Malolactic fermentation induced by silica-alginate encapsulated *Oenococcus oeni* with different inoculation regimes

G. SIMÓ[1], E. FERNÁNDEZ-FERNÁNDEZ[1], J. VILA-CRESPO[2], V. RUIPÉREZ[2] and J.M. RODRÍGUEZ-NOGALES[1]

[1] Food Technology Department, University of Valladolid, School of Agricultural Engineering, Palencia, Spain; [2] Microbiology Department, School of Agricultural Engineering, University of Valladolid, Palencia, Spain
Corresponding author: Dr J.M. Rodríguez-Nogales, email rjosem@iaf.uva.es

Abstract

Background and Aims: The encapsulation of *Oenococcus oeni* into silica-alginate (Si-ALG) gels has been previously confirmed as a suitable strategy for a successful malolactic fermentation (MLF). The aim of this study was to evaluate the effect of two inoculation strategies (simultaneous vs sequential) on the performance of MLF with encapsulated *O. oeni* under winemaking conditions.

Methods and Results: Sequential inoculation of Si-ALG biocapsules successfully achieved a complete MLF in high ethanol wines, while free and ALG encapsulated bacteria failed. A simultaneous inoculation with Si-ALG biocapsules provided a significant reduction in time to complete MLF in high sugar and low pH musts. The regime of inoculation did not modify the chemical composition of the wines.

Conclusions: Either sequential or simultaneous inoculation of Si-ALG biocapsules constitute an effective alternative to free bacteria to undertake MLF under stressful conditions.

Significance of the Study: Effective winemaking protocols based on sequential or simultaneous induction of MLF with inoculated Si-ALG encapsulated *O. oeni* have been accomplished. These are of potential interest for winemaking in both warm and cool regions.

Keywords: *co-inoculation, immobilisation, inoculation timing, silicate, wine*

Introduction

Malolactic fermentation (MLF) is a biotechnological process that takes place during the making of most red and some white wines. Malolactic fermentation may occur spontaneously or may be induced by inoculation of selected lactic acid bacteria (LAB), mainly cultures of *Oenococcus oeni*, and involves the conversion of L-malic acid into L-lactic acid and CO_2 (Bauer and Dicks 2004), causing a de-acidification of wine. This process is recommended because an improvement of sensory characteristics (Boido et al. 2009, Antalick et al. 2012) and a microbial stabilisation of wine are achieved (Bartowsky 2005).

Development and metabolism of LAB in wine are complicated because of the stressful conditions of wine [high ethanol concentration, low pH, scarce nutrients, low temperature and the presence of SO_2 (Lerm et al. 2010)]. Currently, MLF is also affected by an increase in temperature because of climate change which is leading to higher concentration of grape sugar and production of high ethanol and unbalanced wines (Mira de Orduña 2010, Vila-Crespo et al. 2010). Because of these circumstances, the development of alternatives to improve the adaptation of LAB to these unfavourable winemaking conditions is necessary.

An explored strategy is the encapsulation of LAB because it can protect bacteria against adverse conditions during MLF (Simó et al. 2017a). Moreover, immobilised cell technology can offer other important advantages for the development of MLF such as: (i) reduction of the lag phase of LAB thus improving productivity of the process and decreasing the risk of microbial contamination; (ii) easy recovery of immobilised bacteria enabling reduction of SO_2 level, a potential allergen; (iii) possibility of reutilisation of encapsulated bacteria in successive lots of wine; and (iv) opportunity of continuous processing (Kourkoutas et al. 2004, 2010, Zhang and Lovitt 2006, Vila-Crespo et al. 2010, Rodríguez-Nogales et al. 2013). Currently, the major challenges for successful application of encapsulated cells are the high cost of the encapsulation process and the difficulty of producing them on a large scale, under aseptic conditions and with high metabolic activity and cell viability. Despite this, some applications for winemaking have been implemented using encapsulated yeasts (Simó et al. 2017a)

Cell encapsulation into alginate (ALG) hydrogel has received special attention because of its simplicity, low cost and mild working conditions (Lee et al. 2004, Bouallagui and Sayadi 2006, Safarik et al. 2008, Satora et al. 2009, Huang et al. 2016, Shin et al. 2016). Low chemical stability and mechanical robustness, however, of ALG hydrogel discourage its implementation for industrial application. Our research group is implementing an innovative process for adaptation of *O. oeni* to the stressful environment of wine based on bacterial encapsulation using robust biocomposites of silica-alginate (Si-ALG) (Simó et al. 2017b). An improvement of ALG gel stability and robustness has been achieved using colloidal silica and silicates. Inclusion of *O. oeni* into these inorganic–organic hydrogels markedly improved its capacity to reduce the concentration of L-malic acid in high ethanol as well as in low pH wines, and at low fermentation temperature (Simó et al. 2017c). These results confirmed

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the greater tolerance of Si-ALG encapsulated LAB to the unfavourable environment of wine.

Together with the encapsulation of *O. oeni*, the time of bacterial inoculation also plays a key role in bacterial adaptation to unfavourable conditions in winemaking. Traditionally, bacterial inoculum is added after alcoholic fermentation (AF) (sequential AF/MLF), and MLF takes place when the bacterial population increases to about 10^6 CFU/mL. Inoculation of high ethanol as well as low pH wines, however, with selected cultures of LAB after completion of AF can cause a large reduction in viable LAB and an increase in the lag phase. Therefore, sluggish or stuck MLF (Zapparoli et al. 2009) and a rise in the risk of spoilage by other wine microorganisms may occur (Di Toro et al. 2015).

Direct inoculation of LAB into grape must at the beginning of AF (simultaneous AF/MLF) is an alternative that enables a gradual adaptation of bacteria to the increasing ethanol concentration in a medium rich in nutrients. This practice is cautiously used by oenologists, fearing that wine quality may be compromised because of the capacity of LAB to consume must sugars and produce acetic acid as a consequence of their heterofermentative metabolism (Antalick et al. 2013). Moreover, an interruption of AF could take place before sugar depletion (Jussier et al. 2006) by a specific yeast strain-bacteria interaction, because yeast growth could be reduced by LAB (Mendoza et al. 2011).

Conversely, recent studies have highlighted the enhanced capacity of *O. oeni* to complete MLF and reduce fermentation time in simultaneous AF/MLF without a notable increase in wine volatile acidity (VA) (Jussier et al. 2006, Zapparoli et al. 2009, Pan et al. 2011, Abrahamse and Bartowsky 2012, Cañas et al. 2012, 2014, Knoll et al. 2012, Antalick et al. 2013, Taniasuri et al. 2016, Tristezza et al. 2016). Furthermore, wines obtained by simultaneous AF/MLF showed higher red fruit and ripe fruit notes (Jussier et al. 2006, Massera et al. 2009, Abrahamse and Bartowsky 2012, Tristezza et al. 2016, Versari et al. 2016). From a technical point of view, wines elaborated by simultaneous AF/MLF would be ready sooner for downstream processes, such as racking, fining and SO_2 addition, improving microbial stability and winemaking efficiency (Jussier et al. 2006).

The aim of this study was to evaluate, for the first time, the performance of free and encapsulated *O. oeni* inoculated at two stages of the winemaking process: (i) at the end of AF (sequential AF/MLF); and (ii) at the beginning of AF (simultaneous AF/MLF) to induce MLF under different winemaking conditions. The trials were carried out with *O. oeni* encapsulated into gels of ALG and Si-ALG.

Materials and methods

Immobilisation of LAB

Oenococcus oeni strain LALVIN VP 41 MBR (Lallemand, Blagnac, France) was encapsulated into both Si-ALG and ALG gels. A procedure based on the mixture of derivatives of silicon with sodium ALG before gelation in the presence of Ca^{2+} was carried out to encapsulate bacteria into Si-ALG biocomposites (Simó et al. 2017b). The pH of a solution of 1.23 mol/L colloidal silica (Ludox HS40, Sigma-Aldrich, Madrid, Spain) and 0.06 mol/L sodium silicate (Sigma-Aldrich) in water was adjusted to 6.29 by adding 2 N HCl. Then, sodium ALG (Panreac, Barcelona, Spain) was mixed with the silica solution until a final concentration of 2% (w/v). Later on, *O. oeni* was added at ~3×10^9 CFU/g of gel. Before adding bacteria to the siliceous material-ALG solution and according to the manufacturer's instructions, freeze-dried bacteria were rehydrated in 20 times their mass of sterilised chlorine-free water at 20°C for a maximum of 15 min. The well-mixed siliceous material-ALG-cell suspension was extruded from a 10 mL sterile syringe (BD 166 Plastipak, Toledo, Spain) into a sterile 0.2 mol/L $CaCl_2$ solution under continuous agitation (260 rpm) at 22°C. The diameter of the nozzle used was 1.78 mm. The height from which the suspension was dripped into the gelation bath was maintained at 20 cm. Capsules were kept in this solution for 2 h and washed with water at 22°C. The encapsulation of bacteria into ALG hydrogels was similar to that reported above but omitting the addition of colloidal silica and sodium silicate. The Si-ALG and ALG encapsulated bacteria were used immediately after preparation.

Fermentation protocols

Wines of this study were prepared from Cabernet Sauvignon grapes from the 2017 harvest in the experimental winery of the School of Agricultural Engineering, University of Valladolid, Palencia, Spain. Grapes were destemmed and crushed, and SO_2 was added (30 mg/L). Wines were made using grape must without skins or seeds. The composition of the grape must [pH 3.9, 22.4°Brix, 3.7 g/L TA (expressed as tartaric acid), 2.2 g/L of malic acid, 15 mg/L of free SO_2] was modified in order to obtain the required experimental conditions. First, sugar concentration was adjusted in two lots of must to: (i) 20.8°Brix by addition of a volume of distilled water; and (ii) 25.3°Brix by adding the same volume of a solution of sucrose. Thus, the composition of the rest of must components remained equal in both musts. Afterwards, a pH value of 3.0 and 3.8 was obtained using concentrated acid or basic solutions. Finally, the concentration of L-malic acid was adjusted to 3.0 g/L with L-malic acid (Sigma-Aldrich). Four lots of grape must with following composition were sterilised by filtration (0.2 μm, cellulose nitrate membrane): must #1, 25.3°Brix and pH 3.0; must #2, 25.3°Brix and pH 3.8; must #3, 20.8°Brix and pH 3.0; and must #4, 20.8°Brix and pH 3.8. Then, musts were inoculated with 0.25 g/L of rehydrated *Saccharomyces cerevisiae* (Lalvin Clos, Lallemand). The completion of AF was estimated by the measurement of the reducing sugar concentration. Free and encapsulated *O. oeni* (into ALG and Si-ALG hydrogels) were inoculated at a concentration of ~9×10^7 CFU/mL of must/wine: (i) 2 days after the yeast inoculation (simultaneous AF/MLF); and (ii) at the end of AF (sequential AF/MLF). A Control wine without inoculating bacteria was also made. The AF and MLF fermentations were carried out in 100 mL Erlenmeyer flasks containing 50 mL of must/wine at 22°C and in duplicate. The flasks were closed with Müller valves, filled with pure sulfuric acid.

Analytical procedures

The AF was monitored by gravimetric determination, evaluating the loss of mass of the flasks because of the production of CO_2. The degradation of L-malic acid concentration during MLF was monitored with an enzymatic kit (TDI, Barcelona, Spain). The methods described by the Organisation Internationale de la Vigne et du Vin (OIV) (2017) were employed for the determination of pH, VA, reducing sugars and alcohol concentration. Colour parameters were determined at 420, 520 and 620 nm. Total polyphenol index (TPI) was quantified at 280 nm (Lan Optics 2000 UV, Labolan, Spain). Analytical determinations were in triplicate.

Statistical analysis

All statistical analyses were undertaken with the SPSS v. 17.0 statistical package (SPPS, Chicago, IL, USA). Statistical differences were determined by a variance analysis followed by a Tukey's post-hoc test ($P < 0.05$).

Results and discussion

Alcoholic fermentation

The AF was completed in about 10 days in all MLF inoculation treatments, independent of the composition of the grape must (Figures 1–2). Similar fermentative kinetics were found in all samples made by either simultaneous or sequential AF/MLF. The kinetics of AF were not affected by the presence of free or encapsulated bacteria during simultaneous AF/MLF (Figure 2). As reported by other authors (Abrahamse and Bartowsky 2012, Knoll et al. 2012, Tristezza et al. 2016), the presence of *O. oeni* during AF in simultaneous AF/MLF assays does not appear to influence the rate of yeast fermentation. The ability of wine LAB, however, to inhibit AF has been reported, having an adverse impact on the production and the quality of the wine (Alexandre et al. 2004). The mechanisms of yeast inhibition by bacteria involve the formation of metabolites, such as acetic acid (Huang et al. 1996) and bacteriocin-like inhibitors (Yurdugül and Bozoglu 2002), and the depletion of either certain nutrients or survival factors required by yeasts (Alexandre et al. 2004). The degradation of yeast cell wall by extracellular bacterial β-1,3-glucanase activity has been also reported as another factor contributing to AF inhibition (Guilloux-Benatier et al. 2000). This disparity in results indicates that the selection of a suitable yeast–bacteria combination avoids the competition for the nutrients and the synthesis of metabolites with potential inhibitory properties (Alexandre et al. 2004, Guzzon et al. 2013), and plays an important role for a successful AF. Our results have demonstrated that the yeast and bacterial strains used in this study were compatible with a simultaneous AF/MLF strategy.

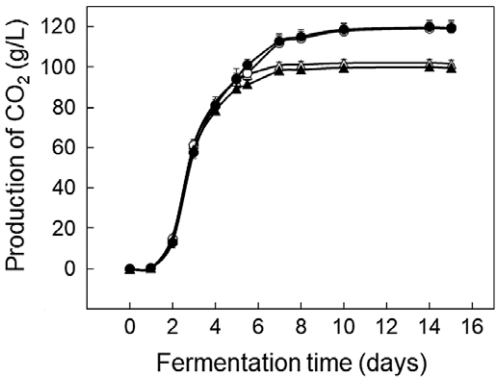

Figure 1. Production of CO_2 during alcoholic fermentation (AF) of Cabernet Sauvignon musts (sequential AF/MLF) at 22°C. Must #1 (25.3°Brix, pH 3.0) (●), must #2 (25.3°Brix, pH 3.8) (○), must #3 (20.8°Brix, pH 3.0) (▲), and must #4 (20.8°Brix, pH 3.8) (△). Standard errors are represented as error bars ($n = 6$).

Malolactic fermentation

The dynamics of MLF were measured along the winemaking processes in both regimes, the sequential AF/MLF (Figure 3) and the simultaneous AF/MLF (Figure 4). The effect of grape must composition (TSS and pH), type of inoculum (encapsulated or free bacteria) and inoculation time (sequential and simultaneous AF/MLF) on the average L-malic acid conversion were analysed 8 days after bacterial inoculation (Figure 5). Significant differences between groups ($P < 0.05$) were found for the four principal variables (TSS, pH, and type of inoculum and inoculation time) and for the following interactions between principal variables: TSS × type of inoculum, TSS × inoculation time and type of inoculum × inoculation time.

The kinetics of MLF were dependent on the composition of the grape must (Figures 3,4). L-Malic acid concentration did not change with respect to the initial value (data not shown) in Control wines (without LAB), demonstrating that MLF development was because of the bacterial inoculum in the remaining samples. The highest average L-malic acid conversion was achieved in wines produced with low sugar grape must (90%) compared with high sugar grape must (66%) (Figure 5). Winemaking with high sugar musts leads to high ethanol wines, which may cause problems with the induction of MLF (Zapparoli et al. 2009). Ethanol is one of the most important factors that affect negatively the metabolism of LAB (Lerm et al. 2010, Sumby et al. 2014). It has been reported that ethanol provokes disruption of cell membrane structure and alteration of membrane fluidity (Betteridge et al. 2015). The average L-malic acid conversion

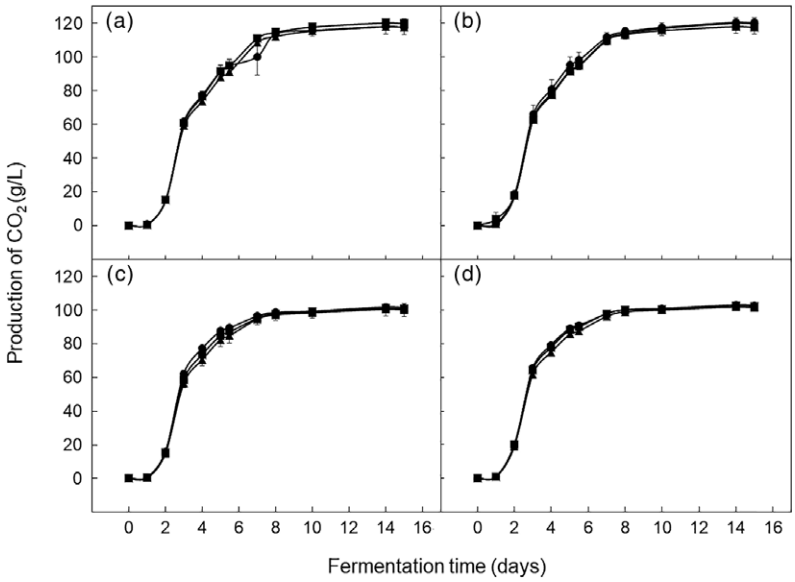

Figure 2. Production of CO_2 during alcoholic fermentation (AF) of Cabernet Sauvignon musts inoculated with free (▲), and alginate (■) and silica-alginate (●) encapsulated *Oenococcus oeni* at the beginning of AF (simultaneous AF/MLF) at 22°C. (a) Must #1 (25.3°Brix, pH 3.0), (b) must #2 (25.3°Brix, pH 3.8), (c) must #3 (20.8°Brix, pH 3.0) and (d) must #4 (20.8°Brix, pH 3.8). Standard errors are represented as error bars ($n = 2$).

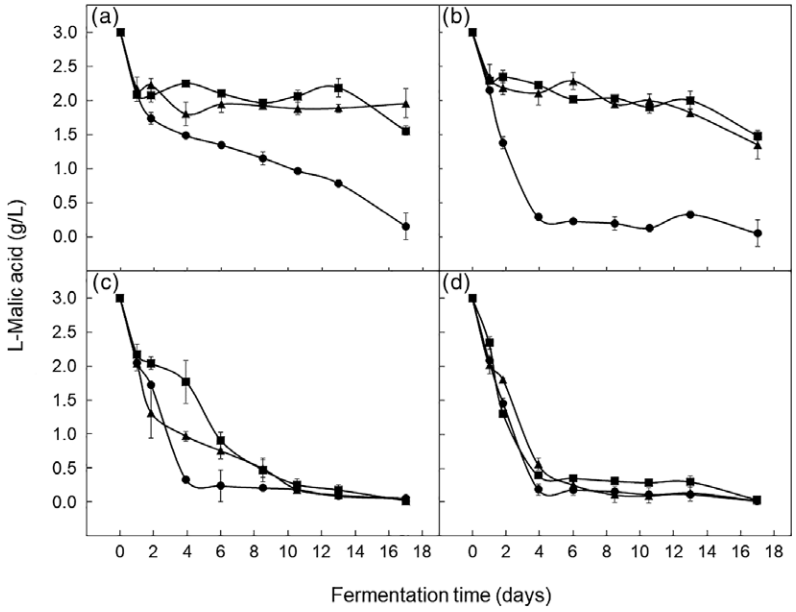

Figure 3. L-Malic acid consumption in Cabernet Sauvignon wines inoculated with free (▲), and alginate (■) and silica-alginate (●) encapsulated *Oenococcus oeni* after alcoholic fermentation (AF) (sequential AF/MLF) at 22°C. (a) Wine from must #1 (25.3°Brix, pH 3.0), (b) wine from must #2 (25.3°Brix, pH 3.8), (c) wine from must #3 (20.8°Brix, pH 3.0) and (d) wine from must #4 (20.8°Brix, pH 3.8). Standard errors are represented as error bars (n = 2).

was reduced from 84 to 71% when pH was reduced from 3.8 to 3.0 (Figure 5). It has been reported that growth of *O. oeni* and development of MLF are negatively influenced by values of pH lower than 3.5 (Rosi et al. 2003, Betteridge et al. 2015), while the highest MLF activity is achieved at pH values of about 3.5–4.0 (Bauer and Dicks 2004).

The combined effect of TSS and pH on malic acid conversion in a sequential AF/MLF regime is shown in Figure 3. A slow reduction of L-malic acid concentration was observed in the wine from grape must #1, characterised by high sugar concentration and low pH. Under these unfavourable conditions, only Si-ALG encapsulated bacteria were able to complete MLF. L-Malic acid, however, was not completely depleted in wines inoculated with ALG encapsulated and free bacteria after 17 days, achieving a low L-malic acid conversion of 48 and 35%, respectively. Conversely, faster L-malic acid depletion was observed during winemaking of grape musts #2, #3 and #4 using Si-ALG encapsulated bacteria (90.7, 89.0 and 94.0% of malic acid conversion, respectively). Moreover, the kinetics of L-malic acid consumption in must #4 were similar with the three types of inoculum, achieving L-malic acid conversion of 96–98% after 8 days. The MLF kinetics of the ALG encapsulated and free bacteria were notably improved in the wines from low sugar musts (#3 and #4) compared with that of wines from high sugar musts (#1 and #2). Solieri et al. (2010) indicated that a low pH value of 3.0–3.2 is the main factor affecting MLF, regardless of ethanol concentration (10–13%). Our results indicated, however, that both low pH and high TSS of must negatively affected malic acid conversion (Figure 3). Similar results were found in Riesling and Chardonnay wines (9.8% of alcohol) when the reduction of pH from 3.8 to 3.2 increased the duration of MLF by up to 34 days, while at pH 3.2 and 11.8% of alcohol a partial MLF was observed (Knoll et al. 2011).

Compared to other inoculation treatments, complete MLF was observed after 4 days (musts #2, #3 and #4) and 17 days (must #1) inoculated with Si-ALG encapsulated bacteria. Slow MLF was found with free bacteria, which completed MLF in wines from musts #3 and #4 after 10 and 6 days, respectively, and they were unable to complete MLF in wines from musts #1 and #2 after 17 days.

Simultaneous AF/MLF had a positive influence on the average of L-malic acid conversion inoculating either

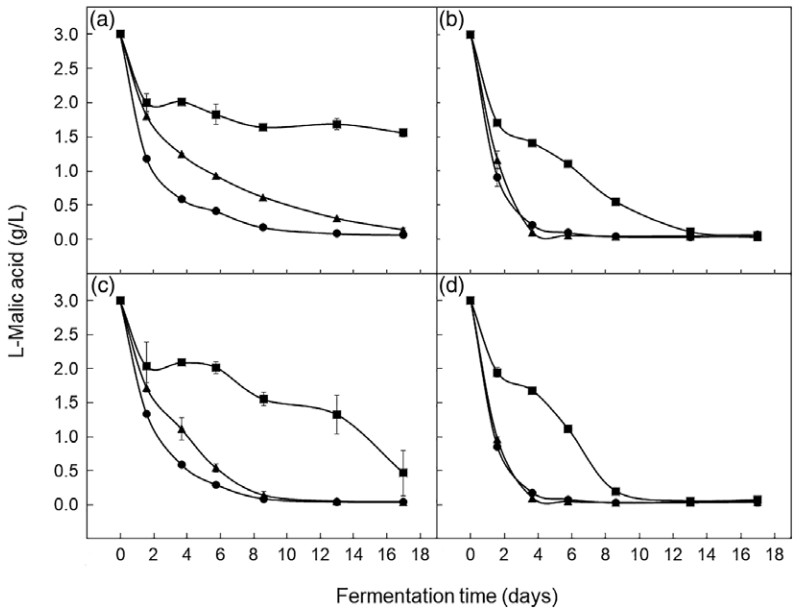

Figure 4. L-Malic acid consumption during winemaking of Cabernet Sauvignon musts inoculated with free (▲), and alginate (■) and silica-alginate (●) encapsulated *Oenococcus oeni* at the beginning of alcoholic fermentation (AF) (simultaneous AF/MLF) at 22°C. (a) Must #1 (25.3°Brix, pH 3.0), (b) must #2 (25.3°Brix, pH 3.8), (c) must #3 (20.8°Brix, pH 3.0) and (d) must #4 (20.8°Brix, pH 3.8). Standard errors are represented as error bars (n = 2).

encapsulated or free bacteria, increasing this parameter from 70 (sequential regime) to 86% (simultaneous regime) (Figure 5). This practice markedly improved MLF behaviour in high sugar grape must (Figure 5c), where an average L-malic conversion of 83% was achieved in comparison with 49% obtained by the sequential regime. Nevertheless, these results were not found in low sugar grape musts where a significant difference for the average of L-malic acid conversion between both inoculation strategies was not observed after 8 days of bacterial inoculation. The combined effect of pH and TSS on malic acid conversion using a simultaneous AF/MLF regimen is shown in Figure 4. In every trial MLF succeeded, regardless of the inoculation treatment; except for musts at pH 3.0 (#1 and #3) inoculated with ALG encapsulated bacteria. In grape must #1, when conditions were more restrictive, simultaneous AF/MLF improved markedly the L-malic acid depletion of grape must inoculated with free and Si-ALG bacteria. L-Malic acid conversion assayed after 8 days increased from 61 and 36% (sequential AF/MLF) to 94 and 79% (simultaneous AF/MLF) with Si-ALG encapsulated and free bacteria, respectively. Simultaneous AF/MLF inoculated with Si-ALG encapsulated and free bacteria resulted in a complete consumption of L-malic acid in 13 and 17 days, respectively. As mentioned before, however, when Si-ALG encapsulated bacteria were inoculated in a sequential inoculation, they required up to 17 days to complete MLF process, while free bacteria were unable to successfully complete the conversion of L-malic acid (Figure 3). In musts #2, #3 and #4, the dynamics of MLF in the simultaneous inoculation were similar using Si-ALG encapsulated and free bacteria, where complete consumption of L-malic acid required 6, 8 and 6 days, respectively.

These results proved that simultaneous AF/MLF was the most efficient inoculation strategy, consistent with other studies (Abrahamse and Bartowsky 2012, Antalick et al. 2013, Cañas et al. 2014, Homich et al. 2016, Tristezza et al. 2016, Versari et al. 2016). Under these winemaking conditions, bacteria were able to induce MLF without a phase of adaptation in grape must. Simultaneous AF/MLF resulted in a valid strategy to allow a gradual bacterial adaptation to the ethanol of the must produced during AF (Zapparoli et al. 2009). During this period, *O. oeni* may be able to activate some cellular mechanisms to adapt to unfavourable conditions, such as low pH and increasing ethanol concentration.

The type of inoculum affected significantly the average of L-malic acid conversion, providing the best results using Si-ALG encapsulated bacteria (90%), followed by free bacteria (79%) and ALG encapsulated bacteria (65%) (Figure 5). In high sugar grape must (Figure 5b), the performance of Si-ALG encapsulated bacteria was superior (87% of conversion) to that obtained inoculating free bacteria (62% of conversion). Both types of inoculum, however, showed similar performance (93–95% of conversion) in low sugar grape must. Differences in performance of Si-ALG encapsulated and free bacteria were observed between sequential and simultaneous AF/MLF (Figure 5f). When bacteria were inoculated after AF (sequential AF/MLF), the highest average of L-malic acid conversion was found with Si-ALG encapsulated bacteria, however, free and Si-ALG encapsulated bacteria did not show a significant difference in simultaneous AF/MLF

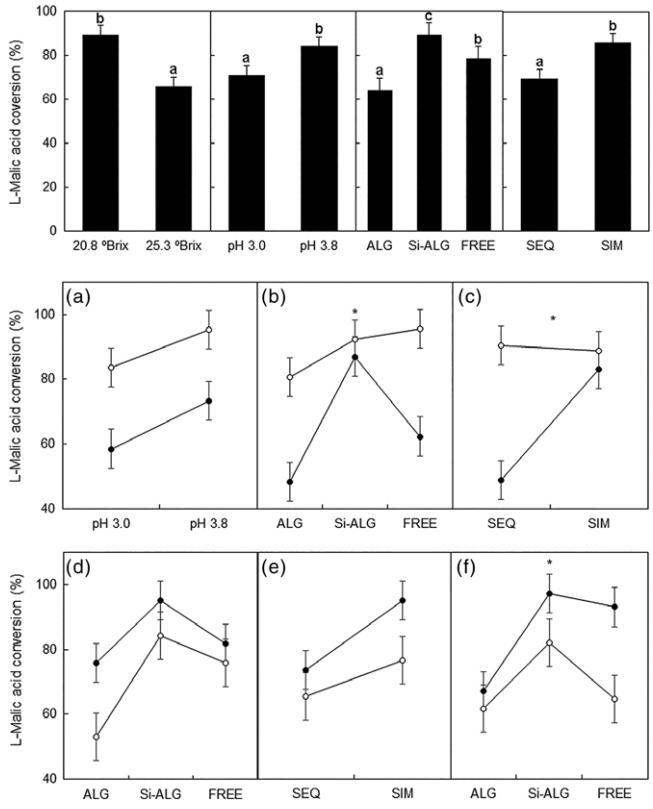

Figure 5. Effect of the grape must composition (TSS and pH), the type of inoculum [alginate (ALG) and silica-alginate (Si-ALG) encapsulated or free bacteria], and the inoculation time [sequential (SEQ) and simultaneous (SIM) AF/MLF] on the average of L-malic acid conversion after 8 days of bacterial inoculation. Standard errors are represented as error bars. Different letters indicate a significant difference at $P < 0.05$. Effect on L-malic consumption depending on (a) pH, (b) type of inoculum and (c) inoculation time of musts with 20.8°Brix (○) and 25.3°Brix (●). Effect on L-malic consumption depending on (d) type of inoculum and (e) inoculation time of musts with pH 3.0 (○) and pH 3.8 (●). Effect on L-malic consumption depending on (f) the type of inoculum performing sequential (○) and simultaneous (●) AF/MLF. *Statistically significant interaction between principal variables ($P < 0.05$).

Table 1. Average of basic chemical composition of Cabernet Sauvignon wines after malolactic fermentation inoculated with free, and alginate and silica-alginate encapsulated *Oenococcus oeni* using simultaneous and sequential alcoholic fermentation/malolactic fermentation.

Factors	Levels	Reducing sugars (g/L)	Alcohol degree (%)	Volatile acid (g/L)	pH	TPI	Colour intensity†	Tonality‡
TSS	20.8	2.83 ± 1.00a	11.8 ± 0.1a	0.21 ± 0.06a	3.41 ± 0.29a	19.2 ± 0.6b	3.26 ± 0.08a	0.97 ± 0.26b
	25.3	4.37 ± 1.06b	14.7 ± 0.1b	0.23 ± 0.05b	3.37 ± 0.33a	18.7 ± 0.3a	3.22 ± 0.05a	0.90 ± 0.23a
pH	3.0	4.17 ± 1.63b	13.3 ± 0.3b	0.24 ± 0.05b	3.12 ± 0.15a	18.9 ± 0.5a	3.65 ± 0.05b	0.49 ± 0.12a
	3.8	3.03 ± 0.74a	13.7 ± 0.3a	0.19 ± 0.06a	3.65 ± 0.15b	19.0 ± 0.9a	2.73 ± 0.05a	1.08 ± 0.18b
Type of inoculum	ALG	3.46 ± 0.42a	13.3 ± 0.4a	0.21 ± 0.04a	3.31 ± 0.20a	19.1 ± 0.3b	3.12 ± 0.03a	0.91 ± 0.22a
	Si-ALG	3.94 ± 1.57a	13.3 ± 0.4a	0.22 ± 0.07a	3.37 ± 0.18ab	18.6 ± 0.5a	3.42 ± 0.06a	0.87 ± 0.17a
	Free	3.38 ± 0.51a	13.3 ± 0.4a	0.22 ± 0.07a	3.47 ± 0.25b	19.2 ± 0.9b	3.31 ± 0.07a	1.02 ± 0.25b
Inoculation time	Sequential AF/MLF	3.72 ± 1.45a	13.3 ± 0.3a	0.23 ± 0.06b	3.34 ± 0.31a	19.2 ± 0.6b	3.32 ± 0.07a	0.96 ± 0.23a
	Simultaneous AF/MLF	3.47 ± 1.32a	13.3 ± 0.3a	0.21 ± 0.06a	3.42 ± 0.30a	18.7 ± 0.8a	3.39 ± 0.07a	0.90 ± 0.19a

Values represent means ± standard error for each level of the factors (n = 12 for TSS, type of inoculum and inoculation time; n = 8 for type of inoculum) and different letters mean a significant difference for each individual factor at $P < 0.05$. AF, alcoholic fermentation; ALG, alginate; MLF, malolactic fermentation; Si-ALG, silica-alginate; TPI, total polyphenol index. †Colour intensity was calculated as $A_{420} + A_{520} + A_{620}$. ‡Tonality was calculated as A_{420}/A_{520}.

inoculation. As previously mentioned, similar L-malic acid evolution by simultaneous AF/MLF was observed inoculating Si-ALG and free bacteria, except for high acid and high sugar grape musts where the depletion of L-malic acid was faster in wines inoculated with Si-ALG bacteria than those inoculated with free bacteria (Figure 4).

The encapsulation of bacteria and yeasts into porous matrices has been applied to improve their catalytic activities in alcohol beverage production (Kourkoutas et al. 2004). A recent study has highlighted that the encapsulation of *O. oeni* into biocomposites of Si-ALG enhanced notably its malolactic activity as compared with that of ALG encapsulated as well as free bacteria (Simó et al. 2017b). The inclusion of siliceous material into ALG capsules increased the tolerance of bacteria towards the stressful environment of wine (low pH, high ethanol and low nutrients) and at low fermentation temperature (Simó et al. 2017c). Rodríguez-Nogales et al. (2013) observed a significant increase in MLF efficiency in high ethanol wines inoculated with encapsulated *O. oeni* into polyvinyl alcohol hydrogel. The success of MLF under winemaking conditions when inoculating Si-ALG encapsulated LAB could be attributed to several factors. The organic–inorganic matrix could offer protection from the harsh environmental conditions of wine, such as alcohol, pH and inhibitors (Kourkoutas et al. 2010, Kosseva 2011). Moreover, an increase in the level of saturated fatty acids of the hydrogel encapsulated cell membrane has been observed in response to ethanol stress, providing a better ethanol tolerance to cells (Junter and Jouenne 2004). Furthermore, it has been reported that cell–cell contact in high cell density matrices enhanced the cell resistance against alcohol stress (Norton et al. 1995). Finally, the non-gelling liquid ALG matrix could improve the stability of the hydration layer around the cell affording protection (Sun et al. 2007).

Wine composition

The composition of the 24 wines after MLF is summarised in Table S1. The average of the final wine composition following MLF for each level of the factors TSS and pH of must, type of inoculum and inoculation time is shown in Table 1. Wines produced with high sugar and low pH grape musts presented a higher value of reducing sugars with an average value of 4.37 and 4.17 g/L, respectively. Wines produced from low pH grape must showed a lower average value of alcohol (13.3%) than those elaborated with high pH grape must (13.7%). Significant differences among type of inoculum and between inoculation regimes were found neither for reducing sugars nor for alcohol concentration, indicating that AF was not affected by the type of inoculum (free or encapsulated bacteria) or by the bacterial inoculation time. All the wines can be considered 'dry' because their reducing sugar level fell below 5 g/L.

The concentration of VA was of neither technological nor legal significance (Table S1), because the sensory threshold and maximum legal concentration for acetic acid range around 1 g/L (Jussier et al. 2006). The type of inoculum, free or encapsulated bacteria, did not affect the average concentration of VA (Table 1). There was a slight increase in VA in wines elaborated with high sugar and high pH grape musts; however, the impact of this difference is limited from a technological and sensory point of view. Sequential inoculation slightly increased the average concentration of VA compared to that of simultaneous inoculation. The results of our study and those of previous studies (Rosi et al. 2003, Knoll et al. 2011, Abrahamse and Bartowsky 2012, Cañas et al. 2012, Homich et al. 2016, Tristezza et al. 2016, Versari et al. 2016) found no evidence for the increase in acetic acid

production because of LAB metabolism in fermenting grape juice using simultaneous AF/MLF. Our results are also consistent with those obtained in a recent study with *S. cerevisiae* and *O. oeni* co-immobilised in ALG gels where inoculation either in simultaneous or in sequential AF/MLF regime did not affect VA (Bleve et al. 2016).

Little difference was found for pH among wines inoculated with free and encapsulated bacteria. This slight difference did not present technological significance. Jussier et al. (2006) found no significant difference with regard to the final value of wine pH between sequential and simultaneous AF/MLF.

The concentration of phenolic substances and colour tonality and intensity values are important factors in the evaluation of wine quality. Overall, there were negligible changes in the TPI and in the colour of wine because of the TSS of grape must and the inoculation time. Wine colour was also affected by must pH, showing the highest colour intensity and the lowest tonality at pH 3.0. The red colour in wine is strongly affected by pH and depends on the proportion of anthocyanin in the flavylium state. As the acidity of wine increases, the proportion of red flavylium anthocyanins also rises (Castañeda-Ovando et al. 2009). Previously, it was shown that similar levels of TPI, colour intensity and tonality were found in wines elaborated by sequential and simultaneous AF/MLF (Abrahamse and Bartowsky 2012, Versari et al. 2016). The use of encapsulated LAB into hydrogels to induce MLF could dilute metabolites and wine colour because ALG and Si-ALG capsules are hydrogels formed by water (about 96–99%) and tend to be dehydrated in the presence of ethanol (Simó et al. 2017b). Moreover, ALG gels have some capacity to adsorb phenolic substances (Massalha et al. 2007). In our study, we did not notice a notable difference in TPI and wine colour characteristics because of the type of inoculum. These results are consistent with the study carried out by Bleve et al. (2016) with *S. cerevisiae* and *O. oeni* co-immobilised in ALG gels. The influence of type of inoculum, however, on colour components should be verified in wines elaborated with skins and under real winemaking conditions.

Conclusions

Inoculation of Si-ALG encapsulated *O. oeni* after AF (sequential AF/MLF) involves a substantial advantage over free bacteria in high sugar as well as in low pH grape must, either enabling to conclude MLF or sensitively reducing the time of this process. A simultaneous AF/MLF strategy may be an alternative to traditional oenological approaches, completing both AF and MLF in a shorter time, and causing no modification in AF kinetics, no increase in VA and no significant variation in basic wine composition. In addition, simultaneous inoculation of yeast and Si-ALG encapsulated bacteria into must of unfavourable composition markedly enhanced the development of MLF compared with free bacteria. These results open the possibility of using both practices (bacterial encapsulation and concomitant yeast–bacteria inoculation), together with a potential recycling of Si-ALG encapsulated *O. oeni* and an implementation of a continuous process. Further studies will examine these factors under commercial winemaking conditions, evaluating the influence of Si-ALG encapsulated bacteria on volatile and sensory characteristics of wines.

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References

Abrahamse, C.E. and Bartowsky, E.J. (2012) Timing of malolactic fermentation inoculation in Shiraz grape must and wine: influence on chemical composition. World Journal of Microbiology and Biotechnology **28**, 255–265.

Alexandre, H., Costello, P.J., Remize, F., Guzzo, J. and Guilloux-Benatier, M. (2004) *Saccharomyces cerevisiae-Oenococcus oeni* interactions in wine: current knowledge and perspectives. International Journal of Food Microbiology **93**, 141–154.

Antalick, G., Perello, M. and De Revel, G. (2012) Characterization of fruity aroma modifications in red wines during malolactic fermentation. Journal of Agricultural and Food Chemistry **60**, 12371–12383.

Antalick, G., Perello, M.C. and De Revel, G. (2013) Co-inoculation with yeast and LAB under winery conditions: modification of the aromatic profile of Merlot wines. South African Journal of Enology and Viticulture **34**, 223–232.

Bartowsky, E.J. (2005) *Oenococcus oeni* and malolactic fermentation – moving into the molecular arena. Australian Journal of Grape and Wine Research **11**, 174–187.

Bauer, R. and Dicks, L.M.T. (2004) Control of malolactic fermentation in wine. A review. South African Journal of Enology and Viticulture **25**, 74–88.

Betteridge, A., Grbin, P. and Jiranek, V. (2015) Improving *Oenococcus oeni* to overcome challenges of wine malolactic fermentation. Trends in Biotechnology **33**, 547–553.

Bleve, G., Tufariello, M., Vetrano, C., Mita, G. and Grieco, F. (2016) Simultaneous alcoholic and malolactic fermentations by *Saccharomyces cerevisiae* and *Oenococcus oeni* cells co-immobilized in alginate beads. Frontiers in Microbiology **7**, 943.

Boido, E., Medina, K., Fariña, L., Carrau, F., Versini, G. and Dellacassa, E. (2009) The effect of bacterial strain and aging on the secondary volatile metabolites produced during malolactic fermentation of Tannat red wine. Journal of Agricultural and Food Chemistry **57**, 6271–6278.

Bouallagui, Z. and Sayadi, S. (2006) Production of high hydroxytyrosol yields via tyrosol conversion by *Pseudomonas aeruginosa* immobilized resting cells. Journal of Agricultural and Food Chemistry **54**, 9906–9911.

Cañas, P.M.I., Pérez-Martín, F., Romero, E.G., Prieto, S.S. and Herreros, M.d.l.L.P. (2012) Influence of inoculation time of an autochthonous selected malolactic bacterium on volatile and sensory profile of Tempranillo and Merlot wines. International Journal of Food Microbiology **156**, 245–254.

Cañas, P.M.I., Romero, E.G., Pérez-Martín, F., Seseña, S. and Palop, M.L. (2014) Sequential inoculation versus co-inoculation in Cabernet Franc wine fermentation. Food Science and Technology International **21**, 203–212.

Castañeda-Ovando, A., Pacheco-Hernández, M.de L., Páez-Hernández, M.E., Rodríguez, J.A. and Galán-Vidal, C.A. (2009) Chemical studies of anthocyanins: a review. Food Chemistry **113**, 859–871.

Di Toro, M.R., Capozzi, V., Beneduce, L., Alexandre, H., Tristezza, M., Durante, M., Tufariello, M., Grieco, F. and Spano, G. (2015) Intraspecific biodiversity and 'spoilage potential' of *Brettanomyces bruxellensis* in Apulian wines. LWT – Food Science and Technology **60**, 102–108.

Guilloux-Benatier, M., Pageault, O., Man, A. and Feuillat, M. (2000) Lysis of yeast cells by *Oenococcus oeni* enzymes. Journal of Industrial Microbiology and Biotechnology **25**, 193–197.

Guzzon, R., Villega, T.R., Pedron, M., Malacarne, M., Nicolini, G. and Larcher, R. (2013) Simultaneous yeast-bacteria inoculum. A feasible solution for the management of oenological fermentation in red must with low nitrogen content. Annals of Microbiology **63**, 805–808.

Homich, L.J., Scheinberg, J.A., Elias, R.J. and Gardner, D.M. (2016) Effects of co-inoculation on wine-quality attributes of the high-acid, red hybrid variety chambourcin. American Journal of Enology and Viticulture **67**, 245–250.

Huang, Y.C., Edwards, C.G., Peterson, J.C. and Haag, K.M. (1996) Relationship between sluggish fermentations and the antagonism of yeast by lactic acid bacteria. American Journal of Enology and Viticulture **47**, 1–10.

Huang, M.P., Wu, M., Xu, Q.S., Mo, D.J. and Feng, J.X. (2016) Highly efficient synthesis of fructooligosaccharides by extracellular fructooligosaccharide-producing enzymes and immobilized cells of *Aspergillus aculeatus* M105 and purification and biochemical characterization of a fructosyltransferase. Journal of Agricultural and Food Chemistry **64**, 6425–6432.

Junter, G.A. and Jouenne, T. (2004) Immobilized viable microbial cells: from the process to the proteome… or the cart before the horse. Biotechnology Advances **22**, 633–658.
Jussier, D., Morneau, A.D. and De Orduña, R.M. (2006) Effect of simultaneous inoculation with yeast and bacteria on fermentation kinetics and key wine parameters of cool-climate Chardonnay. Applied and Environmental Microbiology **72**, 221–227.
Knoll, C., Fritsch, S., Schnell, S., Grossmann, M., Rauhut, D. and Du Toit, M. (2011) Influence of pH and ethanol on malolactic fermentation and volatile aroma compound composition in white wines. LWT – Food Science and Technology **44**, 2077–2086.
Knoll, C., Fritsch, S., Schnell, S., Grossmann, M., Krieger-Weber, S., du, Toit, M. and Rauhut, D. (2012) Impact of different malolactic fermentation inoculation scenarios on Riesling wine aroma. World Journal of Microbiology and Biotechnology **28**, 1143–1153.
Kosseva, M.R. (2011) Immobilization of microbial cells in food fermentation processes. Food and Bioprocess Technology **4**, 1089–1118.
Kourkoutas, Y., Manojlović, V. and Nedović, V.A. (2010) Immobilization of microbial cells for alcoholic and malolactic fermentation of wine and cider. Zuidam N. and Nedovic V., eds. Encapsulation technologies for active food ingredients and food processing (Springer: New York, NY) pp. 327–343.
Kourkoutas, Y., Bekatorou, A., Banat, I.M., Marchant, R. and Koutinas, A.A. (2004) Immobilization technologies and support materials suitable in alcohol beverages production: a review. Food Microbiology **21**, 377–397.
Lee, J.S., Cha, D.S. and Park, H.J. (2004) Survival of freeze-dried *Lactobacillus bulgaricus* KFRI 673 in chitosan-coated calcium alginate microparticles. Journal of Agricultural and Food Chemistry **52**, 7300–7305.
Lerm, E., Engelbrecht, L. and du, Toit, M. (2010) Malolactic fermentation: the ABC's of MLF. South African Journal of Enology and Viticulture **31**, 186–212.
Massalha, N., Basheer, S. and Sabbah, I. (2007) Effect of adsorption and bead size of immobilized biomass on the rate of biodegradation of phenol at high concentration levels. Industrial and Engineering Chemistry Research **46**, 6820–6824.
Massera, A., Soria, A., Catania, C., Krieger, S. and Combina, M. (2009) Simultaneous inoculation of Malbec (*Vitis vinifera*) musts with yeast and bacteria: effects on fermentation performance, sensory and sanitary attributes of wines. Food Technology and Biotechnology **47**, 192–201.
Mendoza, L.M., Merín, M.G., Morata, V.I. and Farías, M.E. (2011) Characterization of wines produced by mixed culture of autochthonous yeasts and *Oenococcus oeni* from the northwest region of Argentina. Journal of Industrial Microbiology and Biotechnology **38**, 1777–1785.
Mira de Orduña, R. (2010) Climate change associated effects on grape and wine quality and production. Food Research International **43**, 1844–1855.
Norton, S., Watson, K. and D'Amore, T. (1995) Ethanol tolerance of immobilized brewers' yeast cells. Applied Microbiology and Biotechnology **43**, 18–24.
Organisation Internationale de la Vigne et du Vin (2017) Compendium of international methods of wine and must analysis (Organisation Internationale de la Vigne et du Vin: Paris, France).
Pan, W., Jussier, D., Terrade, N., Yada, R.Y. and Mira de Orduña, R. (2011) Kinetics of sugars, organic acids and acetaldehyde during simultaneous yeast-bacterial fermentations of white wine at different pH values. Food Research International **44**, 660–666.
Rodríguez-Nogales, J.M., Vila-Crespo, J. and Fernández-Fernández, E. (2013) Immobilization of *Oenococcus oeni* in Lentikats® to develop malolactic fermentation in wines. Biotechnology Progress **29**, 60–65.
Rosi, I., Fia, G. and Canuti, V. (2003) Influence of different pH values and inoculation time on the growth and malolactic activity of a strain of *Oenococcus oeni*. Australian Journal of Grape and Wine Research **9**, 194–199.
Safarik, I., Sabatkova, Z. and Safarikova, M. (2008) Hydrogen peroxide removal with magnetically responsive *Saccharomyces cerevisiae* cells. Journal of Agricultural and Food Chemistry **56**, 7925–7928.
Satora, P., Tarko, T., Duda-Chodak, A., Sroka, P., Tuszynski, T. and Czepielik, M. (2009) Influence of prefermentative treatments and fermentation on the antioxidant and volatile profiles of apple wines. Journal of Agricultural and Food Chemistry **57**, 11209–11217.
Shin, K.C., Sim, D.H., Seo, M.J. and Oh, D.K. (2016) Increased production of food-grade D-tagatose from D-galactose by permeabilized and immobilized cells of *Corynebacterium glutamicum*, a GRAS host, expressing D-galactose isomerase from *Geobacillus thermodenitrificans*. Journal of Agricultural and Food Chemistry **64**, 8146–8153.
Simó, G., Fernández-Fernández, E., Vila-Crespo, J., Ruipérez, V. and Rodríguez-Nogales, J.M. (2017a) Research progress in coating techniques of alginate gel polymer for cell encapsulation. Carbohydrate Polymers **170**, 1–14.
Simó, G., Vila-Crespo, J., Fernández-Fernández, E., Ruipérez, V. and Rodríguez-Nogales, J.M. (2017b) Highly efficient malolactic fermentation of red wine using encapsulated bacteria in a robust biocomposite of silica-alginate. Journal of Agricultural and Food Chemistry **65**, 5188–5197.
Simó, G., Fernández-Fernández, E., Vila-Crespo, J., Ruipérez, V. and Rodríguez-Nogales, J.M. (2017c) Silica-alginate encapsulated bacteria to enhance malolactic fermentation performance in a stressful environment. Australian Journal of Grape and Wine Research **23**, 342–349.
Solieri, L., Genova, F., De Paola, M. and Giudici, P. (2010) Characterization and technological properties of *Oenococcus oeni* strains from wine spontaneous malolactic fermentations: a framework for selection of new starter cultures. Journal of Applied Microbiology **108**, 285–298.
Sumby, K.M., Grbin, P.R. and Jiranek, V. (2014) Implications of new research and technologies for malolactic fermentation in wine. Applied Microbiology and Biotechnology **98**, 8111–8132.
Sun, Z.J., Lv, G.J., Li, S.Y., Yu, W.T., Wang, W., Xie, Y.B. and Ma, X. (2007) Differential role of microenvironment in microencapsulation for improved cell tolerance to stress. Applied Microbiology and Biotechnology **75**, 1419–1427.
Taniasuri, F., Lee, P.R. and Liu, S.Q. (2016) Induction of simultaneous and sequential malolactic fermentation in Durian wine. International Journal of Food Microbiology **230**, 1–9.
Tristezza, M., di, Feo, L., Tufariello, M., Grieco, F., Capozzi, V., Spano, G., Mita, G. and Grieco, F. (2016) Simultaneous inoculation of yeasts and lactic acid bacteria: effects on fermentation dynamics and chemical composition of Negroamaro wine. LWT – Food Science and Technology **66**, 406–412.
Versari, A., Patrizi, C., Parpinello, G.P., Mattioli, A.U., Pasini, L., Meglioli, M. and Longhini, G. (2016) Effect of co-inoculation with yeast and bacteria on chemical and sensory characteristics of commercial Cabernet Franc red wine from Switzerland. Journal of Chemical Technology and Biotechnology **91**, 876–882.
Vila-Crespo, J., Rodriguez-Nogales, J.M., Fernández-Fernández, E. and Hernanz-Moral, M.C. (2010) Strategies for the enhancement of malolactic fermentation in the new climate conditions. Méndez-Vilas A., ed. Current research, technology and education topics in applied microbiology and microbial biotechnology (Formatex Research Center: Badajoz, Spain) pp. 920–929.
Yurdugül, S. and Bozoglu, F. (2002) Studies on an inhibitor produced by lactic acid bacteria of wines on the control of malolactic fermentation. European Food Research and Technology **215**, 38–41.
Zapparoli, G., Tosi, E., Azzolini, M., Vagnoli, P. and Krieger, S. (2009) Bacterial inoculation strategies for the achievement of malolactic fermentation in high-alcohol wines. South African Journal of Enology and Viticulture **30**, 49–55.
Zhang, D. and Lovitt, R.W. (2006) Strategies for enhanced malolactic fermentation in wine and cider maturation. Journal of Chemical Technology and Biotechnology **81**, 1130–1140.

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Supporting information

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Table S1. Basic composition of the 24 wines after MLF inoculated with free and alginate (ALG) and silica-alginate (Si-ALG) encapsulated *Oenococcus oeni* using simultaneous and sequential AF/MLF of Cabernet Sauvignon.