce organic acids like pyruvic acid, *α*-ketoglutaric acid, and fumaric acid (Englezos et al., 2018a; Magyar, Nyitrai-Sárdy, Leskó, Pomázi, & Kállay, 2014). In the present study, some of the couples with *Starm. bacillaris* FC54 produced an increase of more than 1.0 g/L in total acidity compared to the pure culture fermentations. This increase has both organoleptic and enological implications. It is well known that one of the challenges of producing quality wine in warm viticultural regions is to maintain a suitable acid balance because the acids naturally found in the berry degrade with heat (Godden, Wilkes, & Johnson, 2015). In this context, mixed fermentations carried out by appropriately chosen strains of *Starm. bacillaris* and *S. cerevisiae* can be used as biological solution to current challenges in winemaking.

The mixed culture ferments with *Starm. bacillaris* and *S. cerevisiae* significantly impact the volatile compounds during alcoholic fermentation (Englezos et al., 2017; Whitener et al., 2017; Zara et al., 2014). In this study, the concentration of fatty acids, esters and higher alcohols, which are well known volatile compounds contributing to the wine organoleptic profile, was significantly different between pure and mixed culture fermentations. Interestingly, all 37 volatile compounds detected were found to have significantly different concentrations between the pure and mixed culture fermentations, although it was not always for all strain-strain couples. In general, the results of this study highlighted an increase in volatile compounds in the mixed culture fermentations compared to the pure culture fermentations. This disagrees with the findings of Englezos et al. (2018b) who found that mixed culture fermentations with *Starm. bacillaris* provided a significantly lower concentration of volatile compounds. These differences emphasize the importance of *S. cerevisiae* strain choice in mixed culture fermentation with *Starm. bacillaris* and suggest that, when a limited number of mixed culture couples are tested, there is a risk of missing the enological potential of a non-*Saccharomyces* yeast.

Additionally, in this study, it was possible to note a general increase of some specific higher alcohols in the majority or in all mixed fermented samples compared to their respective pure fermented control wines. Isoamyl alcohol, which was found in higher concentrations in all mixed fermentations (except for FC54 and Opale 2.0), can have two possible origins: the transamination of leucine and decarboxylation of the resulting α-

ketoacid via the Ehrlich pathway, forming first an aldehyde that can be reduced to the corresponding alcohol (Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008), or it can derive from central carbon metabolism through pyruvate (Swiegers, Bartowsky, Henschke, & Pretorius, 2005). Similarly, isobutanol, found in higher concentration in all mixed fermentations (except for FC54 and D80; FC54 and RBS133), can derive from valine catabolism through the Ehrlich pathway or from pyruvate through the central carbon metabolism. Other leucine and valine derivates were found in greater concentration in mixed trials compared to pure samples. More specifically, isovaleric acid (found in higher concentration in all mixed samples) is described as leucine derivate and isobutyric acid (detected in higher concentration in all mixed trials) is a valine derivate through the transamination and decarboxilation of the relative α-ketoacid via the Ehrlich pathway with an oxidation of the corresponding aldehyde in an acid instead of a reduction (Hazelwood et al., 2008). This tendency to increase these higher alcohols and acids was partially noted in a previous study carried out by Nisiotou et al. (2018), who detected more isobutanol and isoamyl alcohol in sequential fermentation performed with *Starm bacillaris* and *S. cerevisiae* compared to pure *S. cerevisiae* fermentation.

The increase of hexanol (except for FC54 and QA23; FC54 and Exence) and its relative ethyl ester, ethyl hexanoate (except for FC54 and OKAY), in mixed fermented wines is in accordance with previous findings (Englezos et al., 2016) for both compounds; Nisiotou et al. 2018 and Rolle et al. 2018 for ethyl hexanoate). The lower levels of ethyl acetate in all mixed fermentations were already noted by Nisiotou et al. (2018). The significantly higher levels of citronellol, compound belonging to the chemical family of monoterpenes, in all mixed fermentations compared to the respective pure can be explained by the *β*-glucosidase activity of *Starm. bacillaris* FC54 found by Englezos et al. (2017). This enzymatic activity might have influenced the concentration of citronellol through hydrolysis of glycosylated precursors. In addition, Whitener et al. (2016) have found in Sauvignon blanc wine fermented by *Starm. bacillaris* and *S. cerevisiae* a greater concentration of some monoterpenes like geraniol, nerol, linalool, and ocimene, compared to pure *S. cerevisiae* fermentation. It is necessary to emphasise that the terpene profile of a wine is strongly dependent by the genotype of grape cultivar. In addition, it cannot be excluded a yeast biosynthesis of this compound as described by Carrau et al. (2005) for *S. cerevisiae*.

5. Conclusion

To our knowledge, this is the first time that a set of commercial *S. cerevisiae* strains were tested as partners of a non-*Saccharomyces* yeast in mixed culture fermentations. The results obtained highlighted that *S. cerevisiae* strain choice plays a key role and affects yeast growth dynamics, sugar consumption and final composition of the wines in terms of technological parameters and volatile organic compounds. These findings should allow greater understanding and management of the production of target metabolites to improve wine characteristics.

Acknowledgements

The authors would like to thank Paola Vagnoli (Lallemand Italia) for providing *S. cerevisiae* strains used in this study.

References

Andujar-Ortiz, I., Moreno-Arribas, M. V., Martín-Álvarez, P. J., & Pozo-Bayón, M. A. (2009). Analytical performance of three commonly used extraction methods for the gas chromatography–mass spectrometry analysis of wine volatile compounds. *Journal of Chromatography A*, *1216*(43), 7351-7357.

Belda, I., Ruiz, J., Esteban-Fernández, A., Navascués, E., Marquina, D., Santos, A., & Moreno-Arribas, M. (2017). Microbial contribution to wine aroma and its intended use for wine quality improvement. *Molecules*, *22*(2), 189.

Boulton, R. B., Singleton, V. L., Bisson, L. F., & Kunkee, R. E. (1996). Principles and practices of winemaking. New York: Chapman & Hall (Chapter 12).

Carrau, F. M., Medina, K., Boido, E., Farina, L., Gaggero, C., Dellacassa, E., Versini, G., & Henschke, P. A. (2005). De novo synthesis of monoterpenes by *Saccharomyces cerevisiae* wine yeasts. *FEMS Microbiology Letters*, *243*(1), 107-115.

Ciani, M., Comitini, F., Mannazzu, I., & Domizio, P. (2010). Controlled mixed culture fermentation: a new perspective on the use of non−*Saccharomyces* yeasts in winemaking. *FEMS Yeast Research*, *10*(2), 123-133.

Ciani, M., & Comitini, F. (2015). Yeast interactions in multi-starter wine fermentation. *Current Opinion in Food Science*, *1*, 1-6.

Di Maio, S., Genna, G., Gandolfo, V., Amore, G., Ciaccio, M., & Oliva, D. (2016). Presence of *Candida zemplinina* in Sicilian musts and selection of a strain for wine mixed fermentations. *South African Journal of Enology and Viticulture, 33*(1), 80-87.

Domizio, P., Liu, Y., Bisson, L. F., & Barile, D. (2014). Use of non-*Saccharomyces* wine yeasts as novel sources of mannoproteins in wine. *Food Microbiology*, *43*, 5-15.

Englezos, V., Rantsiou, K., Cravero, F., Torchio, F., Ortiz-Julien, A., Gerbi, V., Rolle, L., & Cocolin, L. (2016). *Starmerella bacillaris* and *Saccharomyces cerevisiae* mixed fermentations to reduce ethanol content in wine. *Applied Microbiology and Biotechnology*, *100*, 5515-5526.

Englezos, V., Giacosa, S., Rantsiou, K., Rolle, L., & Cocolin, L. (2017). *Starmerella bacillaris* in winemaking: opportunities and risks. *Current Opinion in Food Science*, *17*, 30-35.

Englezos, V., Cocolin, L., Rantsiou, K., Ortiz-Julien, A., Bloem, A., Dequin, S., & Camarasa, C. (2018a). Specific phenotypic traits of *Starmerella bacillaris* regarding nitrogen source consumption and central carbon metabolites production during wine fermentation. *Applied and Environmental Microbiology*, AEM-00797.

Englezos, V., Cravero, F., Torchio, F., Rantsiou, K., Ortiz-Julien, A., Lambri, M., Gerbi, V., Rolle, L., & Cocolin, L. (2018b) Oxygen availability and strain combination modulate yeast growth dynamics in mixed culture fermentations of grape must with *Starmerella bacillaris* and *Saccharomyces cerevisiae*. *Food Microbiology*, *69*, 179-188

Fleet, G. H. (2003). Yeasts interactions and flavour. *International Journal of Food Microbiology*, *86*, 11-22.

Fleet, G. H. (2008). Wine yeasts for the future. *FEMS Yeast Research*, *8*, 979-995.

Giaramida, P., Ponticello, G., Di Maio, S., Squadrito, M., Genna, G., Barone, E., Scacco, A., Corona, O., Amore, G., di Stefano, R., & Oliva, D. (2016). *Candida zemplinina* for production of wines with less alcohol and more glycerol. *South African Journal of Enology and Viticulture*, *34*(2), 204-211.

Gobbi, M., Comitini, F., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., & Ciani, M. (2013). *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in simultaneous and sequential co-fermentation: a strategy to enhance acidity and improve the overall quality of wine. *Food Microbiology*, *33*(2),271-281.

Godden, P., Wilkes, E., & Johnson, D. (2015). Trends in the composition of Australian wine 1984–2014. *Australian journal of grape and wine research*, *21*(1), pp.741-753.

Hazelwood, L. A., Daran, J. M., van Maris, A. J., Pronk, J. T., & Dickinson, J. R. (2008). The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. *Applied and environmental microbiology*, 74(8), 2259-2266.

Jolly, N. P., Varela, C., & Pretorius, I. S. (2014). Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Research*, *14*(2), 215-237.

Magyar, I., Nyitrai-Sárdy, D., Leskó, A., Pomázi, A., & Kállay, M. (2014). Anaerobic organic acid metabolism of *Candida zemplinina* in comparison with *Saccharomyces* wine yeasts. *International Journal of Food Microbiology, 178*, 1-6.

Mangani, S., Buscioni, G., Collina, L., Bocci, E., & Vincenzini, M. (2011). Effects of microbial populations on anthocyanin profile of Sangiovese wines produced in Tuscany, Italy. *American Journal of Enology and Viticulture*, *62*, 487-494.

Mate J. J., & Maicas, S. (2016) Application of non-*Saccharomyces* yeasts to wine-making process. *Fermentation*, *2*, 14.

Nisiotou, A., Sgouros, G., Mallouchos, A., Nisiotis, C. S., Michaelidis, C., Tassou, C., & Banilas, G. (2018). The use of indigenous *Saccharomyces cerevisiae* and *Starmerella bacillaris* strains as a tool to create chemical complexity in local wines. *Food Research International*, *111*, 498-508.

OIV (2015). Recueil international des méthodes d'analyse des vins et des moûts. Paris, France: Organisation Internationale de la Vigne et du Vin.

Padilla, B., Gil, J. V., & Manzanares, P. (2016). Past and future of non-*Saccharomyces* yeasts: From spoilage microorganisms to biotechnological tools for improving wine aroma complexity. *Frontiers in Microbiology*, *7*, 411.

Rantsiou, K., Englezos, V., Torchio, F., Risse, P. A., Cravero, F., Gerbi, V., Rolle L., & Cocolin, L. (2017). Modeling the fermentation behavior of *Starmerella bacillaris*. *American journal of Enology and Viticulture*, *68*, 378-385.

Rolle, L., Englezos, V., Torchio, F., Cravero, F., Segade, S. R., Rantsiou, K., Giacosa, S., Gambuti, A., Gerbi, V., & Cocolin, L. (2018). Ethanol reduction in red wines by technological and microbiological approaches: a comparative study. *Australian Journal of Grape and Wine Research*, *24*(1), 62-74.

Room, R., Babor, T., & Rehm, J. (2005). Alcohol and public health. *Lancet*, *365*(9458), 519-530.

Suzzi, G., Schirone, M., Sergi, M., Marianella, R. M., Fasoli, G., Aguzzi, I., & Tofalo, R. (2012). Multistarter from organic viticulture for red wine Montepulciano d' Abruzzo production. *Frontiers in Microbiology*, *3*, 135.

Swiegers, J. H., Bartowsky, E. J., Henschke, P. A., & Pretorius, I. S. (2005). Yeast and bacterial modulation of wine aroma and flavour. *Australian Journal of Grape and Wine Research*, *11*, 139-173.

Tofalo, R., Schirone, M., Torriani, S., Rantsiou, K., Cocolin, L., Perpetuini, G., & Suzzi, G. (2012). Diversity of *Candida zemplinina* strains from grapes and Italian wines. *Food Microbiology*, *29*, 18-26.

Varela, C. (2016). The impact of non-*Saccharomyces* yeasts in the production of alcoholic beverages. *Applied Microbiology and Biotechnology*, *100*, 9861-9874.

Whitener, M. E. B., Stanstrup, J., Panzeri, V., Carlin, S., Divol, B., Du Toit, M., & Vrhovsek, U. (2016). Untangling the wine metabolome by combining untargeted SPME–GCxGC-TOF-MS and sensory analysis to profile Sauvignon blanc co-fermented with seven different yeasts. *Metabolomics*, *12*(3), 53.

Whitener, M. E. B., Stanstrup, J., Carlin, S., Divol, B., Du Toit, M., & Vrhovskek, U. (2017). Effect of non-*Saccharomyces* yeasts on the volatile chemical profile of Shiraz wines. *Australian Journal of Grape and Wine Research*, *23*, 179-192.

Zara, G., Mannazzu, I., Del Caro, A., Budroni, M., Pinna, M. B., Murru, M., Farris, G. A., & Zara, S. (2014). Wine quality improvement through the combined utilisation of yeast hulls and *Candida zemplinina*/*S Saccharomyces cerevisiae* mixed starter cultures. *Australian Journal of Grape and Wine Research*, *20*(2), 199-207.

Table 1

Standard chemical parameters in wine produced by pure and mixed fermentations

Yeast	Acetic acid (g/L)	Glycerol (g/L)	Ethanol (% v/v)	Glycerol yield[a]	Ethanol yield[b]	pH	Total acidity (g/L as tartaric acid)
D80	0.34 ± 0.01	7.48 ± 0.01	13.96 ± 0.07	0.03 ± 0.01	0.60 ± 0.1	3.24 ± 0.01	6.68 ± 0.05
FC54 and D80	0.14 ± 0.01	11.60 ± 0.07	13.47 ± 0.06	0.05 ± 0.02	0.58 ± 0.0	3.15 ± 0.01	7.73 ± 0.07
Sign.	***	***	***	***	***	***	***
QA23	0.23 ± 0.01	8.23 ± 0.03	13.94 ± 0.04	0.04 ± 0.00	0.60 ± 0.00	3.24 ± 0.02	6.71 ± 0.02
FC54 and QA23	0.14 ± 0.01	11.45 ± 0.22	13.44 ± 0.12	0.05 ± 0.00	0.58 ± 0.01	3.18 ± 0.01	7.18 ± 0.06
Sign.	***	***	**	***	**	**	***
RBS133	0.30 ± 0.01	8.91 ± 0.05	13.71 ± 0.01	0.04 ± 0.00	0.59 ± 0.00	3.22 ± 0.01	7.01 ± 0.24
FC54 and RBS133	0.14 ± 0.02	12.35 ± 0.67	13.34 ± 0.10	0.05 ± 0.00	0.57 ± 0.00	3.17 ± 0.02	8.26 ± 0.08
Sign.	***	***	**	***	**	***	***
D254	0.25 ± 0.01	7.73 ± 0.07	13.94 ± 0.03	0.03 ± 0.00	0.61 ± 0.0	3.19 ± 0.01	7.11 ± 0.02
FC54 and D254	0.16 ± 0.01	12.48 ± 0.28	13.33 ± 0.05	0.05 ± 0.00	0.57 ± 0.1	3.18 ± 0.01	7.07 ± 0.02
Sign.	***	***	***	***	***	NS	*
OPALE 2.0	0.25 ± 0.01	9.85 ± 0.07	13.69 ± 0.03	0.04 ± 0.00	0.59 ± 0.00	3.20 ± 0.01	7.01 ± 0.01
FC54 and OPALE 2.0	0.15 ± 0.01	12.94 ± 0.31	13.15 ± 0.19	0.06 ± 0.00	0.57 ± 0.01	3.18 ± 0.02	7.56 ± 0.04
Sign.	***	***	*	***	**	*	**
VRB	0.21 ± 0.02	8.43 ± 0.07	13.66 ± 0.28	0.04 ± 0.00	0.59 ± 0.01	3.20 ± 0.02	7.11 ± 0.04
FC54 and VRB	0.15 ± 0.03	13.59 ± 1.02	13.20 ± 0.13	0.06 ± 0.00	0.57 ± 0.01	3.17 ± 0.01	7.94 ± 0.11
Sign.	NS	*	NS	*	NS	*	***
RHONE 2056	0.34 ± 0.01	8.49 ± 0.03	13.84 ± 0.05	0.04 ± 0.01	0.60 ± 0.01	3.20 ± 0.01	6.92 ± 0.06
FC54 and RHONE 2056	0.10 ± 0.02	14.49 ± 0.08	13.13 ± 0.05	0.06 ± 0.00	0.57 ± 0.00	3.16 ± 0.03	7.49 ± 0.07
Sign.	***	***	***	***	***	*	*
CHARME FRUITY	0.25 ± 0.01	8.21 ± 0.12	13.92 ± 0.04	0.04 ± 0.01	0.62 ± 0.02	3.20 ± 0.01	6.86 ± 0.13
FC54 and CHARME FRUITY	0.12 ± 0.01	14.54 ± 0.29	13.11 ± 0.02	0.06 ± 0.00	0.57 ± 0.00	3.14 ± 0.01	7.36 ± 0.07
Sign.	***	***	***	***	***	***	**
EXENCE	0.19 ± 0.02	9.21 ± 0.15	13.74 ± 0.04	0.04 ± 0.01	0.59 ± 0.00	3.22 ± 0.01	6.84 ± 0.01
FC54 and EXENCE	0.12 ± 0.02	14.94 ± 0.37	13.11 ± 0.06	0.06 ± 0.00	0.56 ± 0.00	3.13 ± 0.02	7.54 ± 0.03
Sign.	*	***	***	***	***	**	***
OKAY	0.16 ± 0.01	9.91 ± 0.03	13.71 ± 0.02	0.04 ± 0.00	0.59 ± 0.01	3.19 ± 0.01	6.87 ± 0.09
FC54 and OKAY	0.15 ± 0.01	12.97 ± 0.23	13.28 ± 0.02	0.06 ± 0.00	0.57 ± 0.00	3.15 ± 0.02	7.85 ± 0.09
Sign.	NS	**	***	**	***	***	***

The concentration of sugars prior to fermentation was 234 g/L, consisting of 118 g/L glucose and 116 g/L fructose. Values are expressed as mean ± standard deviation of three independent replicates. *Sign.*: *, **, *** and "NS" indicate significance at $p < 0.05$, 0.01, 0.001 and no significant differences, respectively, between pure and mixed culture fermentations. [a]Glycerol

yield: glycerol production/sugar consumption. [b]Ethanol yield: ethanol production/sugar consumption.

Figure captions

Fig. 1 Growth dynamics of yeasts during pure and mixed culture fermentations. *Starmerella bacillaris* strain FC54 (white circle) and *Saccharomyces cerevisiae* (black circle). Counts are the mean CFU/mL values ± standard deviations of three independent experiments.

Fig. 2 Evolution of metabolites during pure and mixed culture fermentations. Glucose (black circle), fructose (white circle), ethanol (white diamond) and glycerol (black diamond). Data are the mean ± standard deviations of three independent experiments.

Fig. 3 Dendrogram of similarity for pure and mixed fermented wines based on the standard chemical parameters (A) and volatile compounds (B).

Fig. 4 Score plot (A) and loading plots of the first (B) and second (C) principal component corresponding to PCA of the standard chemical parameters in pure and mixed fermented wines.

Fig. 5 Score plot (A) and loading plots of the first (B) and second (C) principal component corresponding to PCA of volatile compounds in pure and mixed fermented wines.

Fig. 1

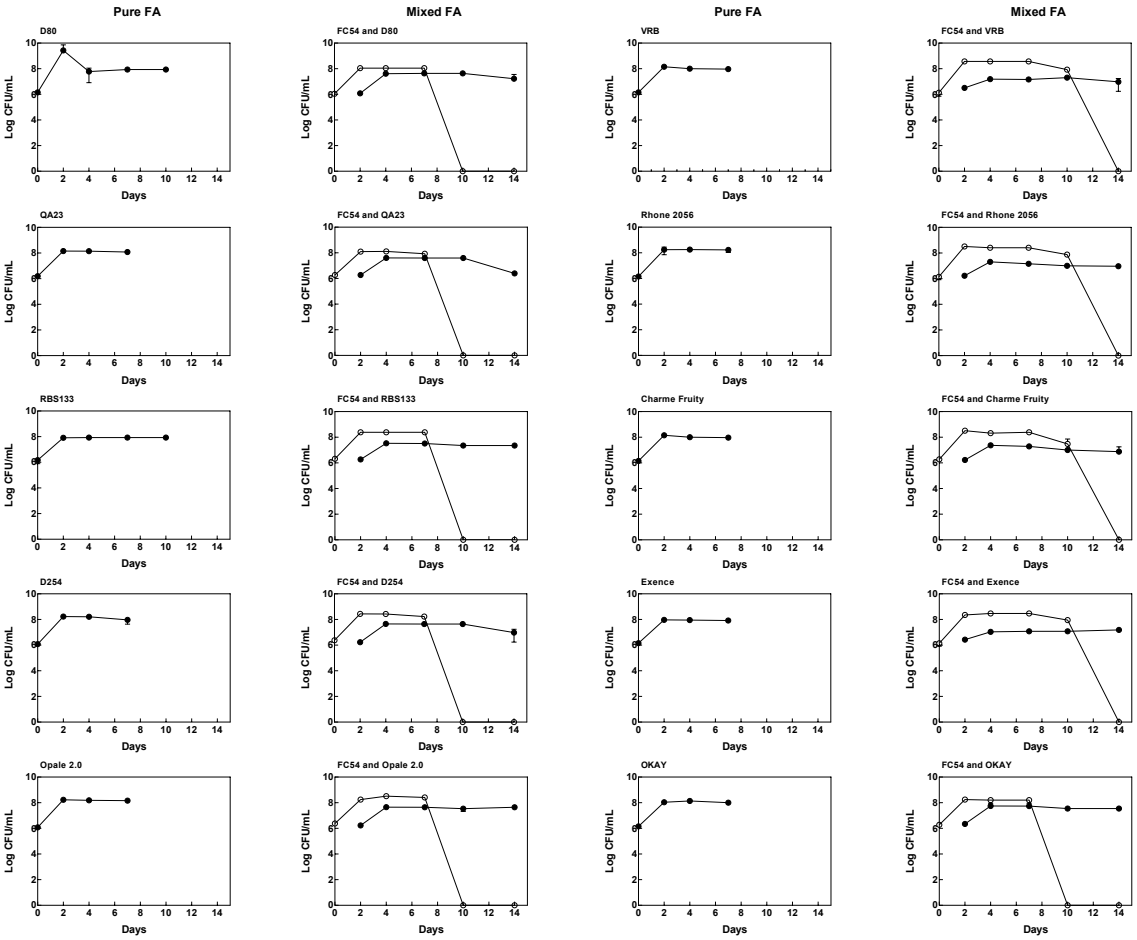

Pure FA
Mixed FA
Pure FA
Mixed FA
D80
FC54 and D80
VRB
FC54 and VRB
QA23
FC54 and QA23
Rhone 2056
FC54 and Rhone 2056
RBS133
FC54 and RBS133
Charme Fruity
FC54 and Charme Fruity
D254
FC54 and D254
Exence
FC54 and Exence
Opale 2.0
FC54 and Opale 2.0
OKAY
FC54 and OKAY
Log CFU/mL
Days

Fig. 2

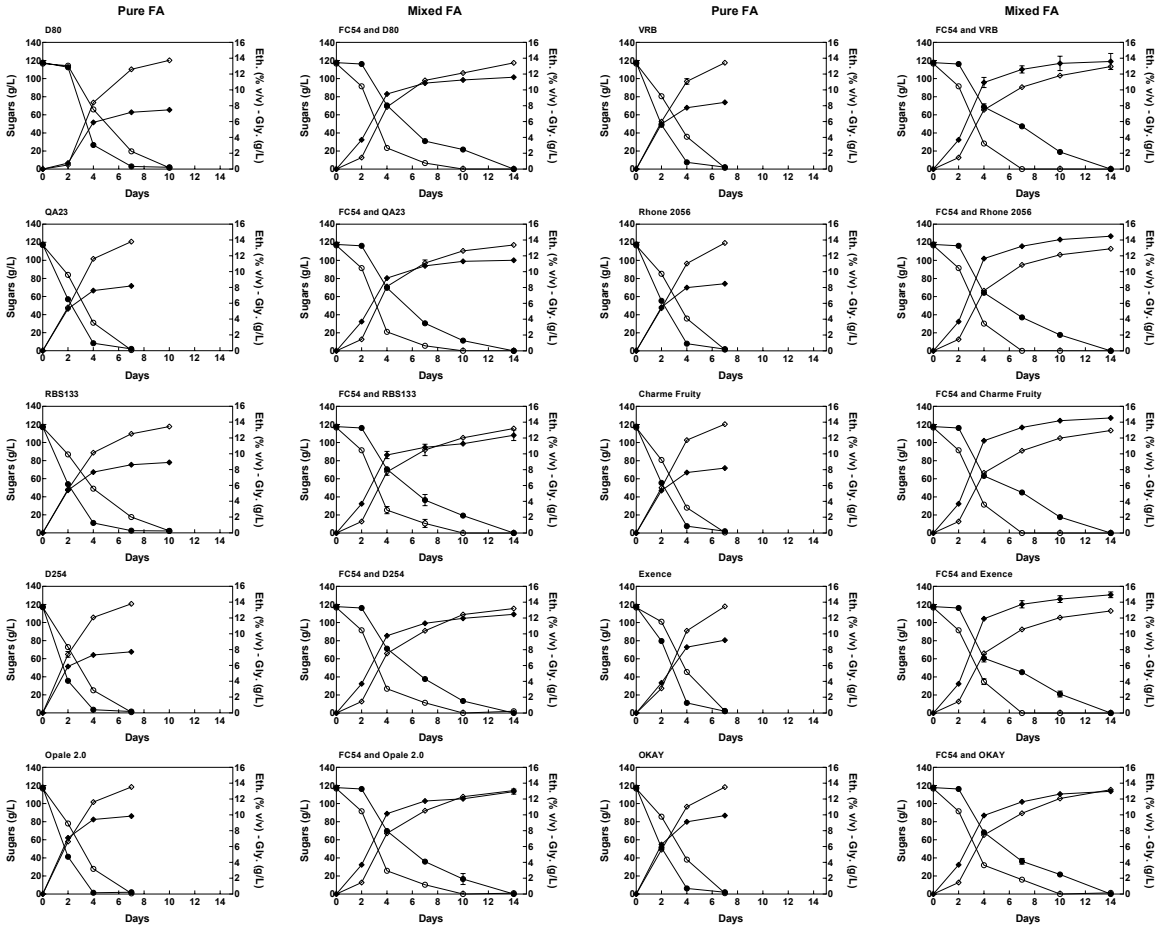

Pure FA
Mixed FA
Pure FA
Mixed FA
D80
FC54 and D80
VRB
FC54 and VRB
QA23
FC54 and QA23
Rhone 2056
FC54 and Rhone 2056
RBS133
FC54 and RBS133
Charme Fruity
FC54 and Charme Fruity
D254
FC54 and D254
Exence
FC54 and Exence
Opale 2.0
FC54 and Opale 2.0
OKAY
FC54 and OKAY
Sugars (g/L)
Eth. (% v/v) - Gly. (g/L)
Days

Fig. 3

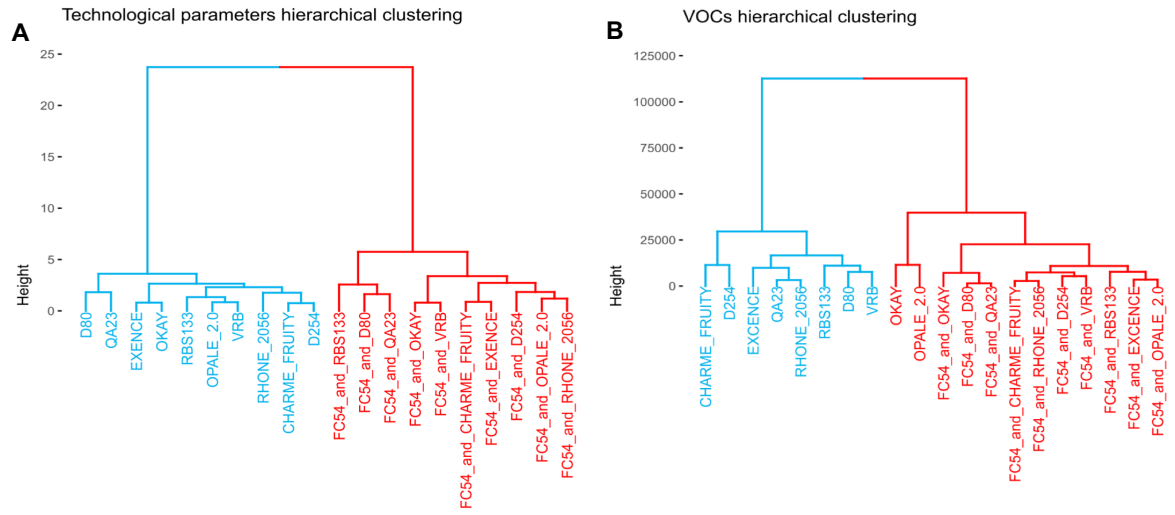

A
Technological parameters hierarchical clustering
Height
25
20
15
10
5
0
D80
QA23
EXENCE
OKAY
RBS133
OPALE_2.0
VRB
RHONE_2056
CHARME_FRUITY
D254
FC54_and_RBS133
FC54_and_D80
FC54_and_QA23
FC54_and_OKAY
FC54_and_VRB
FC54_and_CHARME_FRUITY
FC54_and_EXENCE
FC54_and_D254
FC54_and_OPALE_2.0
FC54_and_RHONE_2056
B
VOCs hierarchical clustering
Height
125000
100000
75000
50000
25000
0
CHARME_FRUITY
D254
EXCENCE
QA23
RHONE_2056
RBS133
D80
VRB
OKAY
OPALE_2.0
FC54_and_OKAY
FC54_and_D80
FC54_and_QA23
FC54_and_CHARME_FRUITY
FC54_and_RHONE_2056
FC54_and_D254
FC54_and_VRB
FC54_and_RBS133
FC54_and_EXENCE
FC54_and_OPALE_2.0

Fig. 4

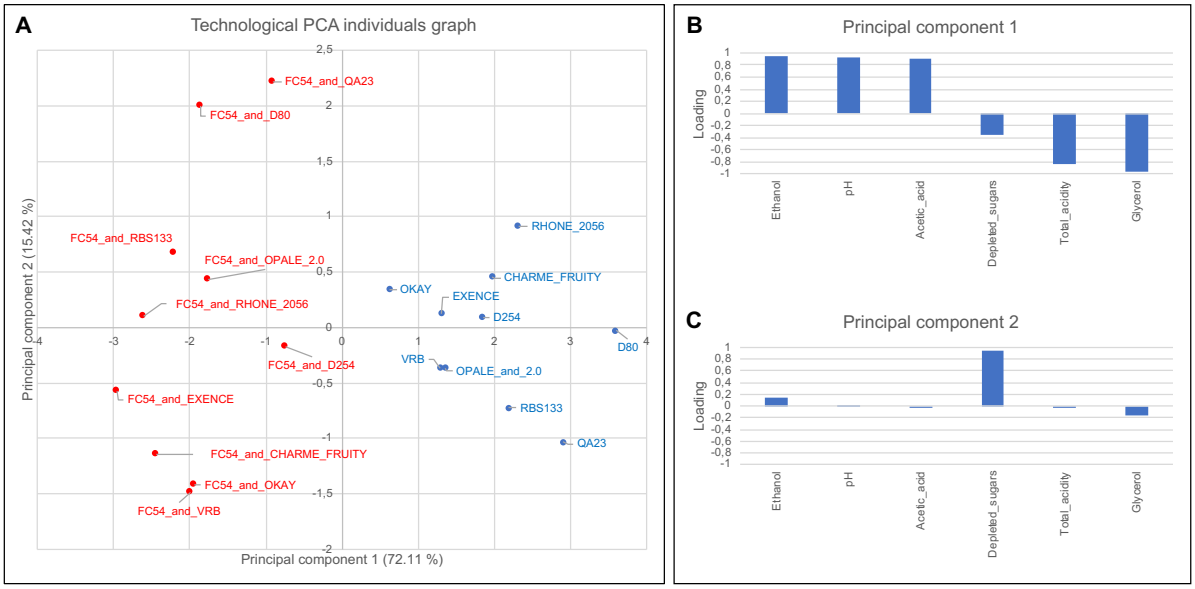
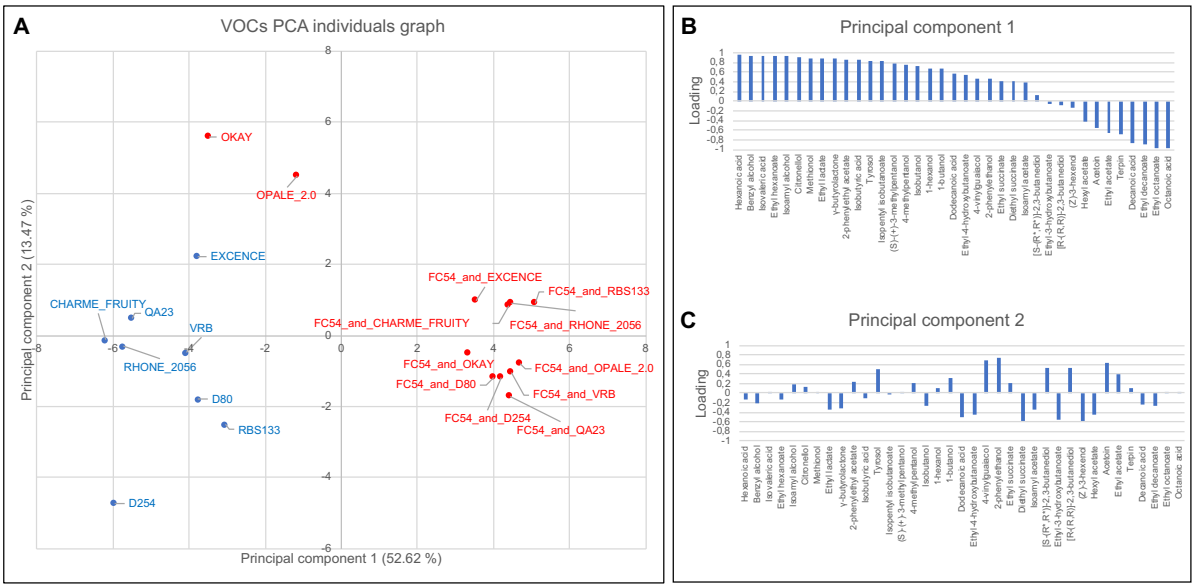

A
Technological PCA individuals graph
Principal component 2 (15.42 %)
Principal component 1 (72.11 %)
FC54_and_QA23
FC54_and_D80
FC54_and_RBS133
FC54_and_OPALE_2.0
FC54_and_RHONE_2056
FC54_and_D254
FC54_and_EXENCE
FC54_and_CHARME_FRUITY
FC54_and_OKAY
FC54_and_VRB
RHONE_2056
CHARME_FRUITY
OKAY
EXENCE
D254
D80
VRB
OPALE_and_2.0
RBS133
QA23
B
Principal component 1
Loading
Ethanol
pH
Acetic_acid
Depleted_sugars
Total_acidity
Glycerol
C
Principal component 2
Loading
Ethanol
pH
Acetic_acid
Depleted_sugars
Total_acidity
Glycerol

Fig. 5


A
VOCs PCA individuals graph
OKAY
OPALE_2.0
EXCENCE
CHARME_FRUITY
QA23
VRB
RHONE_2056
D80
RBS133
D254
FC54_and_EXCENCE
FC54_and_RBS133
FC54_and_CHARME_FRUITY
FC54_and_RHONE_2056
FC54_and_OKAY
FC54_and_OPALE_2.0
FC54_and_D80
FC54_and_VRB
FC54_and_D254
FC54_and_QA23
Principal component 2 (13.47 %)
Principal component 1 (52.62 %)
B
Principal component 1
Loading
C
Principal component 2
Loading