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Vasileios Englezos[1§], Fabrizio Torchio[2§], Francesco Cravero[1], Fabio Marengo[1], Simone Giacosa[1], Vincenzo Gerbi[1], Kalliopi Rantsiou[1], Luca Rolle[1] and Luca Cocolin[1*]

[1]University of Torino, Department of Agricultural, Forest and Food Science, University of Turin, Agricultural Microbiology and Food Technology Sector, Largo Paolo Braccini 2, 10095 Grugliasco, Torino, Italy.

[2]Istituto di Enologia e Ingegneria Agro-Alimentare, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84, 29122 Piacenza, Italy.

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

§The authors contributed equally to this paper.

ABSTRACT

In recent years there is an increasing global interest for the use of selected non-*Saccharomyces* yeasts by the winemaking industry, mainly due to their positive contribution to the wine complexity. In this study, *Starmerella bacillaris* (synonym *Candida zemplinina*) and *Saccharomyces cerevisiae* were evaluated in mixed (co-inoculated and sequentially) inoculated fermentations with the aim of improving the aroma profile of Barbera wine. The different inoculation protocols and combination of strains tested, influenced the interactions and the fermentation behaviour of the two yeast species. The wines produced with mixed cultures contained higher amounts of glycerol and pleasant esters compared to the wine fermented with *S. cerevisiae* alone. The use of mixed culture fermentations with selected yeast strains and appropriate inoculation strategies could be considered as a tool to enhance the aroma profile of wines produced from non-floral grape varieties like Barbera.

Keywords: Non-*Saccharomyces*; *Starmerella bacillaris*; Wine fermentation; Mixed fermentation; Aroma

1. Introduction

The Barbera grape is Piedmont's most widely planted red variety of *Vitis vinifera* L. Barbera vineyards are located mainly in two big areas, which produce the most outstanding wines of this grape variety, the region near the town of Alba (Barbera d'Alba) and Asti (Barbera d'Asti). Even if the Nebbiolo-based wines (Barolo and Barbaresco) are considered as the most renowned red wines of this region, Barbera is thde quintessential "wine of the people". It is meant to be enjoyed young during the meals. The sensory quality of young fresh wines, produced from non-aromatic grape varieties, like Barbera, depends greatly on numerous chemical constituents, mainly extracted during the pre-fermentation and fermentation process (Delfini et al., 2001).

Among other parameters, the volatile aroma compounds need special attention since it has a substantial influence on the wine quality and its acceptance by the wine consumers (Bruwer, Saliba, & Miller, 2011; Swiegers, Bartowksy, Henschke, & Pretorius, 2005). Aroma is considered as one of the main parameters that is affected by innumerable variations during wine production, ranging from viticulture to winemaking. Particularly the nature and amount of the volatile compounds can be influenced by environmental factors, cultivar and vineyard management, fermentation conditions and lastly by the microbial community consisting of non-*Saccharomyces* and *S. cerevisiae* yeast species which take over the fermentation (Fleet, 2003; González-Barreiro, Rial-Ortero, Cancho-Grande, & Simal-Gándara, 2015; Lambrechts & Pretorius, 2000; Swiegers, Francis, Herderich, & Pretorius, 2006).

Wine yeasts found on grapes and consequently in the grape juice, have a strong impact on the wine quality and composition, since are responsible for the production

of hundreds of secondary products, which contribute collectively, or individually, to the wine character and composition (Fleet 2003; Lambrechts & Pretorius, 2000; Romano, Fiore, Paraggio, Caruso, & Capece, 2003).

Wine production is based on spontaneous or inoculated fermentation and in both cases, the dominance of *S. cerevisiae,* either indigenous or inoculated, is desired in order to ensure a complete consumption of sugars. However, the presence of non-*Saccharomyces* yeast has been documented (Fleet, 2008), at significant levels (up to 10^7 -10^8 CFU/mL), during fermentation progress and for longer periods than previously thought (Bokulich, Swadener, Sakamoto, Mills, & Bisson, 2015; Cocolin, & Mills 2003). Few years ago, it was believed that the presence of non-*Saccharomyces* yeasts, could make the wine defective due to the production of metabolites of unpleasant origin (Romano, Suzzi, Comi, & Zironi, 1993). Nowadays, this trend is changing and the inoculation of mixed cultures of selected non-*Saccharomyces* yeasts in combination with highly fermentative *S. cerevisiae* strains able to ensure the complete consumption of sugars, is gaining attention and considered as an up-to-date inoculation strategy to enhance wine complexity and avoid unwanted compounds to be produced (Ciani & Comitini, 2015; Fleet, 2008; Jolly, Varela, & Pretorius, 2013) In this context, over the last years there has been an increasing interest regarding non-*Saccharomyces* yeasts and in order to improve the chemical composition and sensory aspect of the wines (Andorrà, Berradre, Mas, Esteve-Zarzoso, & Guillamón, 2012; Gobbi et al., 2013; Sadoudi et al., 2012; Soden, Francis, Oakey, & Henschke, 2000).

The increasing interest of winemakers in improving the complexity of young fresh wines produced from non-aromatic grape varieties requires further effort into understanding the metabolic profiles of specific non-*Saccharomyces* yeast species. To

gain an insight into the contribution of these species to wine aroma, the aim of this work was to evaluate the use of controlled multi-starter fermentation cultures of *Starmerella bacillaris* and *Saccharomyces cerevisiae* to enhance the analytical composition of Barbera wine. Two inoculation protocols were investigated: i) inoculation of both species at the beginning of the fermentation process (co-inoculation), and ii) inoculation of *S. cerevisiae* two days after *Starm. bacillaris* inoculation (sequential inoculation). Control wines were also produced by fermenting the same must with each of the *S. cerevisiae* and *Starm. bacillaris* strains in pure culture. Metabolic profiles of wines produced were compared, in order to highlight the effect of the inoculation strategy and strain selection on the final product.

2. Materials and Methods

2.1. Yeast strains

Four *Starm. bacillaris* (FC54, BC60, EFR3B and C.z 02) and two *S. cerevisiae* (ScBa49 and ScBa50) strains from the yeast culture collection of the Department of Agricultural, Forest and Food Science (DISAFA, University of Turin, Italy) were used in this study. *Starm. bacillaris* strains were isolated from grape and musts of different varieties and were selected for their oenological attributes in laboratory scale fermentations (Englezos et al., 2015).

2.2. Must preparation

Barbera grapes were harvested, destemmed and crushed. The must with grape skins was heated to 60 °C for 1h to promote color extraction in a process called *thermovinification* (Boulton, Singleton, Bisson, & Kunkee, 1996) and to deactivate indigenous yeast populations already present in the must. The grape juice was then separated using a stainless steel sieve, cooled down and frozen at -20 ^{o}C until use. The efficiency of the pasteurization was checked by plating 100 μL of the treated must on WLN medium (Biogenetics, Milan, Italy) and then incubated at 28 °C for 5 days. The unfermented must had the following composition: pH 3.20; titratable acidity 5.39 (expressed as g/L of tartaric acid); sugar concentration 244.4 g/L.

2.3. Inoculation procedure

For each strain, an aliquot of a stock in YPD broth (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose with 20 % glycerol), stored at – 80 ^{o}C was streaked onto a WLN medium, 48 h before the preparation of inoculum. Afterwards, one fresh single colony was selected to inoculate 10 mL of sterile must. After 24 h of incubation at 25 °C, 30 mL of sterile must were added to the activated inoculum and then incubated for another 24 h at the same temperature. Finally, the preadapted inoculum was added in 360 mL of fresh sterile must.

2.4. Microfermentation trials

Three sets of fermentations were performed: inoculation of each *Starm. bacillaris* and S. *cerevisiae* strains in pure culture fermentations, simultaneous

inoculation of both yeast species (co-inoculation) and inoculation of *S. cerevisiae* after 48 hours from the *Starm. bacillaris* inoculation (sequential inoculation). Mixed fermentations were carried out, using 8 different combinations of *Starm. bacillaris* and *S. cerevisiae*, according to the experimental plan reported in Table 1. Fermentations were carried in 500 mL sterile glass bottles, containing 400 mL of sterile must per bottle. Pure and mixed culture fermentations were inoculated with the abovementioned preadapted cultures, to achieve an initial cell population of about 1 x 10^6 cells/mL which was determined through plate counts on WLN medium. The bottles were equipped with sterile glass air locks containing sterile paraffin oil, to allow the carbon dioxide evolved during the fermentation process to escape from the fermenting juice. Fermentations were performed twice, under static conditions at 25 ± 1 °C (semi-anaerobic conditions). Fermentations were stopped when the weight loss remained stable for two days. Wines from both pure and mixed fermentations were then refrigerated for two days at 4 °C to remove solid parts. Afterwards, a solution of potassium metabisulfite was added to the wines, to achieve a total sulfur dioxide concentration of 50 mg/L, which were stored at -4 °C until analysis.

2.5.Microbiological Analyses

From each bottle, 1 mL samples were collected in duplicate at 0, 1, 2, 4, 7, 14 and 21 days from the beginning of fermentation to evaluate the viable cell populations. One hundred microliter aliquots of serial dilutions were plated on WLN medium, which allows the visual differentiation of the two yeast species. Plates were incubated

at 28 °C and the two types colonies were visually differentiated as described previously by Rantsiou et al. (2012) and subsequently counted.

2.6. Chemical analyses

2.6.1. Determination of standard chemical parameters

The production of glycerol, alcohol and acetic acid, as well as glucose and fructose consumption, were directly quantified by HPLC using an Agilent 1260 Infinity HPLC system (Milford, MA, USA), equipped with a UV detector set to 210 nm and a refractive index detector, as described in Rolle et al. (2012). Fermentation purity was calculated as the amount of acetic acid produced in relationship to ethanol produced (acetic acid (g/L) / ethanol (% v/v)) (Ciani, & Macarelli, 1998).

2.6.2. Volatile compounds determination

Volatile aroma compounds from wines produced using pure and mixed cultures of yeasts, were directly analysed by Head Space Solid Phase Micro-Extraction (HS-SPME), coupled by Gas Chromatography-Mass Spectroscopy (GC-MS) as previously reported (Rolle, Torchio, Giacosa, & Río Segade, 2015; Whitener et al., 2015), with some modifications. Five mL of each wine sample were placed into 20 mL glass vials with a headspace screw cap containing, 5 mL of water, 2 g of NaCl and 1-heptanol solution (200 μL of 15.5 mg/L solution in 10% v/v ethanol) as an internal standard (IS). The sealed vials were carefully shaken to dissolve the NaCl before the

analysis. Silicon septa (Supelco, Bellefonte, PA, USA) were used with 18 mm diameter screw caps to seal the glass vials. The fiber used for the extraction of the volatile compounds was the 50/30 μm DVB/CAR/PDMS fiber (Supelco) and the procedure was performed with Gerstel MPS2 XL auto sampler (Gerstel, Baltimore, MD, USA). The sample vial was placed at 40 °C for 10 min, then the SPME were exposed to the headspace of the capped vial for 20 min at 40 °C. Afterwards the fiber was inserted into the injection port of the GC apparatus for the thermal desorption. The thermal adsorption of the analytes from the fibre was carried out, in splitless mode at 250 °C for 5 min.

The analyses were performed using an Agilent 7890C gas chromatograph (GC) (Little Falls, DE, USA) equipped with an Agilent 5795 mass selective detector (MS) and a DB-WAX capillary column (30 m x 0.25 mm inner diameter, 0.25 μm film thickness, J&W Scientific Inc., Folsom, CA, USA). Helium was used as carrier gas, with a flow-rate of 1 mL/min. The injection port temperature was 250 °C, the ion source temperature was 150 °C and interface was 280 °C. The GC oven program used was as follows: 40 °C for 5 min, and an increase to 200 °C (at a rate of 2 °C/min) for 10 min followed by an increase of 5 °C/min to 220 °C. The detection was carried out by electron impact mass spectroscopy in total ion current (TIC) mode, using ionization energy of 70 eV. The analyses were performed in a scan range between m/z 33-330. Identification of the volatile compounds was carried out using mass spectra and retention indices, reported in the literature and in the database (http://webbook.nist.gov/chemistry/) and pure standards when available (2,3-butanediol isomers mixture, 2-ethyl hexanol, 2-methyl-1-propanol, 1-octanol, 2-phenylethanol, diethyl succinate, ethyl acetate, ethyl decanoate, ethyl dodecanoate, ethyl heptanoate, ethyl hexanoate, ethyl nonanoate, ethyl octanoate, ethyl

phenylacetate, hexanal, hexanoic acid, hexyl acetate, linalool, methyl decanoate, methyl salicylate, octanoic acid and β-damascenone were supplied by Sigma [Milan, Italy]).

For semi-quantification purposes, the relative peak area of each identified compound was measured and then compared with the relative peak area of the added internal standard.

2.7. Statistical analysis

The quantities of the metabolites produced were subjected to one-way ANOVA to uncover statistical differences between the wines produced from the different inoculation protocols. The significant differences among the data obtained were established through the use of Tukey-b test, at $p < 0.05$. Principal Component Analysis (PCA) based on the concentration of the volatile compounds formed from each inoculation strategy and couple of strains tested, was also carried out in order to enlighten the relationship between samples and variables. Statistical analyses were performed with the statistical software package IBM SPSS Statistics (version 21.0, IBM Corp., Armonk, NY, USA).

3. Results and discussion

3.1. Yeast growth during fermentation

The growth dynamics of the pure culture fermentations conducted with *Starm. bacillaris* and *S. cerevisiae* are summarized in Fig.1. As can be seen, both species grew equally reaching a cell population of around 10^8 CFU/mL in two days. The cell

population remained stable for seven days and then started to decline, with plate counts ranging from 10^6 to 10^7 CFU/mL for *S. cerevisiae* strains, probably due to the nutrient depletion (Cramer, Vlassides, & Block, 2002) or/and the presence of significant levels of alcohol present (Alexandre & Charpentier, 1998), while *Starm. bacillaris* population became undetectable after 21 days.

In Fig. 2, the growth dynamics of the mixed culture fermentations are illustrated. Remarkably, all couples showed comparable growth dynamics. When both yeasts were co-inoculated (Fig. 2, panel A) the two population dynamics were similar in all cases (for all couples of strains tested). They achieved the stationary phase (almost 5 x 10^7 CFU/mL) in two days. A remarkable decrease of *Starm. bacillaris* population was registered on day 4, while *S. cerevisiae* population remained stable throughout the whole period. The early death of *Starm. bacillaris* cells appeared to be the result of the antagonistic effect of *S. cerevisiae* strains upon non-*Saccharomyces* yeasts, as also reported by Andorrà et al. (2010) in mixed fermentations with *Starm. bacillaris*, *H. uvarum* and *S. cerevisiae*.

A completely different picture emerged when *S. cerevisiae* was sequentially inoculated (Fig. 2, panel B). Compared to fermentations which *S. cerevisiae* strains pure inoculated, *Starm. bacillaris* caused a small reduction in rate of growth and the maximum population (remaining below 10^8 CFU/mL), achieved by both *S. cerevisiae* strains. There appeared to be an inhibitory effect of *Starm. bacillaris*, probably due to the high consumption of nutrients prior to *S. cerevisiae* inoculation, in agreement with the findings of Englezos et al. (2016). The cell viability of both yeasts, remained relative high during the fermentation and started to decrease from day 14 onwards. The capability of the *Starm. bacillaris* to dominate *S. cerevisiae* strains and persist up

to the middle-end phase of the fermentation process was previously observed in laboratory scale fermentations (Cocolin, & Mills 2003; Rantsiou et al., 2012).

3.2. Aroma composition of the wines

The main objective of this study was to evaluate how the inoculation strategy and strain selection could modulate the production of volatile aroma compounds during fermentation. The results are shown in Table 2, as an average value for each inoculation protocol applied in this study. Chromatographic analysis allowed the identification of 42 volatiles compounds (Supplementary Fig. 1) belonging to seven chemical families, including 9 alcohols, 19 esters, 4 fatty acids, 4 aldehydes and ketones, 2 terpenes and C13-norisoprenoids, 2 sulfur compounds and 1 lactone.

By comparing these secondary aroma compounds, all the fermentation protocols produced the same levels of alcohols, in concentrations that could enhance the desirable complexity in the wines (Rapp & Versini, 1991). The total alcohol concentration in the mixed fermentations was found to be very similar to this occurring in pure fermentations with *S. cerevisiae*, mainly due to the contribution of the main aromatic alcohols 2-phenylethanol and isoamyl alcohol, in accordance with the results reported by Sadoudi et al. (2012). On the other hand, aliphatic alcohols (1-propanol, 2-methyl-1-propanol, hexanol and 2-ethyl-hexanol) were detected in significantly higher concentrations, in the fermentations in which *Starm. bacillaris* was involved (Andorrà et al., 2010; Zara et al., 2014).

Total esters concentration was not affected by the presence of *Starm. bacillaris* and all the wines showed the same esters production pattern. Ethyl acetate, the most significant ester present in the wines (Swiegers et al., 2005), was generally produced

in relative low quantitates, well below the spoilage and threshold values of 150 and 12 mg/L, respectively, reported in the literature for the red wines (Corison, Ough, Berg, & Nelson, 1979). In this context, low production of this ester (below 70 mg/L) is considered positive for the wine aroma and complexity, since it is associated with fruity, solvent and balsamic descriptors (Rapp, Pretorius, & Kugler, 1992). Both mixed and pure fermentations with *S. cerevisiae* produced wines with significant increased concentrations of 2-phenyl acetate (Andorrà et al., 2010), which contribute to the overall flavour of the young wines and thus the wines could be characterized by higher complexity, in accordance with Lambrechts & Pretorius (2000). The concentration of some pleasant esters, such as hexyl acetate, ethyl hexanoate, ethyl heptanoate, ethyl dodecanoate and ethyl butanoate was significantly higher in the mixed fermentations (Fig. 3), highlighting an important positive interaction between the two species, as previously reported by Andorrà et al. (2010) in Macabeo must.

The concentration of specific aldehydes and ketones (2-nonanone and 4-methyl- benzaldehyde) was also found to be significantly higher in the wines produced by mixed fermentations and due to the low threshold values of these compounds they could enhance the overall aroma and bouquet of the wines (Lambrechts & Pretorius, 2000). The production of free terpenes and C13-norisoprenoids was also found to be significantly higher in the mixed fermentations compared to the pure *S. cerevisiae* fermentations, as already reported in Sadoudi et al. (2012) and Whitener et al. (2016). Regarding the fatty acids production, no significant differences were observed in the wines, with only exception of the dodecanoic acid, which was produced in higher quantities in the co-inoculated trials. Finally, the concentration sulphur compounds

and lactones increased significantly in pure culture fermentations with *Starm. bacillaris* and *S. cerevisiae* respectively.

The aroma values of 30 compounds (Table 2), which differences among the inoculation protocols were significant were analysed using a Principal Component Analysis (PCA), in order to evaluate the correlation among samples and aroma compounds. The first two components obtained explained the 63 % of the total variance, while the replicates were clustered quite well indicating a high experimental reproducibility (Fig. 3). The first principal component (PC1) was correlated positively with the most important esters, isoamyl alcohol and 2-phenylethanol. The second principal component (PC2) was correlated positively to diethyl succinate, 2-phenyl ethyl acetate and negatively to hexanol, 2-ethyl hexanol, β-damascenone and 1-propanol.

As it can be seen from the PCA output, the samples were classified into two groups (Fig. 3, Panel B). One group clustered mixed fermentations (both co-inoculated and sequentially inoculated) and pure fermentations conducted by *Starm. bacillaris* strains, while the other group included the pure fermentations performed by *S. cerevisiae* strains. Wines produced from mixed fermentations, showed a homogeneous distribution in the PCA plot independent the couple of strains used, with only exception a major part of wines produced from sequentially inoculated cultures which were grouped closer to pure fermented wines with *Starm. bacillaris*. Based on these results, it can be speculated that *Starm. bacillaris* is effective in impacting and modulating the aroma profiles of the wines produced from the mixed fermentations, in agreement with general observations that non-*Saccharomyces* yeasts could enhance the organoleptic complexity of the wines (Jolly et al., 2013).

3.3. Analytical profiles of the wines produced by the pure and mixed cultures

The chemical composition of the wines produced from pure and mixed cultures are shown in Table 3. Pure culture fermentations of the *Starm. bacillaris* strains exhibited a clear fructophilic pattern, leaving only glucose in the medium (29.3 – 35.9 g/L) confirming the clear fructophilic character of this species (Rantsiou et al., 2012). Concerning, ethanol production all the wines reached significant values ranging from 11.8 to 12.2 (% v/v). On the other hand, *S. cerevisiae* strains exhibited more complete utilization of sugars and produced less glycerol (7.1 – 7.3 g/L) than the wines fermented by *Starm. bacillaris* in pure culture, in agreement with previous studies (Englezos et al., 2015; Suzzi et al., 2012). Concerning acetic acid production all the strains gave values not greater than 0.50 g/L.

Fermentations conducted by using a combination of *Starm. bacillaris* and *S. cerevisiae*, independent from the inoculation strategy applied, produced partially fermented wines (86 – 97 % total sugar consumption) (Table 4). Compared to sequential inoculated fermentations, co-inoculated fermentations produced wines with less residual sugars. This data was in accordance with the plate count results, since *Starm. bacillaris* in sequential fermentations affected *S. cerevisiae* growth and subsequently its metabolic activity. The inhibitory effect observed in these fermentations, validate the observations by Englezos et al. (2016, accepted manuscript). Comparing residual sugar composition, co-inoculated fermentations fermented fructose at a lower rate, leaving a higher residual fructose concentration, compared to sequential inoculated wines, probably due to the competitive ability of *S.*

cerevisiae over *Starm. bacillaris* yeast cells. Chemical analyses values for the co-inoculated wines did not vary substantially from those of *S. cerevisiae* strains in pure cultures, in agreement with Englezos et al. (2016, accepted manuscript) and Soden et al. (2000). On the contrary, sequential inoculated wines were very similar in composition to the wines produced by *Starm. bacillaris* in pure culture. Glycerol concentration slightly increased (8.8 – 9.8 g/L), when compared to the pure culture *S. cerevisiae* wines (Romboli, Mangani, Buscioni, Granchi, & Vincenzini, 2015; Suzzi et al. 2012). The acetic acid production ranged from moderate amounts to values up to 0.4 g/L and fermentation purities were also very low (0.02 – 0.03).

4. Conclusion

Here, new information about the influence of *Starm. bacillaris* and *S. cerevisiae* mixed fermentations on the formation of volatile aroma compounds was presented. The results obtained revealed significant differences between the two yeast species and high similarities among the two inoculation protocols investigated in this study. As shown, mixed culture fermentations resulted in greater complexity due to the higher production of volatile compounds, independently of the couple tested. A better knowledge of the environmental factors (such as nitrogen composition and concentration), which modulate the yeast growth, will allow a greater understanding and management of the production of specific metabolites during the alcoholic fermentation.

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Table 1 Experimental plan used in this study

Pure fermentations[1]	Mixed fermentations[1,2]	
	Co-inoculation	Sequential inoculations
Strains	Couples	Couples
S. cerevisiae	FC54 and ScBa49	FC54 and ScBa49
ScBa49	FC54 and ScBa50	FC54 and ScBa50
ScBa50	EFR3B and ScBa49	EFR3B and ScBa49
Starm. bacillaris	EFR3B and ScBa50	EFR3B and ScBa50
FC54	C.z 02 and ScBa49	C.z 02 and ScBa49
EFR3B	C.z 02 and ScBa50	C.z 02 and ScBa50
C.z 02	BC60 and ScBa49	BC60 and ScBa49
BC60	BC60 and ScBa50	BC60 and ScBa50

[1]Inoculum size: 1.0 x 10^6 cells/mL

[2]Inoculum ratio: 1:1

Table 2 Semi-quantitative concentration of the volatile compounds (ratio among volatile compounds and internal standards) produced from the different inoculation protocols.

Compounds	***Kovats index***	*S. cerevisiae*	*Starm. bacillaris*	Co-inoculation	Sequential inoculation	Sig[1]	Odour description[2]
Alcohols							
(R,R)-2,3-Butanediol	1552	0.615 ± 0.010^{b}	0.525 ± 0.052^{ab}	0.433 ± 0.068^{ab}	0.472 ± 0.050^{ab}	**	Butter, creamy
(R,S-meso)-2,3-Butanediol	1587	0.164 ± 0.035^{b}	0.148 ± 0.016^{ab}	0.122 ± 0.016^{a}	0.134 ± 0.013^{ab}	*	Butter, creamy
2-Ethyl-hexanol	1501	0.005 ± 0.002^{a}	0.016 ± 0.002^{c}	0.013 ± 0.002^{b}	0.013 ± 0.002^{bc}	***	Sweet, floral , citrus
Hexanol	1367	0.145 ± 0.006^{a}	0.195 ± 0.003^{b}	0.193 ± 0.011^{b}	0.205 ± 0.006^{b}	***	Resin, flower, green
Isoamyl alcohol	1231	5.712 ± 0.153^{ab}	3.908 ± 0.416^{a}	6.466 ± 1.467^{b}	7.935 ± 2.033^{b}	**	Fusel, fruity, banana
2-Methyl-1-propanol	1113	0.002 ± 0.001^{a}	0.298 ± 0.021^{b}	0.380 ± 0.087^{b}	0.515 ± 0.176^{c}	***	Ethereal
1-Octanol	1568	0.019 ± 0.001	0.021 ± 0.003	0.018 ± 0.006	0.023 ± 0.002	NS	Floral, citrus, rose
2-Phenylethanol	1885	7.08 ± 0.688^{ab}	4.468 ± 0.599^{a}	7.460 ± 2.787^{ab}	10.116 ± 3.483^{b}	*	Floral, rose, sweet
1-Propanol	1052	0.003 ± 0.001^{a}	0.013 ± 0.005^{b}	0.013 ± 0.006^{b}	0.011 ± 0.003^{b}	**	Alcohol, pungent
Σ Alcohols		13.745 ± 0.971^{ab}	9.596 ± 0.965^{a}	15.097 ± 4.367^{ab}	19.422 ± 5.691^{b}	**	
Esters							
Diethyl succinate	1684	0.113 ± 0.010^{b}	0.053 ± 0.018^{a}	0.068 ± 0.029^{a}	0.089 ± 0.024^{ab}	**	Fruity
Ethyl acetate	nd	5.654 ± 0.021^{a}	6.132 ± 0.340^{a}	6.83 ± 1.229^{ab}	8.072 ± 0.968^{b}	**	Vanish, nail polish, fruity
Ethyl butanoate	1040	0.110 ± 0.008^{a}	0.111 ± 0.013^{a}	0.171 ± 0.034^{b}	0.203 ± 0.035^{b}	***	Sweet, fruity
Ethyl decanoate	1648	18.462 ± 3.763	18.065 ± 5.151	21.989 ± 9.634	20.830 ± 4.338	NS	Waxy, fruity, apple, grape
Ethyl 9-decenoate	1697	0.119 ± 0.011	0.082 ± 0.047	0.108 ± 0.055	0.129 ± 0.035	NS	Fruity
Ethyl dodecanoate	1834	3.244 ± 0.527^{a}	4.602 ± 0.942^{ab}	6.426 ± 3.597^{ab}	7.682 ± 0.928^{b}	*	Sweet, waxy
Ethyl heptanoate	1344	0.003 ± 0.001^{a}	0.008 ± 0.003^{a}	0.010 ± 0.002	0.011 ± 0.003^{b}	**	Fruity, cognac
Ethyl hexadecanoate	2122	0.354 ± 0.047^{b}	0.325 ± 0.016^{b}	0.203 ± 0.085^{a}	0.427 ± 0.092^{b}	***	Waxy

Ethyl hexanoate	1249	1.685 ± 0.172[a]	2.709 ± 0.511[ab]	3.521 ± 0.783[bc]	4.027 ± 0.865[c]	***	Apple peel, fruit
Ethyl nonanoate	1543	0.016 ± 0.003	0.014 ± 0.006	0.016 ± 0.005	0.016 ± 0.003	NS	Fruity, rose, waxy
Ethyl octanoate	1445	16.577 ± 2.700	17.434 ± 5.562	22.392 ± 6.055	21.939 ± 5.577	NS	Fruity, fatty
Hexyl acetate	1286	0.209 ± 0.019[a]	0.293 ± 0.079[ab]	0.425 ± 0.148[b]	0.451 ± 0.153[b]	*	Fruit, herb
Isobutyl decanoate	1758	0.002 ± 0.002[a]	0.005 ± 0.002[ab]	0.011 ± 0.006[b]	0.011 ± 0.005[b]	*	Cognac, brandy, apricot,
Methyl decanoate	1599	0.047 ± 0.010	0.043 ± 0.013	0.047 ± 0.016	0.045 ± 0.011	NS	Winey, fruity, floral
3-Methyl-1-butyl acetate	1130	2.705 ± 0.604[ab]	1.662 ± 0.493[a]	3.159 ± 0.902[ab]	3.76 ± 1.287[ab]	*	Fruity, banana
2-Methylbuthyl octanoate	1664	0.040 ± 0.008[a]	0.039 ± 0.008[a]	0.103 ± 0.066[b]	0.097 ± 0.029[b]	*	Fruity
3-Methylbutyl pentadecanoate	1846	0.072 ± 0.011[a]	0.098 ± 0.008[ab]	0.272 ± 0.185[b]	0.279 ± 0.063[b]	*	Fruity
Nerolidyl acetate	1971	0.068 ± 0.002[b]	0.039 ± 0.008[a]	0.029 ± 0.010[a]	0.040 ± 0.006[a]	***	Floral, woody
2-Phenylethyl acetate	1815	1.128 ± 0.150[b]	0.519 ± 0.116[a]	1.118 ± 0.441[b]	1.398 ± 0.409[b]	**	Floral, rose, sweet, honey
Σ Esters		50.609 ± 7.226	52.236 ± 11.261	66.894 ± 21.566	69.507 ± 12.556	NS	
Fatty acids							
Decanoic acid	2138	0.527 ± 0.013	0.385 ± 0.037	0.736 ± 0.335	0.495 ± 0.180	NS	Fatty, rancid
Dodecanoic acid	2266	0.014 ± 0.006[a]	0.014 ± 0.006[a]	0.082 ± 0.05[b]	0.039 ± 0.026[ab]	**	Fatty
Hexanoic acid	1838	0.205 ± 0.006	0.197 ± 0.006	0.256 ± 0.076	0.243 ± 0.029	NS	Cheese, sweaty, fatty
Octanoic acid	1986	0.580 ± 0.002	0.493 ± 0.010	0.66 ± 0.253	0.525 ± 0.139	NS	Fatty, rancid, cheese
Σ Fatty acids		1.326 ± 0.013	1.091 ± 0.043	1.733 ± 0.689	1.300 ± 0.358	NS	
Aldehydes and ketones							
Decanal	1506	0.008 ± 0.005	0.006 ± 0.003	0.008 ± 0.002	0.008 ± 0.003	NS	Soap, orange, peel, tallow
4-Methyl-benzaldehyde	1653	0.011 ± 0.003[a]	0.035 ± 0.01[b]	0.034 ± 0.01[b]	0.047 ± 0.018[b]	**	Almond
3-Methyl butanone	nd	0.111 ± 0.045	0.174 ± 0.089	0.188 ± 0.079	0.205 ± 0.045	NS	Camphor
2-Nonanone	1395	0.014 ± 0.005[a]	0.024 ± 0.008[ab]	0.042 ± 0.013[c]	0.034 ± 0.01[bc]	**	Green fruity, soap,
Σ Aldehydes and ketones		0.145 ± 0.047[a]	0.240 ± 0.079[ab]	0.272 ± 0.072[b]	0.293 ± 0.055[b]	**	

Terpenes and C13-norisoprenoid						
Linalool	1556	0.021 ± 0.001[a]	0.024 ± 0.002[a]	0.024 ± 0.003[ab]	0.027 ± 0.003[b]	**	Flower, lavender
β-Damascenone	1820	0.003 ± 0.003[a]	0.006 ± 0.002[b]	0.008 ± 0.002[b]	0.008 ± 0.002[b]	**	Apple, rose, honey
Σ Terpenes and C13-norisoprenoid		0.024 ± 0.003[a]	0.031 ± 0.003[b]	0.032 ± 0.003[bc]	0.035 ± 0.003[c]	**	
Sulphur compounds							
Benzothiazole	1916	0.01 ± 0.002[a]	0.077 ± 0.014[c]	0.039 ± 0.013[b]	0.048 ± 0.016[b]	***	Gasoline, rubber
3-(Methylthio)-1-propanol	1727	0.058 ± 0.018[c]	0.021 ± 0.003[a]	0.035 ± 0.008[ab]	0.039 ± 0.006[b]	***	Cauliflower, cabbage
Σ Sulphur compounds		0.066 ± 0.021[a]	0.098 ± 0.018[b]	0.072 ± 0.013[ab]	0.087 ± 0.019[ab]	*	
Lactones							
γ-Butyrolactone	1633	0.116 ± 0.014[b]	0.072 ± 0.006[a]	0.122 ± 0.021[ab]	0.092 ± 0.018[b]	**	Caramel, sweet

All data are expressed as average value ± standard deviation (n = 4 for *S. cerevisiae*, n= 8 for *Starm. bacillaris*, n=16 for co-inoculated and sequentially inoculated fermentations). Different Latin letters within the same row indicate significant differences among the applied inoculation protocols, according to the Tukey-b test ($p < 0.05$). nd: not determinable

[1]Sig: *, **, *** and NS indicate significance at $p < 0.05$, $p < 0.01$, $p < 0.001$ and not significant respectively.

[2]Odor descriptions were taken from http://www.flavornet.com and http://www.thegoodscentscompany.com.

Table 3 Mean concentration of organic acids, glycerol, ethanol and sugars in grape juice and wines produced from pure cultures.

Treatment	Residual sugars (g/L)	Glucose (g/L)	Fructose (g/L)	Ethanol (% v/v)	Glycerol (g/L)	Acetic acid (g/L)	Fermentation purity[1]
Must	244.4 ± 1.2	120.6 ± 0.6	123.8 ± 0.6	< 0.1	< 0.1	< 0.1	< 0.1
S. cerevisiae							
ScBa49	4.2 ± 0.5	0.9 ± 0.4	3.1 ± 0.4	14.2 ± 0.1	7.1 ± 0.3	0.35 ± 0.2	0.025 ± 0.002
ScBa50	3.8 ± 0.2	0.9 ± 0.2	2.9 ± 0.4	14.3 ± 0.1	7.3 ± 0.2	0.33 ± 0.3	0.024 ± 0.004
Starm. bacillaris							
FC54	30.9 ± 6.0	30.4 ± 5.3	0.5 ± 0.7	12.2 ± 0.0	11.8 ± 0.8	0.36 ± 0.04	0.030 ± 0.004
EFR3B	37.2 ± 0.6	35.7 ± 1.3	1.5 ± 0.7	11.8 ± 0.1	10.9 ± 0.7	0.35 ± 0.02	0.030 ± 0.002
C.z 02	29.8 ± 4.1	29.3 ± 3.4	0.5 ± 0.7	12.2 ± 0.3	11.7 ± 0.6	0.45 ± 0.05	0.037 ± 0.003
BC60	36.4 ± 4.9	35.9 ± 4.2	0.5 ± 0.7	12.1 ± 0.4	11.9 ± 0.6	0.40 ± 0.04	0.033 ± 0.004

[1]Fermentation purity: acetic acid (g/L) ÷ ethanol (% v/v).

All data are expressed as average value ± standard deviation (n = 2).

Table 4 Mean concentration of organic acids, glycerol, ethanol and sugars in the wines produced from mixed cultures.

Treatment	Glucose (g/L)	Fructose (g/L)	Glucose/fructose (-)	Ethanol (% v/v)	Glycerol (g/L)	Acetic acid (g/L)	Fermentation purity[1]	Sugar consumption (%)
Co-inoculation								
FC54 and ScBa49	2.5 ± 1.3	6.5 ± 0.7	0.37 ± 0.15	13.8 ± 0.1	7.0 ± 0.1	0.34 ± 0.03	0.024 ± 0.002	96 ± 1
FC54 and ScBa50	1.7 ± 1.2	5.3 ± 2.4	0.31 ± 0.09	14.1 ± 0.2	7.4 ± 0.2	0.33 ± 0.05	0.024 ± 0.004	97 ± 1
EFR3B and ScBa49	2.2 ± 0.1	16.6 ± 0.5	0.13 ± 0.01	13.3 ± 0.2	7.3 ± 0.2	0.42 ± 0.07	0.032 ± 0.006	92 ± 0
EFR3B and ScBa50	2.2 ± 0.7	17.0 ± 4.3	0.13 ± 0.01	13.3 ± 0.4	7.5 ± 0.1	0.3 ± 0.04	0.022 ± 0.004	92 ± 2
C.z 02 and ScBa49	1.9 ± 0.5	17.3 ± 2.9	0.11 ± 0.01	13.2 ± 0.5	7.2 ± 0.1	0.31 ± 0.03	0.024 ± 0.002	92 ± 1
C.z 02 and ScBa50	0.9 ± 0.2	8.0 ± 5.1	0.14 ± 0.06	13.7 ± 0.2	7.3 ± 0.2	0.3 ± 0.15	0.022 ± 0.011	96 ± 2
BC60 and ScBa49	1.2 ± 0.2	8.5 ± 2.9	0.15 ± 0.03	13.8 ± 0.2	7.7 ± 0.1	0.38 ± 0.02	0.028 ± 0.002	96 ± 1
BC60 and ScBa50	1.0 ± 0.1	9.2 ± 3.2	0.11 ± 0.03	13.8 ± 0.1	7.4 ± 0.1	0.33 ± 0.11	0.024 ± 0.008	96 ± 1
Sequential inoculation								
FC54 and ScBa49	15.0 ± 5.4	1.7 ± 0.7	8.76 ± 0.26	13.3 ± 0.5	8.8 ± 0.1	0.23 ± 0.01	0.017 ± 0.001	93 ± 2
FC54 and ScBa50	23.4 ± 6.5	2.9 ± 1.8	9.37 ± 3.76	12.9 ± 0.6	9.2 ± 0.4	0.36 ± 0.02	0.028 ± 0.001	89 ± 3
EFR3B and ScBa49	25.4 ± 3.1	4.3 ± 0.9	5.98 ± 0.57	12.8 ± 0.2	9.5 ± 0.1	0.32 ± 0.01	0.025 ± 0.001	88 ± 2
EFR3B and ScBa50	25.1 ± 1.0	3.2 ± 0.2	7.96 ± 0.78	12.7 ± 0.1	9.7 ± 0.1	0.40 ± 0.03	0.032 ± 0.003	88 ± 0
C.z 02 and ScBa49	28.0 ± 1.6	5.2 ± 0.4	5.40 ± 0.06	12.3 ± 0.1	9.8 ± 0.1	0.26 ± 0.04	0.021 ± 0.003	86 ± 1
C.z 02 and ScBa50	23.7 ± 0.1	2.4 ± 0.2	9.95 ± 0.77	12.8 ± 0.1	9.6 ± 0.2	0.33 ± 0.08	0.026 ± 0.006	89 ± 0
BC60 and ScBa49	27.1 ± 1.2	5.2 ± 0.7	5.21 ± 0.51	12.5 ± 0.1	9.6 ± 0.5	0.22 ± 0.03	0.017 ± 0.003	87 ± 1
BC60 and ScBa50	22.7 ± 2.3	2.1 ± 0.5	10.72 ± 1.20	12.7 ± 0.3	9.1 ± 0.6	0.41 ± 0.02	0.032 ± 0.001	90 ± 1

[1] Fermentation purity: acetic acid (g/L) ÷ ethanol (% v/v)

The data are expressed as average ± standard deviation

All data are expressed as average value ± standard deviation (n = 2).

Figure captions

Fig. 1. Growth dynamics of *Starm. bacillaris* (A) and *S. cerevisiae* (B) strains in pure cultures. *Starm. bacillaris* strains: FC54 [●], EFR3B [○], C.z 02 [◆] and BC60 [◊], *S. cerevisiae* strains: ScBa49 [■] and ScBa50 [○]. Counts are the mean CFU/mL values ± standard deviations. Data are representative of two independent experiments.

Fig. 2. Growth dynamics of the *Starm. bacillaris* (FC54 [1], EFR3B [2], C.z 02 [3] and BC60 [4]) co-inoculated (panel A) or sequentially (panel B) inoculated with *S. cerevisiae* (ScBa49 and ScBa50) strains. The arrows indicate *S. cerevisiae* inoculation. Counts are the mean CFU/mL values ± standard deviations. Data are representative of two independent experiments. Mixed fermentations: A. *Starm. bacillaris* (•) with *S. cerevisiae* strain ScBa49 (■) and B. *Starm. bacillaris* (○) with *S. cerevisiae* strain ScBa50 (●).

Fig. 3. Score plot (A) and loading plot (B) of the first and second principal components after analysis of the volatile aroma compounds produced from the pure and mixed culture fermentations. Inoculation protocols were represented as: *S. cerevisiae* (■), *Starm. bacillaris* (▲), co-inoculation (○) and sequential inoculated fermentations (◊).

Fig. 1. Supplementary data. Chromatogram of fermented wine. 1 Ethyl acetate, 2 3-Methyl butanone, 3 Ethyl butanoate, 4 1-Propanol, 5 2-Methyl-1-propanol, 6 3-Methyl-1-butyl acetate, 7 Isoamyl alcohol, 8 Ethyl hexanoate, 9 Hexyl acetate, 10 Ethyl heptanoate, 11 Hexanol, 12 2-Nonanone, 13 Ethyl octanoate, 14 2-Ethyl-hexanol, 15 Decanal, 16 Ethyl nonanoate, 17 (R,R)-2,3-Butanediol, 18 Linalool, 19 1-Octanol, 20 (R,S-meso)-2,3-Butanediol, 21 Methyl decanoate, 22 γ-Butyrolactone, 23 Ethyl decanoate, 24 4-Methyl-benzaldehyde, 25 2-

Methylbuthyl octanoate, 26 Diethyl succinate, 27 Ethyl 9-decenoate, 28 3-(Methylthio)-1-propanol, 29 Isobutyl decanoate, 30 2-Phenylethyl acetate, 31 β-Damascenone, 32 Ethyl dodecanoate, 33 Hexanoic acid, 34 3-Methylbutyl pentadecanoate, 35 2-Phenylethanol, 36 Benzothiazole, 37 Nerolidyl acetate, 38 Octanoic acid, 39 Ethyl hexadecanoate, 40 Decanoic acid, 41 Dodecanoic acid.

Fig.1.

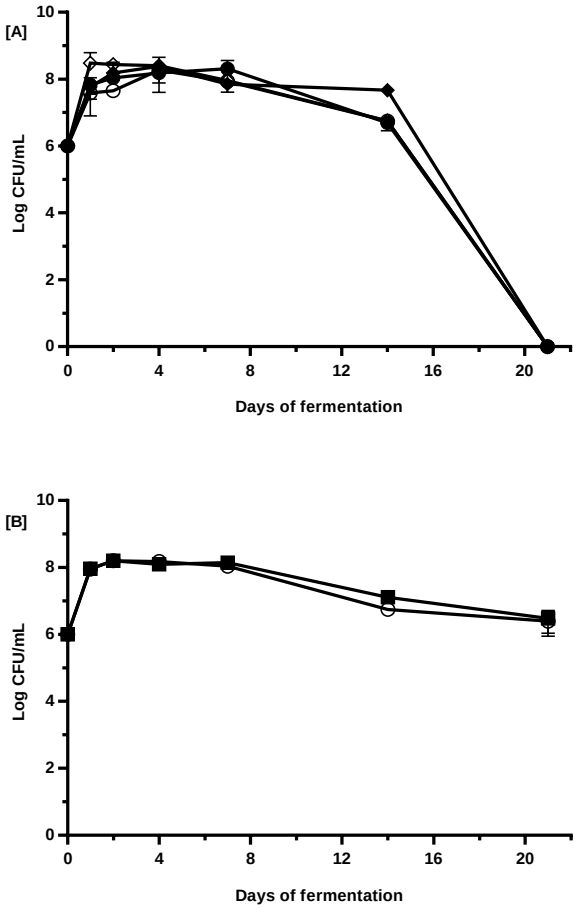
[A]
Log CFU/mL
Days of fermentation
[B]
Log CFU/mL
Days of fermentation

Fig.2.

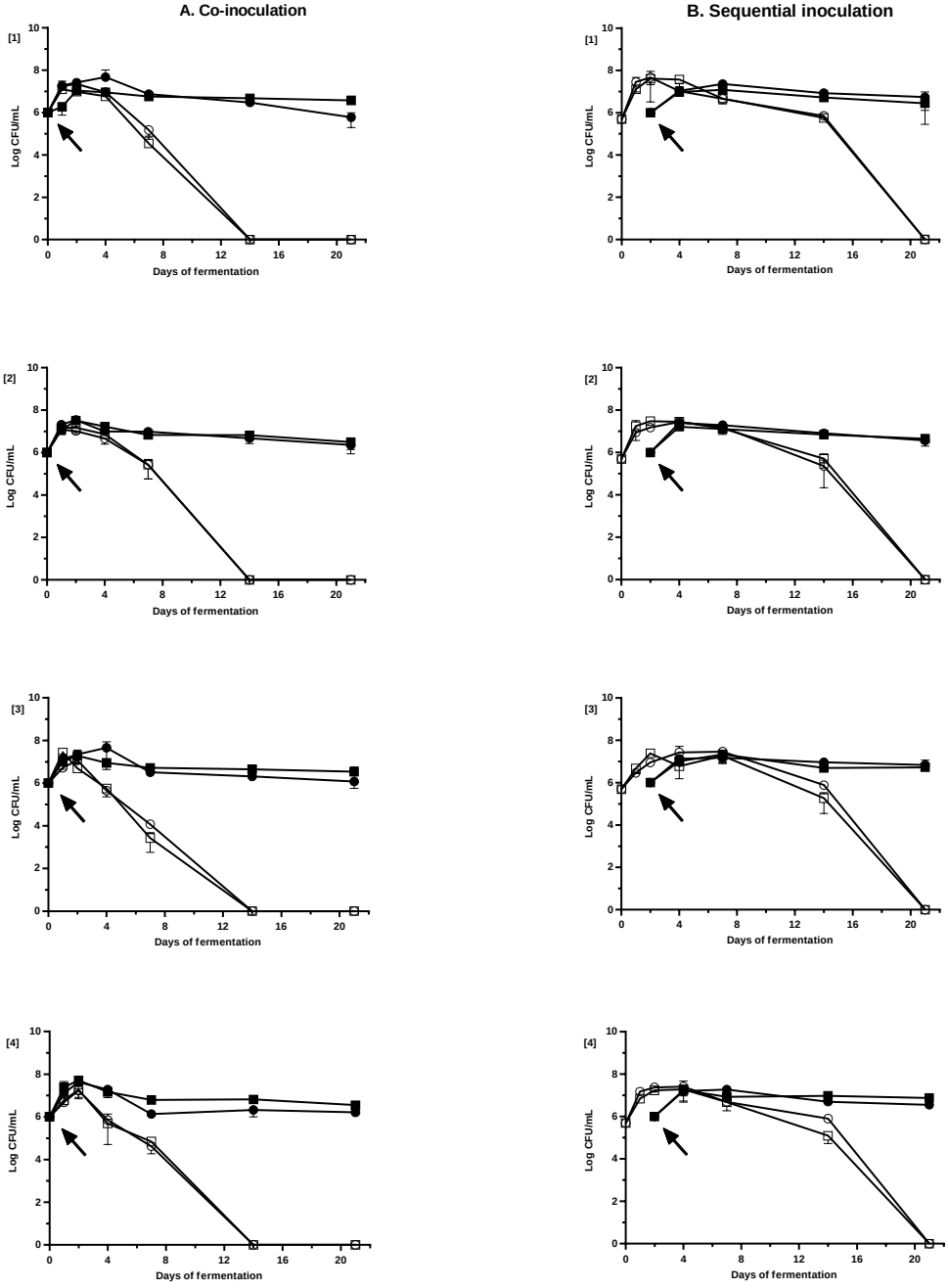
A. Co-inoculation
B. Sequential inoculation
[1]
[2]
[3]
[4]
Log CFU/mL
Days of fermentation

Fig.3.

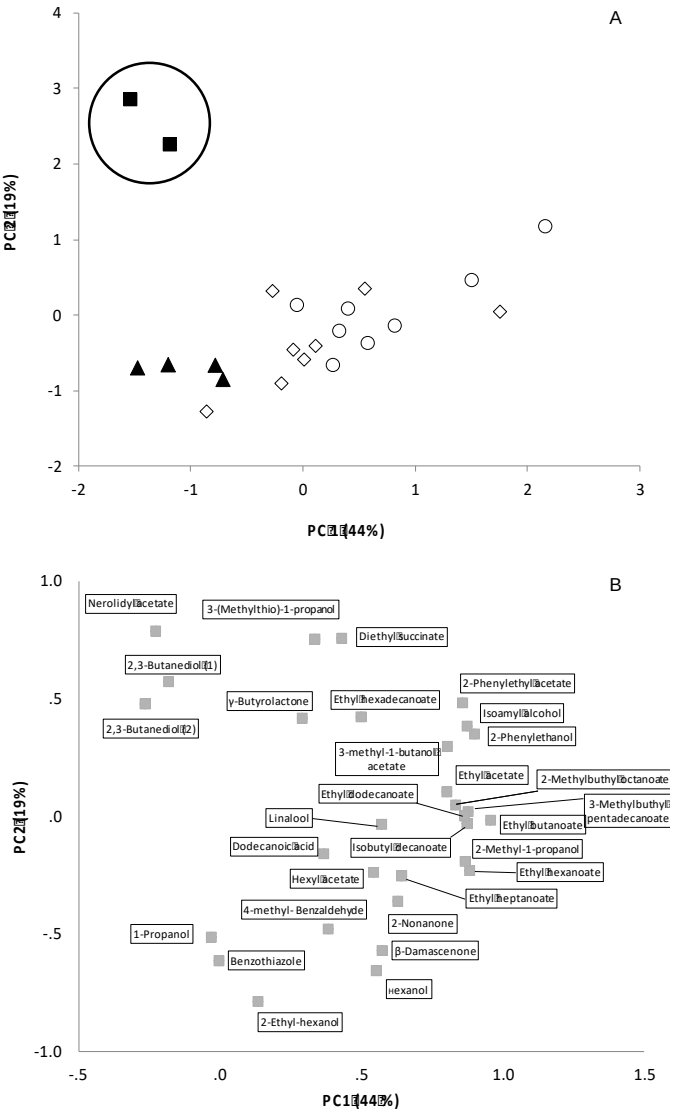
A
PC2 (19%)
PC1 (44%)
B
PC2 (19%)
PC1 (44%)
Nerolidyl acetate
3-(Methylthio)-1-propanol
Diethyl succinate
2,3-Butanediol (1)
2-Phenylethyl acetate
γ-Butyrolactone
Ethyl hexadecanoate
Isoamyl alcohol
2,3-Butanediol (2)
2-Phenylethanol
3-methyl-1-butanol acetate
Ethyl acetate
2-Methylbuthyl octanoate
Ethyl dodecanoate
3-Methylbuthyl pentadecanoate
Linalool
Ethyl butanoate
Dodecanoic acid
Isobutyl decanoate
2-Methyl-1-propanol
Ethyl hexanoate
Hexyl acetate
Ethyl heptanoate
4-methyl- Benzaldehyde
2-Nonanone
1-Propanol
β-Damascenone
Benzothiazole
hexanol
2-Ethyl-hexanol

Fig. 1. Supplementary data.