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Simultaneous inoculation of yeasts and lactic acid bacteria: effects on fermentation dynamics and chemical composition of Negroamaro wine

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ABSTRACT

Traditional vinification process is undertaken with the inoculation of the lactic acid bacteria (LAB) at the end of alcoholic fermentation (AF) to induce malolactic fermentation (MLF). MLF is an important phase during winemaking and the LAB co-inoculation with yeast starter represents a promising approach to enhance the quality and safety of wine. In this investigation we have studied: i) the effect of timing of LAB inoculation on the vinification dynamics and chemical features of Negroamaro wines; ii) the interactions between two commercial yeast and two commercial *Oenococcus oeni* strains. The fermentations dynamics were monitored by microbial counts, quantifying L-malic acid concentration and analyzing the volatile compounds contents in the obtained wines. Our results indicate that simultaneous yeasts/bacteria inoculation at the beginning of vinification reduces the processes duration and simultaneously lowers of volatile acidity. Wine obtained after co-inoculum showed a profile dominated by red and ripe fruits notes associated to esters and to buttery and creamy notes linked to diethyl succinate and ethyl lactate. Furthermore, compatibility specification between commercial yeasts and LAB strains were observed, suggesting the importance of the assessment of microbial-compatibility before their utilization in large-scale vinification.

Keywords: Negroamaro wine; yeast/bacteria coinoculation; alcoholic fermentation; malolactic fermentation

Chemical compounds studied in this article

Malic acid (PubChem CID: 525); Glycerol (PubChem CID: 753); Ethanol (PubChem CID: 702); Lactic acid (PubChem CID: 612); (R,R)-2,3-Butanediol (PubChem CID: 225936); gamma-Butyrolactone(PubChem CID: 7302); Isoamyl acetate (PubChem CID: 31276); Isoamylalcohol (PubChem CID: 31260); 2-Phenylethanol (PubChem CID: 6054); Diethyl succinate (PubChem CID: 31249).

1. Introduction

The malolactic fermentation (MLF) is the conversion of L-malic acid into L-lactic acid and CO_2 implemented by malolactic bacteria (MLB), as a result of their metabolism in wine (Zapparoli et., 2009). This microbiological process causes the de-acidification of wine, since the di-carboxylic malic acid, is transformed into a mono-carboxylic acid such as lactic acid (Bartowsky et al., 2002). Associated with this decarboxylation, other transformations take place, that are important for consumer's safety and the organoleptic characteristic, such as increased stability, color changes and modifications of wine aroma and taste (Bauer & Dicks, 2004). MLF can occur spontaneously by the indigenous flora or through the use of selected starter cultures, that usually belong to the species *Oenococcus oeni* (Capozzi et al., 2010). The advantages of induction of MLF by inoculation of selected MLB consist in the possibility to control the desired/undesired effects, in particular i) to complete degradation of malic acid; ii) to enhance the positive effect on wine bouquet, and iii) to achieve dominance of the starter culture on the undesired wild bacterial strains, often producing biogenic amines (Beneduce et al., 2010). Together with selected microbial resources, also the time of bacteria inoculation plays an important role in defining the wine sensory profile (Zapparoli et al., 2009). Generally the inoculum of the bacteria in the wine is introduced after alcoholic fermentation (AF) (sequential inoculation), when the sugars concentration is low. In fact, a possible undesirable consequence of the hetero-fermentative metabolism of MLB in must is degradation of sugars resulting in the production of acetic acid and lactic acid (Maicas et al., 2002) with the consequent rising of volatile acidity. However, sequential inoculations of LAB starter pose risks: MLF can be sluggish due to the elevated ethanol concentration and to the low pH of wine (Massera et al., 2009). Moreover, with sequential inoculation the antibacterial action of SO_2 is limited because of the decreased addition of this preservative at the end of the alcoholic fermentation (Alexandre et al., 2004), thus increasing the possibility for microorganisms such as *Brettanomyces* spp. to spoil the produced wine (Gerbaux et al., 2009; Di Toro et al., 2015). Therefore, early inoculation of a LAB starter together with yeast directly into the must, in order to stimulate a simultaneous MLF and AF,

has been suggested to overcome these problems and to speed up wine production by reducing the time requested for MLF completion (Zapparoli et al., 2009; Azzolini et al., 2010; Izquierdo Cañas et al., 2012). However, in spite of its many advantages on winemaking process, the co-inoculation approach and, particularly, the unpredictable interactions between *S. cerevisiae* and *O. oeni* strains during grape must fermentation has been poorly investigated (Arnink & Henick-Kling, 2005). Moreover, strain specific yeast-bacteria interactions can also affect the dynamics of the AF, since yeast growth might even be repressed by some LAB strains (Mendoza et al., 2011).

The aim of this study was to compare the performance of four yeast/bacterium combinations when inoculated in two different approaches: simultaneously (co-inoculation), or sequential (yeast followed by the bacteria when AF was close to the end. At the best of our knowledge, we report the first data about the application of a yeasts/bacteria multi-starter approach for the production of Negroamaro wines denoted by high alcohol content and high total acidity, typical of the oenological production of Southern Italy and other similar climates.

2. Materials and methods

2.1 Microorganisms

The following commercially available microorganisms were used for must inoculation: the *Saccharomyces cerevisiae* strains coded as CY1 (Lallemand, USA) and CY2 (Enartis, Italy) and the commercially available *Oenococcus oeni* strains coded as CL1 (Lallemand, USA) and CL2 (Enartis, Italy). The yeast and bacterial starters have been purchased in active dried form. Rehydration and acclimatization procedures were done according to suppliers' instructions. The following codification was adopted to denote the different mixed inocula: A, CY1+CL1; B, CY1+CL2; C, CY2+CL1; D, CY2+CL2.

2.2 Microvinifications and wine analysis

To evaluate strain-specific fermentation performances, the starter cultures were used in micro-fermentations assays to inoculate Negramaro grape must (20.8 ° Babo; 7.2 g/L total acidity; 3.44 g/L malic acid; pH 3.34; free ammonium 163.5 mg/L), following a procedure previously described (De Benedictis et al., 2011). The must was clarified by centrifugation (10 min at 8000 g), sterilized by filtration (0.45 μm membrane) and then supplemented with potassium metabisulphite (70 mg/L). One liter of must was placed in sterile Erlenmeyer 2L flasks and then inoculated at a final concentration of 10^9 CFU/mL of a yeast inoculum pre-cultured in the same must. Malolactic bacteria were inoculated at a final concentration of 10^7 CFU/mL, as follow: i) LAB starter culture was inoculated 24 hours after the yeast inoculation (Versari et al., 2015), in the case of evidence of co-inoculation or ii) bacteria starter cultures were added at the end of AF (15 days after yeast starters inoculation) in the case of traditional inoculum (Capozzi et al., 2010). The starter cultures were prepared and inoculated in the must according to the manufacturer's instructions. The ratio between yeast and MLB starter was equivalent to 100:1. In this study, we used the ratio recommended by starter manufacturers, that allowed us to mime the actual vinery conditions, as already described by several similar investigations (Antalick et al., 2013; Izquierdo Cañas et al., 2012, 2014; Versari et al., 2014). The temperature of the must at the time of inoculation was 24° C, and it ranged between 23° C and 26° C during the experiments. The kinetics of the fermentations were monitored daily by gravimetric determinations, evaluating the loss of weight due to the production of CO_2. Samples were weighted daily to follow the weight loss caused by CO_2 production. When CO_2 evolution stopped (i.e. at constant weight), samples were stored at -20° C, until required for chemical analysis. Each fermentation experiment was carried out by performing three simultaneous independent repetitions.

2.3 Determination of microbial population

The viable count of yeasts was performed by diluting samples serially with 0.1% (wt/vol) peptone water and applying them to agar slants containing WL-agar medium (Sigma, USA) added with 0.1 g/L ampicillin. Plates were incubated at 28° C for 48h. Appropriate dilutions of must and wine were also plated on MRS supplemented with 2% tomato juice pH 4.8, added with 0.05 g/L nystatin. Plates were incubated at 28° C under anaerobic conditions for 5-7 days and isolates were counted in order to quantify LAB (Capozzi et al., 2011).

2.4 Chemical analysis

Wines and musts were analyzed by Fourier Transform Infrared Spectroscopy (FTIR), employing the WineScan Flex (FOSS Analytical, DK). Samples were centrifuged at 8000 rpm for 10 min and then analyzed as previously described (Tristezza et al., 2012). Ethanol was routinely quantified using a specific enzymatic kit (Megazyme, Ireland). Extraction of volatile compounds in wines was carried out by means of solid phase extraction (SPE), according to Tufariello et al. (2014). SPE samples were analyzed using a gas chromatograph 6890N (Agilent Technologies, USA) equipped with DBWax column (60 m, 0.25 mm i.d., 0.25 μm film thickness, Agilent Technologies) and 5975C quadrupole mass spectrometer (Agilent Technologies). The injection was made in the splittless mode, the injector temperature was 250° C. As regards wine volatile compounds, the temperature program was 40° C for 3 min, 4° C min^{-1} to 200° C, 20 min at maximum temperature. Carrier gas (He) flow was at 1.0 mL min^{-1}. Spectra were recorded in the electron impact mode (ionization energy, 70 eV) in a range of 30–500 amu at 3.2 scans/s. A solvent delay time of 10 min was used to avoid overloading the mass spectrometer with solvent. The identification of the volatile compounds was achieved by comparing mass spectra with those of the data system library (NIST 98, P>90%) and retention indexes with published data, or by injection of pure standards. Concentration of each volatile compound is expressed as mg internal standard equivalents L^{-1} wine, obtained by normalizing the compound peak area to that of the internal standard and multiplying by concentration of the internal standard.

2.5 Statistical analysis

Significant differences among samples were determined for each chemical compound by analysis of variance (post-hoc Tukey, $\alpha = 0.05$). Statistical data processing was performed using the free software package PAST (Hammer et al., 2001).

3. Results

3.1 Development of microbial populations during alcoholic fermentation

The alcoholic fermentation performance of the two *S. cerevisiae* strains, as single-, traditional- and sequential-inoculum were assessed, by the daily loss of weight of the flasks in relation to CO_2 production. After 20 days, a stable ethanol concentration in all the samples indicated the end of the alcoholic fermentation. The obtained data showed that the two yeast starter cultures had a similar fermentative performance in all the produced must fermentations (Figure 1). The presence of the bacteria in the early stages of the AF did not affect or inhibit the dynamics of yeast fermentation (Figure 2). In fact, in the inoculated must we observed that the presence of the bacteria does not contrast the development of the yeasts population during fermentation, highlighting the ability of yeast to co-exist with bacteria and the capacity of the latter to better adapt to the environment in a co-inoculation rather than in a sequential inoculum. (Figure 2). In the case of co-inoculation the development profile of the bacterial population shows its gradual acclimatization in the must during the increase of alcohol concentration due to yeasts (Figure 3). However, the two commercial bacterial starter showed a different behavior in presence of the yeast strains used for co-inoculation. In fact, the CL1 strain was able to grow in the presence of both CY1 and CY2 yeast starters at similar level and they maintained a constant concentration (c. 1×10^7 CFU/mL) during the progress of the MLF. In contrast, CL2 strain was unable to successfully grow in presence of both yeast

strains, since its concentration decreased from 1 x 10^7 CFU/mL to 10^4 CFU/mL. (Figure 3). When the bacterial inoculum was carried out at the end of the AF, the CL1 bacterial starter was able to proliferate in both wines produced with CY1 and CY2 yeast starters, showing a comparable behavior to the CL2 strain inoculated in the wine obtained by CY1 starter (Figure 4). On the contrary when the CL2 strain was used to promote MLF in the wine obtained after CY2 fermentation, a continuous decrease in the number of bacteria during the whole period of their monitoring was observed (Figure 4).

3.2 Malolactic fermentation

The dynamics of the MLF process was monitored by recording the transformation of malic acid in lactic acid. When the MLF was promoted by the co-inoculation of yeast with the LAB strains CL1 and CL2, these strains showed different performances (Figure 4). In fact, CL1 strain was able to completely consume the malic acid in about 22 days either in presence of CY1 or of CY2 yeast starters, whereas the CL2 strain did not complete the MLF in both mixed fermentations, resulting in residual malic acid concentration of 0.69 g/L (CY1/CL2 inoculum) and 0.80 g/L (CY2/CL2 inoculum) (Figure 5; Table 1).

The traditional inoculum was performed by adding the LAB starter culture at the end of the AF. The dynamics of MLF carried out by the CL1 strain was similar in both analyzed fermentations (CY1 and CY2), since they had a similar profile and they both resulted in the complete transformation of malic acid in lactic acid 14 days after inoculation (Figure 6). However, the fermentative performances of CL2 strain was strictly dependent on yeast strain used to promote AF. In fact, when CL2 strain was inoculated in the wine produced with CY1 yeast starter, it was able to complete the MLF process in 22 days, whereas it was–unable to successfully complete the conversion of malic in lactic acid when CY2 yeast was used , thus leaving a residual concentration of the former organic acid, corresponding to 2.05 g/L (Figure 6; Table 1)

3.3 Determination of chemical parameters of fermentations

A positive effect on the volatile acidity (VA) was observed when yeasts and bacteria where co-inoculated. In particular, a decrease in acetic acid concentration was achieved, 0.30 g/L for CY1/CL1 co-inoculum and 0.31 g/L for CY2/CL1 co-inoculum, and these values were lower than those (0.49 and 0.51 g/L, respectively) detected in wines produced with the same starters in a sequential approach (Table 1). When CL2 was used as LAB starter a similar VA reduction was obtained in the wine produced by co-inoculum with yeast strain CY1 versus that produced by sequential starter inoculation (0.40 g/L versus 0.54 g/L). No significant variation in VA values was recorded in wine produced with CY2/CL2 strains by both co- and post AF inoculation. The values of citric acid, density, glycerol and pH are unchanged in the three fermentations, indicating that the technique of co-inoculation does not adversely affect the chemistry of the wine compared to the classical MLF induction technique (Table 1).

3.4 Analysis of volatile compounds

The different metabolism of yeast and bacteria can determine changes in volatile chemical composition of wines, including the compounds related to MLF. SPE/GC-MS analysis of the wine produced by the four combinations of yeasts/bacteria starter either sequentially or co-inoculated allowed the identification and quantification of a number of volatile compounds belonging to eight different groups that are by-products of yeast metabolism namely: alcohols, esters, acids and other compounds (Tables 2). Table 2 shows the ester concentrations measured in the wine produced by co-inoculation and those obtained by sequential inoculation. The ester content was higher in wines produced by co-inoculation in all cases, 26,95 mg/L in CY1+CL1 vs 14,45 mg/L in CY1/CL1 inoculated post AF (pAF), 16,28 mg/L in CY1/CL2 vs 12,15 mg/L in CY1/CL2 pAF, 14,93 mg/L in CY2/CL1 vs 10,44 mg/L in CY2/CL1 pAF, 14,36 mg/L in CY2/CL2 vs 8,21 mg/L in CY2/CL2 pAF. The influence of co-inoculation on the chemical composition of wines was even more evident when the concentrations of alcohols and fatty acids were compared with those present in wines

obtained after sequential starters inoculation. Total alcohol and acid concentrations were found to be higher in wines produced by co-inoculation and these compounds are responsible for fruity, sweet, winery and acid sensory notes in wine. Moreover, the concentration of fermentation-derived compounds (Table 2) also varied among the co-inoculated wines. All the esters and alcohols measured were found at higher concentrations in wines produced with CY1/CL1 co-inoculum compared with the other co-inoculated wines.

4. Discussion

One of the most important known benefit of yeasts/LAB simultaneous inoculation consists in the reduction of the total fermentation time (Abrahamse & Bartowsky, 2012). This study corroborated this statement and it is consistent with previous investigations performed on a lab-scale and with experiential winemakers' remarks (Rosi et al., 2006; Massera et al., 2009; Antalick et al., 2013). After co-inoculation, MLF can also occur when AF ended, but still in this case the length of the process is diminished, because of the adaptation of the bacterial starter to the "grape must" environment from the beginning of AF.

Specific interactions between *S. cerevisiae* and *O. oeni* are recognized to happen all through the alcoholic and malolactic fermentations, when co-inoculation of both starter cultures is chosen as strategy (Alexandre et al., 2004). In fact, definite yeasts-bacteria relations might be observed being different to those occurring in post-fermentation inoculations. In our investigation, we used two commercial yeast and two *O. oeni* strains that had been described by the producers to be highly suitable for the use as component of a mixed yeasts/bacteria co-inoculum.

Indeed, the viability of the *S. cerevisiae* starter cultures was not influenced during the simultaneous progress of AF and MLF, indicating that the exponential growth stage of the yeast starter populations was not decreased before reaching the stationary phase (Massera et al., 2009). These evidences are consistent to those obtained in a similar study on Tempranillo and Merlot wines

(Izquierdo Cañas et al., 2012). When the bacterial starters were added, either simultaneously or sequentially, at the end of the AF an initial reduction in their viability was recorded. This evidence was already observed by King & Beelman (1986), after inoculating bacteria in synthetic grape juice and by Muñoz and coworkers (2014), when they added the bacterial starter to musts in the mid of alcoholic fermentation.

The growth level of one of the two bacterial starters used in this study was affected by yeast presence, and the degree of the inhibition depended upon both yeast strain and timing of bacteria inoculation. In fact, when the CL2 bacteria were simultaneously or sequentially inoculated with the CY2 yeast strain, they showed the highest lag phase, the minimal growth and the highest residual malic acid. The yeast strain CY1 affected the growth of CL2 strain when they were early inoculated at the same time, whereas when the bacteria was added post AF a delayed MLF occurred. On the other hand the bacterial starter CL1 successfully carried out MLF process independently from yeast strain or inoculum modality. These evidences confirm the concept that the correct selection of the yeast-bacterium pair is critical for performing a concurrent AF/MLF, as the incompatibility between the two microorganisms can affect both processes (Nehme et al., 2008; Guzzon et al., 2013). This study also confirmed that MLF can take place in the presence of fermentable sugars without a significant increase of acetic acid, it being an interesting findings if we consider that contrasting results were reported about the concentration of acetic acid in a co-inoculation approach (Liu 2002; Knoll et al., 2012; Garofalo et al., 2015a). These variability in scientific literature, in the light of our results, led us to hypothesize that the effect of volatile acidity might be a strain-dependent character. In the experimental tests carried out, the consumption of malic acid occurred during the AF, when the population of bacteria was not in the growth phase. To further support the effectiveness of the yeast-bacteria co-inoculation, it has been considered a volatile acid content of 23% lower than that found in the wine produced by traditional inoculation, resulting in an improving effect on the organoleptic characteristics of the wine (Izquierdo Cañas et al., 2014; Garofalo et al., 2015a).

283 The results reported in this study suggest that the use of co-inoculation for the management of the
284 MLF has a positive influence on fermentation time as well as on aromatic composition of wine. In
285 fact, the considerable effect of yeasts/LAB co-inoculation on the aromatic pattern of produced wine,
286 compared to those obtained by sequential starters inoculation, was clearly shown. Recent
287 investigations have highlighted the variation of the biochemical profile of wine produced by
288 different LAB inoculation procedures (Abrahamse & Bartowsky, 2011; Knoll et al., 2011;
289 Izquierdo Cañas et al., 2012). Our data suggested, in accordance to literature (Antalick et al., 2013),
290 that yeast/LAB co-inoculation could enhance the fruity aroma, thereby increasing the level of
291 esters. Twelve esters were identified and quantified, and wines produced by co-inoculation
292 contained higher concentrations of diethyl and monoethylsuccinate, ethyl lactate, 2-phenylethyl
293 acetate and ethyl esters of fatty acids (Versari et al., 2015). Overall, for all strains tested, co-
294 inoculation resulted in a significant change of the wine esters profile, with ethyl fatty acid esters
295 becoming quantitatively the most representative class of esters. This procedure probably stimulates
296 the formation of mid-chain fatty acids and, hence, the concentration of esters of fatty acids in wines.
297 These compounds were considered to be odorant esters because they had a much higher impact on
298 wine aroma (Fang & Qian, 2005). The presence of 2,3-butanediol indicates that in the case of co-
299 inoculation bacteria were able to perform the degradation of diacetyl, the compound derived from
300 the MLF with high organoleptic impact on wine (Martineau & Henick-Kling, 1995). This
301 compound, if present in the wine at high concentrations, is able to adversely affect the bouquet of
302 the wine conferring aromatic buttery notes that interfere with wines fruity aromas (Bartowsky &
303 and Henschke, 2004). Consequently, applying the technique of co-inoculation it will be possible to
304 produce wines with lower hints of butter and milk, but with the sensory profiles dominated by
305 organoleptic notes related to the grape. A bacterial-mediated modification of yeast by-products is
306 likely to be the molecular mechanism in charge of the increase in butyrolactone concentrations in
307 wines produced by the co-inoculation system compared to the sequential technique (Antalick et al.,

2013). In fact, it has been previously demonstrated that yeasts/LAB interactions promote lactones synthesis during whisky-production process (Wanikawa et al., 2000).

Conclusions

In addition to consistent data on the possible use of autochthonous resources from Apulian region (Cappello et al., 2008; Grieco et al., 2011; Tristezza et al., 2013, 2014; Garofalo et al., 2015b) already published, this study provides the first report on the application of the method of co-inoculation in the winemaking conditions typical of Southern Italy (Puglia) wine production using commercial starter cultures. The present investigation highlighted the needing to assess the real compatibility of commercial yeast bacteria strains, even if they are indicated as suitable for simultaneous fermentations, before they are used for wine production. Furthermore, our data suggest that grape-cultivar-derived extrinsic factors can appreciably modify the intrinsic yeast-bacteria metabolic relation (Costello et al., 2003), even in strain that are described to have compatible interactions

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454 **Captions to figures**

455

456 **Figure 1.** Ethanol concentrations measured during fermentations of Negroamaro must inoculated
457 with bacteria at the beginning of the alcoholic fermentation. A, CY1+CL1; B, CY1+CL2; C,
458 CY2+CL1; D, CY2+CL2.

459

460 **Figure 2.** Yeast populations (CFU/mL) measured during fermentations of Negroamaro must
461 inoculated with bacteria at the beginning of the alcoholic fermentation. A, CY1+CL1; B,
462 CY1+CL2; C, CY2+CL1; D, CY2+CL2.

463

464 **Figure 3.** Bacterial populations (CFU/mL) measured during fermentation of Negroamaro must
465 inoculated with bacteria at the beginning of the alcoholic fermentation. A, CY1+CL1; B,
466 CY1+CL2; C, CY2+CL1; D, CY2+CL2.

467

468 **Figure 4.** Bacterial populations (CFU/mL) measured during fermentation of Negroamaro must in
469 samples inoculated with bacteria at the end of the alcoholic fermentation. A, CY1+CL1; B,
470 CY1+CL2; C, CY2+CL1; D, CY2+CL2.

471

472 **Figure 5.** L-malic acid consumption (g/L) evaluated during vinification of Negroamaro must in
473 samples inoculated with bacteria at the beginning of the alcoholic fermentation. A, CY1+CL1; B,
474 CY1+CL2; C, CY2+CL1; D, CY2+CL2.

475

476 **Figure 6.** L-malic acid consumption (g/L) evaluated during vinification of Negroamaro must in
477 samples inoculated with bacteria at the end of the alcoholic fermentation. A, CY1+CL1; B,
478 CY1+CL2; C, CY2+CL1; D, CY2+CL2.

479

Table 1. Chemical composition of wines at the end of MLF. A, CY1+CL1; B, CY1+CL2; C, CY2+CL1; D, CY2+CL2.

Method	Inoculum	Alcohol	Sugars	TA	VA	pH	Malic	Lactic	Tartaric	Citric	Glycerol
Coinoculation	**A**	13,89	$0,58^{ab}$	$6,01^{bc}$	$0,30^{b}$	$3,44^{de}$	$0,05^{b}$	$1,59^{a}$	2,07	$0,35^{bc}$	$10,12^{a}$
	STD	±0,16	±0,11	±0,37	±0,03	±0,01	±0,03	±0,02	±0,18	±0,00	±0,06
	B	13,78	$0,54^{ab}$	$6,19^{ab}$	$0,40^{e}$	$3,43^{ef}$	$0,69^{c}$	$0,74^{b}$	2,00	$0,38^{ac}$	$10,28^{ae}$
	STD	±0,16	±0,14	±0,21	±0,02	±0,00	±0,22	±0,23	±0,19	±0,02	±0,12
	C	13,82	$0,71^{a}$	$6,07^{bc}$	$0,31^{b}$	$3,47^{ac}$	$0,04^{b}$	$1,63^{a}$	2,03	$0,38^{ac}$	$10,22^{a}$
	STD	±0,29	±0,11	±0,13	±0,06	±0,01	±0,02	±0,01	±0,01	±0,02	±0,10
	D	13,91	$0,55^{ab}$	$6,59^{a}$	$0,64^{c}$	$3,36^{b}$	$0,80^{c}$	$0,58^{b}$	1,97	$0,39^{a}$	$11,17^{c}$
	STD	±0,13	±0,17	±0,15	±0,04	±0,01	±0,07	±0,02	±0,15	±0,01	±0,09
Post AF	**A**	13,82	$0,60^{ab}$	$5,96^{b}$	$0,49^{d}$	$3,44^{df}$	$0,10^{b}$	$1,51^{a}$	1,95	$0,36^{ac}$	$10,37^{ad}$
	STD	±0,16	±0,13	±0,18	±0,01	±0,00	±0,02	±0,02	±0,12	±0,02	±0,13
	B	13,89	$0,68^{a}$	$6,05^{bc}$	$0,54^{e}$	$3,49^{a}$	$0,23^{b}$	$1,06^{c}$	1,94	$0,33^{b}$	$10,57^{de}$
	STD	±0,01	±0,02	±0,01	±0,04	±0,01	±0,04	±0,00	±0,02	±0,01	±0,01
	C	13,87	$0,54^{ab}$	$5,94^{b}$	$0,51^{d}$	$3,46^{cd}$	$0,11^{b}$	$1,54^{a}$	1,95	$0,38^{ac}$	$10,52^{d}$
	STD	±0,16	±0,04	±0,05	±0,03	±0,00	±0,01	±0,00	±0,03	±0,00	±0,13
	D	14,11	$0,28^{b}$	$6,48^{ac}$	$0,59^{a}$	$3,36^{b}$	$2,05^{a}$	$0,57^{b}$	1,92	$0,35^{bc}$	$11,54^{b}$
	STD	±0,13	±0,13	±0,02	±0,05	±0,01	±0,03	±0,02	±0,09	±0,01	±0,08

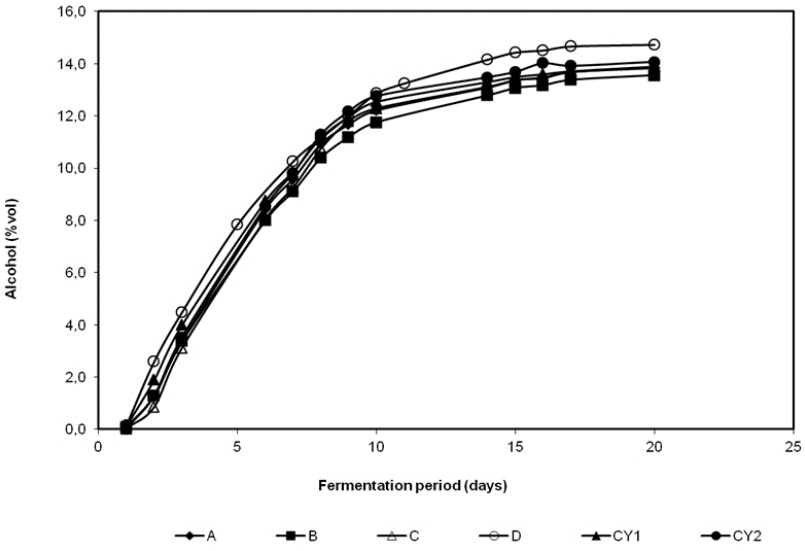
TA, total acidity; VA, volatile acidity. The ethanol concentration is expressed as g/100 mL. The other values are expressed as g/L; the standard deviation values (±) are indicated. Different letters indicate significant differences (α=0.05)

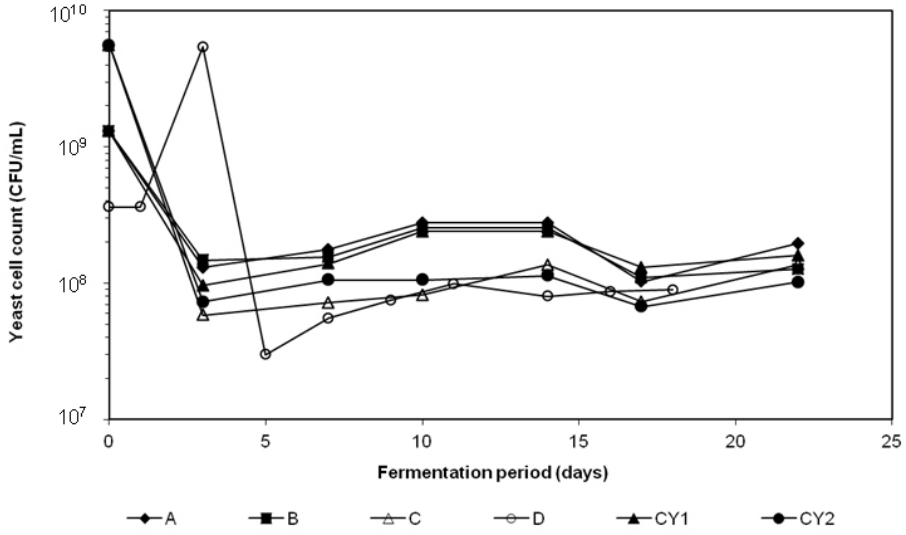
Table 2. Volatile compounds concentration of red wines obtained with co-inoculum and sequential. A, CY1+CL1; B, CY1+CL2; C, CY2+CL1; D, CY2+CL2.

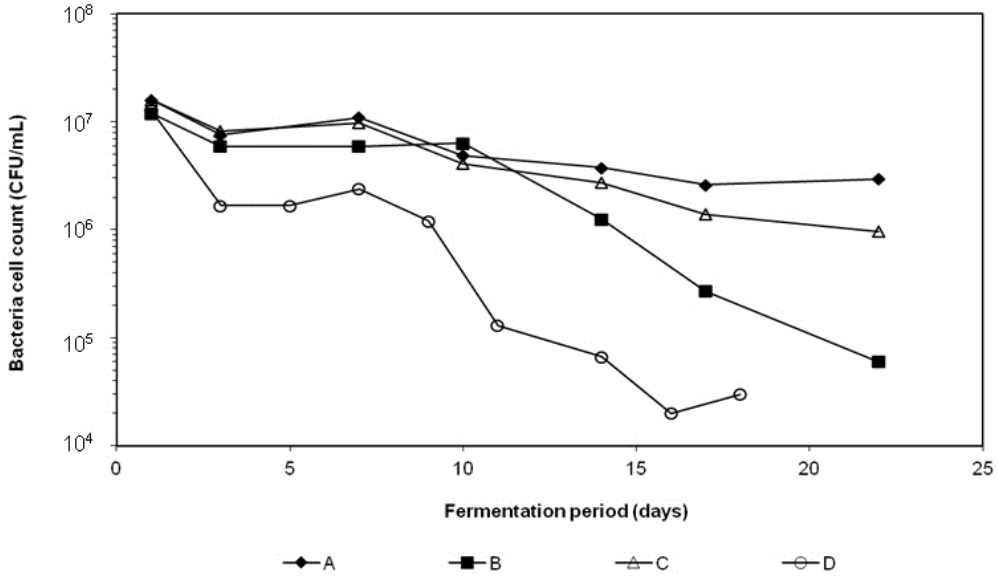
	Co-inoculation								*Sequential*							
Compounds	**A**		**B**		**C**		**D**		**A pAF**		**B pAF**		**C pAF**		**D pAF**	
	Mean*	±SD	**Mean***	±SD	**Mean***	±SD	**Mean***	±SD	**Mean***	±SD	**Mean***	±SD	**Mean***	±SD	**Mean***	±SD
Esters																
diethyl malate	**n.d.**		**0,98a**	0,1	**0,73a**	0,16	**0,51a**	0,47	**0,84a**	0,17	**0,83a**	0,21	**n.d.**		**n.d.**	
diethyl succinate	**3,78c**	0,70	**1,20a**	0,32	**1,89b**	0,18	**1,27a**	0,07	**1,09a**	0,14	**1,01a**	0,18	**0,98a**	0,2	**0,53a**	0,02
ethyl lactate	**4,32b**	0,12	**5,35b**	0,41	**3,78b**	0,29	**4,74b**	0,15	**3,62b**	1,47	**2,87a**	0,74	**2,90a**	0,03	**3,37b**	0,22
monoethyl succinate	**10,90b**	1,33	**5,46a**	0,47	**5,06a**	0,82	**3,90a**	0,39	**4,83a**	0,09	**4,71a**	2,34	**3,55a**	0,55	**2,46a**	0,09
2-phenylethylacetate	**1,20b**	0,13	**0,45a**	0,12	**0,48a**	0,1	**2,17c**	0,12	**0,65a**	0,09	**0,32a**	0,06	**0,38a**	0,03	**0,68a**	0,04
3-hydroxy-ethylbutanoate	**0,61**	0,27	**0,27**	0,03	**0,23**	0,13	**0,26**	0,07	**0,28**	0,02	**0,27**	0,04	**0,19**	0,12	**n.d.**	
ethyl butanoate	**0,80a**	0,16	**0,60a**	0,05	**0,76a**	0,04	**0,43a**	0,18	**0,64a**	0,15	**0,46a**	0,24	**0,57a**	0,08	**0,36a**	0,02
ethyl decanoate	**0,88b**	0,24	**0,19a**	0,01	**n.d.**		**0,29a**	0,08	**0,45b**	0,2	**0,27a**	0,07	**0,28a**	0,14	**0,04a**	0,01
ethyl hexanoate	**1,68b**	0,32	**0,53a**	0,03	**0,70a**	0,06	**0,43a**	0,29	**0,85a**	0,43	**0,49a**	0,25	**0,58a**	0,16	**0,50a**	0,06
ethyl octanoate	**1,97b**	0,49	**0,76a**	0,16	**0,98a**	0,08	**0,07a**	0,01	**0,56a**	0,06	**0,55a**	0,18	**0,75a**	0,03	**n.d.**	
ethyl vanillate	**0,06a**	0,02	**0,07a**	0,06	**0,04a**	0,01	**n.d.**		**0,07a**	0,04	**0,07a**	0,05	**0,03a**	0	**n.d.**	
isoamyl acetate	**0,75a**	0,32	**0,42a**	0,03	**0,28a**	0,03	**0,29a**	0,01	**0,57a**	0,01	**0,3a**	0,05	**0,23a**	0,01	**0,27a**	0,02
Total	**26,95**	4,10	**16,28**	1,79	**14,93**	1,9	**14,36**	1,84	**14,45**	2,87	**12,15**	4,41	**10,44**	1,35	**8,21**	0,48
Alcohols																
1-butanol	**2,28b**	0,13	**0,33a**	0,04	**0,08a**	0,04	**0,13a**	0,06	**0,46a**	0,25	**0,47a**	0,28	**0,04a**	0,01	**0,27a**	0,02
2,3 butanediol (R,R)	**1,59b**	0,63	**3,10c**	0,56	**n.d.**		**n.d.**		**3,32c**	0,02	**2,96c**	0,41	**n.d.**		**0,37a**	0,06
2,3 butanediol (S,S)	**1,41b**	0,28	**1,25b**	0,38	**0,23a**	0,06	**n.d.**		**1,20b**	0,18	**1,21b**	0,26	**n.d.**		**0,07a**	0,03
2-phenylethanol	**40,61**	4,21	**34,55**	1,33	**52,63**	5,12	**32,69**	5,43	**36,51**	2,11	**29,39**	2,29	**30,35**	6,59	**29,24**	4,98
3-hexen-ol (E)	**0,07a**	0,02	**0,42a**	0,05	**0,02a**	0,01	**n.d.**		**0,02a**	0,01	**0,15a**	0,19	**0,03a**	0,01	**n.d.**	
3-hexen-ol (Z)	**0,66b**	0,18	**0,73b**	0,11	**0,13a**	0,04	**0,04a**	0,01	**0,03a**	0,01	**0,03a**	0,01	**0,03a**	0,01	**0,12a**	0,04
hexanol	**0,35a**	0,14	**0,16a**	0,01	**0,16a**	0,01	**0,22a**	0,03	**0,15a**	0,05	**0,19a**	0,02	**0,15a**	0,04	**0,16a**	0,05
isoamylalcohols	**132,4**	5,92	**137,63**	8,11	**135,38**	1,99	**114,36**	8,36	**128,62**	8,6	**128,55**	13,12	**115,44**	11,41	**98,55**	3,29
isobutanol	**13,60b**	3,71	**8,67b**	2,44	**10,42b**	0,37	**5,01a**	1,13	**8,97b**	0,86	**6,65a**	0,23	**6,04a**	1,64	**3,53a**	0,19
propanol	**19,87b**	3,89	**12,54b**	2,42	**4,85a**	0,62	**n.d.**		**14,15b**	2,87	**19,2b**	3,88	**nd**		**3,44a**	0,17
Total	**212,86**	15,22	**199,38**	13,03	**203,90**	7,61	**152,45**	18,02	**193,44**	12,09	**188,81**	16,81	**152,09**	19,71	**135,75**	8,83
Acids																
2-methylpropanoic acid	**0,51b**	0,22	**2,12c**	0,23	**0,14b**	0,08	**0,11b**	0,02	**0,18b**	0,08	**0,17b**	0,04	**0,1a**	0,01	**0,1 a**	0
3-methyl butanoic acid	**1,66b**	0,37	**0,67a**	0,02	**0,51a**	0,19	**0,66a**	0,17	**0,73a**	0,03	**0,67a**	0,05	**0,46a**	0,21	**0,53a**	0,09
benzoic acid	**0,60b**	0,14	**0,28a**	0,16	**0,11a**	0,01	**0,13a**	0,05	**0,24a**	0,2	**0,26a**	0,16	**0,07a**	0,03	**0,07a**	0,03
butanoic acid	**0,15a**	0,06	**0,32a**	0,02	**0,47a**	0,27	**0,28a**	0,08	**0,58a**	0,24	**0,54a**	0,13	**0,28a**	0,12	**0,04a**	0,02

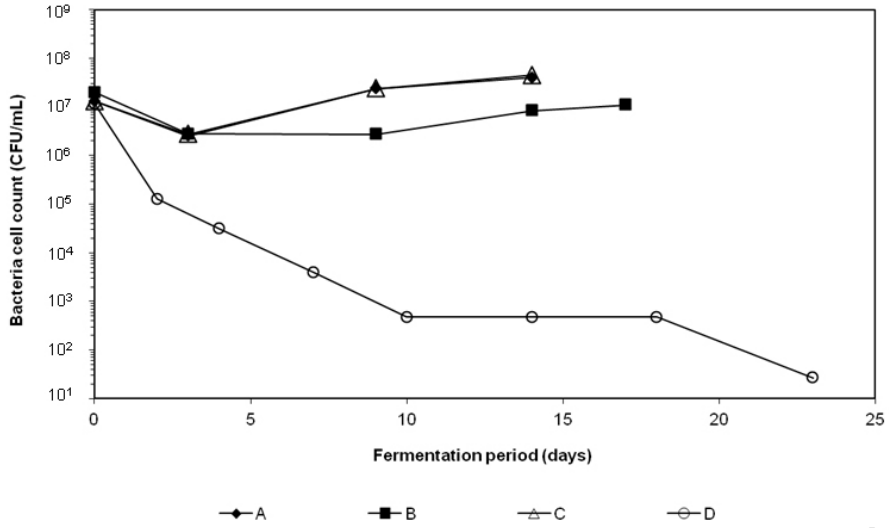
decanoic acid	**0,8c**	0,28	**n.d.**		**0,2b**	0,02	**0,09a**	0,03	**0,58a**	0,18	**0,25a**	0,11	**0,11a**	0,17	**0,13a**	0,03
hexanoic acid	**0,38a**	0,16	**0,42a**	0,02	**1,98c**	0,08	**0,37a**	0,13	**0,49a**	0,05	**0,63a**	0,05	**1,11b**	0,15	**0,44a**	0,03
octanoic acid	**0,4**	0,16	**0,36**	0,11	**0,21**	0,03	**0,4**	0,11	**0,36**	0,15	**0,31**	0,04	**0,31**	0,21	**0,51**	0,05
phenylacetic acid	**0,16a**	0,05	**0,18a**	0,12	**0,13a**	0,01	**0,10a**	0,04	**0,17a**	0,03	**0,18a**	0,06	**0,09a**	0,03	**n.d.**	
propanoic acid	**0,60a**	0,24	**0,23a**	0,05	**2,08b**	0,04	**n.d.**		**n.d.**		**0,05a**	0,03	**n.d.**		**n.d.**	
Total	**5,27**	1,68	**4,58**	0,73	**5,83**	0,73	**2,14**	0,63	**3,31**	0,96	**3,05**	0,67	**2,94**	0,93	**1,82**	0,25
Other Compounds																
acetoin	**1,52b**	0,59	**2,94c**	0,62	**1,66b**	0,15	**n.d.**		**0,43a**	0,12	**0,68a**	0,28	**n.d.**		**n.d.**	
acetovanillone	**0,08a**	0,04	**0,08a**	0,05	**0,05a**	0,01	**0,15a**	0,07	**0,07a**	0,02	**0,08a**	0,05	**0,04a**	0,02	**0,12a**	0,04
benzaldehyde	**0,38b**	0,14	**n.d.**		**0,11a**	0,04	**n.d.**		**0,20a**	0,05	**n.d.**		**n.d.**		**n.d.**	
butyrolactone	**1,84b**	0,69	**1,25b**	0,08	**0,69a**	0,38	**0,30a**	0,06	**0,91b**	0,21	**0,32a**	0,04	**0,38a**	0,25	**0,34a**	0,08
Total	**3,83**	1,46	**4,26**	0,75	**2,41**	0,58	**0,45**	0,13	**1,6**	0,4	**1,07**	0,37	**0,43**	0,27	**0,46**	0,12

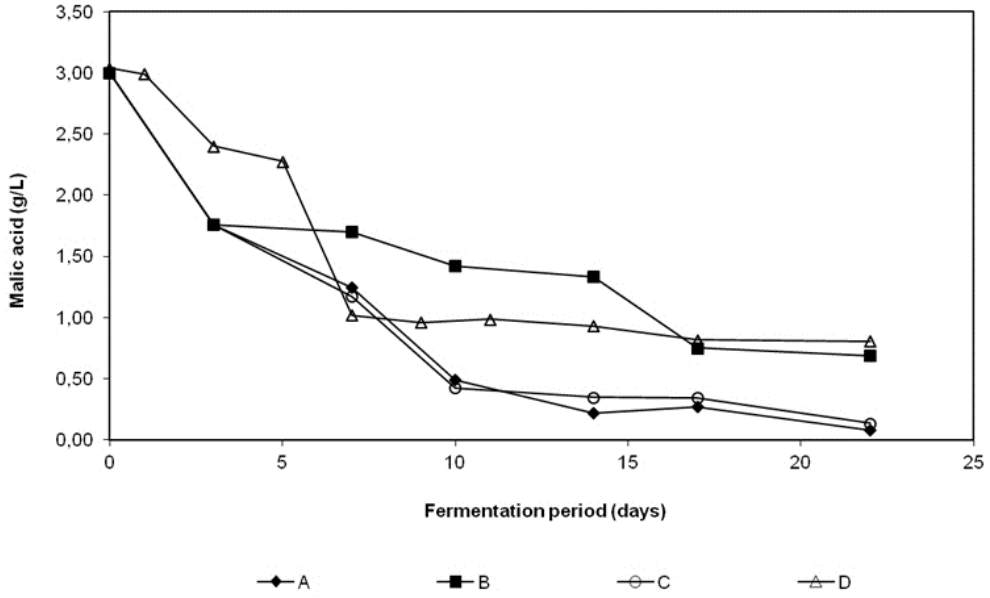
*Data, means of 3 replicates, are expressed as mg/L ± standard deviation(SD);
Nd: not detected.

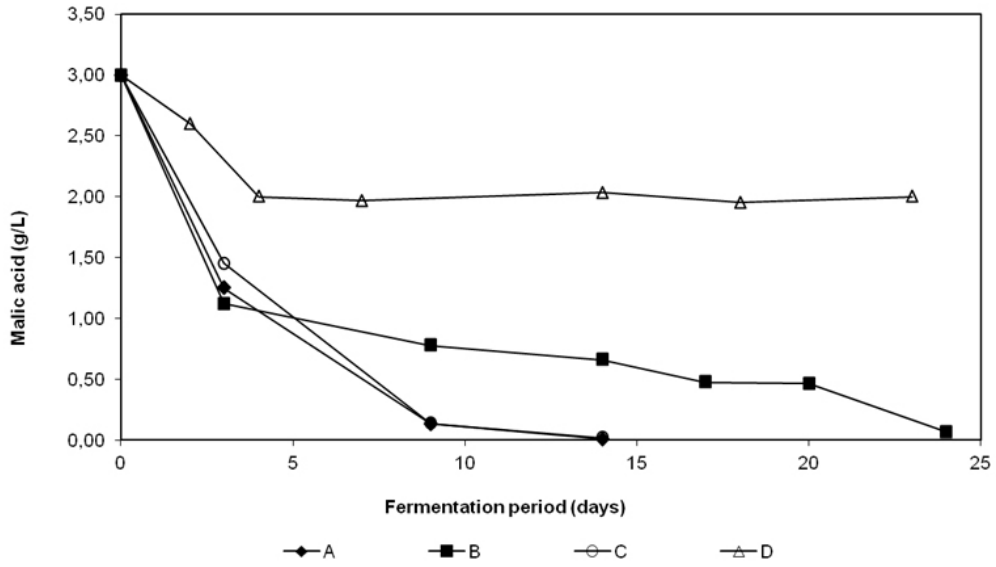












► Yeasts/bacteria co-inoculation is a novel strategy in industrial wine fermentations.

► Sequential inoculation and co-inoculation of yeasts and bacteria approaches are compared.

► The interactions between two yeast and two bacterial strains have been studied.

►Co-inoculation decreases volatile acidity in the produced wines.

► Co-inoculation produces enhancement in wine aroma profile during fermentation.