# Impact of inhibitory peptides released by *Saccharomyces cerevisiae* BDX on the malolactic fermentation performed by *Oenococcus oeni* Vitilactic F

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*Keywords:*
*Saccharomyces cerevisiae*
*Oenococcus oeni*
Malolactic enzyme
Antibacterial yeast peptidic compounds

A B S T R A C T

A previous study has shown that the malolactic fermentation (MLF) was inhibited during sequential fermentations performed with the pair *Saccharomyces cerevisiae* BDX/*Oenococcus oeni* Vitilactic F in synthetic grape juices. A yeast peptidic fraction with an apparent MW of 5–10 kDa was involved in the inhibition. In the present study, the MLF was also inhibited in Cabernet Sauvignon and Syrah wines. The inhibition due to the peptidic fraction was maintained despite high phenolic contents. Kinetic studies showed that the peptidic fraction was gradually released during the alcoholic fermentation (AF). Its highest anti-MLF effect was reached when isolated from late stages of the AF stationary phase. The peptidic fraction was tested *in vitro* on cell-free bacterial cytosolic extracts containing the malolactic enzyme in a pH range between 3.5 and 6.7. Results showed that it was able to directly inhibit the malolactic enzyme activity with an increasing inhibitory kinetic correlated to the AF time at which it was collected.

## 1. Introduction

The production of most red wines and certain white and sparkling wine styles requires two consecutive fermentation steps. The first one is the alcoholic fermentation (AF) and is carried out by yeasts belonging mainly to the *Saccharomyces cerevisiae* species. During this step, the sugars of the grape must (D-Glucose and D-Fructose) are primarily converted into ethanol and $CO_2$. At the end of the AF, these wines are spontaneously or purposely taken through a malolactic fermentation (MLF) step mostly by indigenous or inoculated lactic acid bacteria belonging mainly to the *Oenococcus oeni* species. The MLF, an enzymatic decarboxylation of L-malic acid into L-lactic acid and $CO_2$, reduces wine acidity and improves its sensorial characteristics and its microbial stability (Bartowsky et al., 2002; Lonvaud-Funel, 1999, 2002). However, it is often difficult to trigger and accomplish because of the individual or synergistic antibacterial activity of several physical chemical wine parameters and yeast inhibitory metabolites. Some of these factors have been intensively investigated such as low pH (Britz and Tracey, 1990; Vaillant et al., 1995), inadequate temperature (Britz and Tracey, 1990), nutrient depletion (Patynowski et al., 2002; Remize et al., 2006; Saguir and Manca de Nadra, 2002; Terrade and Mira de Orduna, 2009), endogenous and exogenous $SO_2$ (Carreté et al., 2002; Henick-Kling and Park, 1994; Larsen et al., 2003; Osborne and Edwards, 2006), phenolic compounds (Reguant et al., 2000), high ethanol content (Britz and Tracey, 1990; Vaillant et al., 1995) and medium chain fatty acids (Capucho and San Romao, 1994; Edwards and Beelman, 1987; Lonvaud Funel et al., 1988).

While the anti-MLF role of the previous compounds is already well established, there are gradually growing evidences suggesting the involvement of yeast peptides/proteins in the inhibition of *O. oeni* growth and L-malic acid consumption. So far, few authors have demonstrated the ability of certain *S. cerevisiae* strains to produce anti-MLF compounds of protein nature. Besides, the compounds found presented different MW and were strain dependent. Dick et al. (1992) were the first to isolate two antibacterial cationic proteins produced by the yeast strain R107. One of them had the characteristics of lysozyme and the other one was a small protein with a high pI. Later on, Comitini et al. (2005) found that *S. cerevisiae* F63 was able to produce a proteinaceous compound with a MW >10 kDa that strongly inhibited the growth of *O. oeni* CHR as well as its MLF. Besides, Osborne and Edwards (2007),

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identified a 5.9 kDa peptide produced by *S. cerevisiae* RUBY. Ferm and inhibiting *O. oeni* Viniflora oenos. Mendoza et al. (2010) showed that *S. cerevisiae* mc2 released a proteinaceous compound presenting a MW between 3 and 10 kDa that inhibited the growth of *O. oeni* $X_2L$ but not its ability to consume L-malic acid. Finally, Branco et al. (2014) showed that *S. cerevisiae* CCMI 885 secreted antimicrobial peptides (AMP) that were active against a wide variety of wine-related yeasts in addition to *O. oeni*. However, only the microbial growth was evaluated. These AMP corresponded to different fragments of the C-terminal amino acid sequence of the *S. cerevisiae* glyceraldehyde 3-phosphate dehydrogenase (GAPDH) enzyme. Among these AMP, two main fragments of 1.638 and 1.622 kDa were detected.

Most of the time, the impact of these compounds on the growth of *O. oeni* was the main parameter evaluated and few data concerning the malate consumption was given. The MLF inhibition was often considered as a consequence of the bacterial growth inhibition. Besides, the direct effect of these compounds on the malolactic enzyme activity was never studied.

In a previous work (Nehme et al., 2010), we demonstrated that *S. cerevisiae* BDX (referred to as strain D) was able to produce a peptidic fraction with an apparent MW between 5 and 10 kDa responsible for the strong inhibition of *O. oeni* Vitilactic F (referred to as strain X) during sequential fermentations performed in synthetic grape juices. Sequential fermentations simulate the natural winemaking process by starting the MLF after the accomplishment of the AF. In the present work, we intended to further characterize this peptidic fraction; first by showing its ability to inhibit MLF in natural wines despite high phenolic contents, second by determining the time of its release during AF and third by measuring its direct inhibitory effect on the malolactic enzyme activity.

## 2. Materials and methods

All culture media components were supplied by Sigma-Aldrich (Taufkirchen-Germany) except for the Yeast Extract and the peptone that were supplied by Oxoid (Hampshire-England).

### 2.1. Strains and storage conditions

*S. cerevisiae* BDX and CY3079 and *O. oeni* Vitilactic F used in this work were kindly provided by Lallemand Inc. (Blagnac, France). In previous works, BDX was referred to as strain D, CY3079 as strain A and Vitilactic F as strain X (Nehme et al., 2008, 2010). Yeast stock cultures were kept at 4 °C in YEPD (Yeast Extract Peptone Dextrose) agar slants composed of 10 g/L Yeast Extract, 20 g/L peptone, 20 g/L D-glucose and 20 g/L agar. The bacterial strain was kept frozen at −20 °C in MRS (*De Man, Rogosa and Sharpe*) broth containing 20% glycerol (v/v).

### 2.2. Growth media

#### 2.2.1. Synthetic grape juice medium (SGJ medium)

The medium composition that simulated the natural grape juice consisted of: D-glucose 100 g/L, D-fructose 100 g/L, Yeast Extract 1 g/L, $(NH_4)_2SO_4$ 2 g/L, citric acid 0.3 g/L, L-malic acid 3 g/L, L-tartaric acid 5 g/L, $MgSO_4$ 0.4 g/L, and $KH_2PO_4$ 5 g/L, pH adjusted to 3.5 using a 10 mol/L NaOH solution. The medium was autoclaved before use (121 °C, 20 min).

#### 2.2.2. Cabernet Sauvignon and Syrah grape musts

Cabernet Sauvignon (CS) and Syrah grape varieties were provided by Clos Saint Thomas (Kab Elias, Lebanon) in September 2014. The grapes were destemmed and crushed then submitted to pre-fermentation macerations at 10, 60, 70 and 80 °C for 48 h. The grape musts were sulfitized at a dose of 5 g/hL. A solution of $NaHSO_3$ 100 g/L was used for this purpose. The sugar and L-malic acid concentrations were adjusted to 200 g/L and 3 g/L respectively. The pH was also adjusted to 3.5 using an 85% orthophosphoric acid solution.

#### 2.2.3. Modified MRS medium

The MRS medium was supplemented with L-malic acid (3 g/L) and Tween 80 (1 mL). After autoclaving (121 °C, 15 min), 10% ethanol (v/v) were aseptically added through sterile filter membranes of 0.22 μm cut-off (Elvetec services, Meyzieu-France) and the pH was adjusted to 3.5 using an 85% orthophosphoric acid solution.

### 2.3. Sequential fermentations in natural grape musts

The Cabernet Sauvignon and Syrah grape musts were separately inoculated either by *S. cerevisiae* BDX or by *S. cerevisiae* CY3079 at an initial concentration of $3 \times 10^6$ cells/mL (Thoma counting chamber). The AF was followed until total or cessation of sugar consumption (<2 g/L). The yeast inoculum was beforehand prepared in two steps. First, a preculture of the yeast strain was obtained by reactivating the stock culture in YEPD broth for 24 h. Second, the preculture was used to inoculate a low sugar concentration synthetic grape juice medium: D-Glucose 50 g/L and absence of D-Fructose. This step was carried out for 24 h and provided the yeast inoculum.

After completion of the AF, the wines were subjected to different steps before inoculation of the lactic acid bacteria. First, yeast cells were removed by centrifugation (3000 rpm for 20 min at 4 °C) and the supernatants were recovered. Then, the L-malic acid concentration was measured and readjusted to 3 g/L. Next, the pH was adjusted to 3.5 using a 10 mol/L NaOH solution. Finally, the wines were filtered aseptically through 0.22 μm membranes (Elvetec services) and were inoculated with the malolactic bacteria at an initial concentration of $2 \times 10^6$ cells/mL (Petroff-Hausser counting chamber) corresponding to an initial bacterial biomass of 0.0016 g/L. The MLF was followed until cessation of L-malic acid consumption. The bacterial inoculum was prepared in two steps. First, a preculture of *O. oeni* Vitilactic F was obtained by reactivating the stock culture in MRS broth with 3% ethanol (v/v) added. After 24 h, the preculture was used to inoculate the low sugar concentration synthetic grape juice medium with 6% ethanol (v/v) added. This step provided the bacterial inoculum after an incubation period of 24 h.

All fermentation steps for both AF and MLF were carried out at 22 °C with stirring at 150 rpm in Erlenmeyer flasks.

Yeast and bacterial growth were followed during AF and MLF respectively by measuring their cell concentration (cells/mL) and their dry weight (g/L).

Each sequential fermentation was performed in three independent replicates.

### 2.4. Timing of the release of the 5–10 kDa peptidic fraction produced by S. cerevisiae BDX during AF

The AF was carried out by *S. cerevisiae* BDX for 5 days (120 h) in 2 L of the SGJ medium. Each 24 h, a supernatant of 210 mL was collected and fractionated by ultrafiltration in order to isolate the corresponding 5–10 kDa peptidic fraction. First, the sample was distributed into centrifugal filter units' presenting a cut-off of 10 kDa (Amicon® Ultra-15 with ultracel-10 membrane) and the ultrafiltration was performed at 3500 g for 45 min at 4 °C. Each unit can initially contain a volume of 15 mL. 14 units were filled and at the end of the ultrafiltration, a volume of 0.2 mL was retained by the membrane of each unit. A total retentate of 2.8 mL with a MW ≥ 10 kDa, 75 times concentrated, was obtained. The filtrate ≤10 kDa was then recovered and ultrafiltered using the centrifugal filter units presenting a cut-off of 5 kDa (Corning® Spin-X UF 20 Sigma-Aldrich). The principle was the same and 2.8 mL of a fraction with a MW between 5 and 10 kDa, 75 times concentrated, was obtained. Five fractions were finally collected at the end of the AF (after 120 h). They were referred to as D1 (24 h), D2 (48 h), D3 (72 h), D4 (96 h) and D5 (120 h). The fraction from each day (2.8 mL) was added to a modified MRS medium so as to obtain a final volume of 20 mL and was finally 10.5 times concentrated.

*O. oeni* Vitilactic F was then inoculated into these media at an initial concentration of $2 \times 10^6$ cells/mL and the MLF was carried out at 22 °C with stirring at 150 rpm in Erlenmeyer flasks. The same modified MRS medium without the 5–10 kDa fractions was used as a control for this experiment. The MLF was followed by regular sampling until the cessation of the L-malic acid consumption.

The timing experiment was performed in three independent replicates.

### 2.5. In vitro evaluation of the inhibitory effect of the 5–10 kDa fractions on the malolactic enzyme activity

#### 2.5.1. Preparation of the cell-free bacterial enzymatic extract comprising the malolactic enzyme

The modified MRS medium was inoculated with *O. oeni* Vitilactic F at an initial concentration of $2 \times 10^6$ cells/mL and the bacterial culture was followed for 5 days at 22 °C with stirring at 150 rpm. After a centrifugation at 3500 rpm for 20 min at 4 °C, the supernatant was removed and 0.4 g of the bacterial pellet were weighed and suspended in 800 μL of an appropriate buffer (citrate buffer pH 3.5, phosphate buffer pH 6 or phosphate buffer pH 6.7, buffer concentration 0.1 M). The bacterial cells were then lysed by vortexing them with glass beads of 300 μm diameter (Sigma-Aldrich) in a FastPrep® FP120 bead-beater device (BIO 1010/Savant) at 6 m/s for $3 \times 45$ s at 4 °C. Between 2 consecutive runs, the cells were kept on ice for 1 min. The cell debris was removed by centrifugation at 14,000 g for 10 min at 4 °C (Larsen et al., 2006). The supernatant containing the cell-free enzymatic extract was recovered and the volume completed to 2 mL using the corresponding buffer.

#### 2.5.2. Monitoring of the L-malic acid consumption in vitro

The 2 mL of the enzymatic extract were divided into 2 aliquots of 1 mL each. One was used as a control and the other as a test medium to which the 5–10 kDa fractions were added. The reaction mixture is given in Table 1. The reagents were added to each aliquot in the order listed in the table. The enzymatic reaction was performed at 30 °C for 30 min at pH 6, 40 min at pH 6.7 and 70 min at pH 3.5.

The inhibitory kinetic of the malolactic enzyme activity by the 5–10 kDa fractions was evaluated. The 5 peptidic fractions of 5–10 kDa collected from D1 to D5 (Section 2.4) were tested separately. At the end of each assay, the concentrations of the L-malic acid consumed and the L-lactic acid produced were measured in both aliquots (control and test) and compared.

The inhibitory effect of each 5–10 kDa fraction on the malolactic enzyme activity was performed in three independent replicates.

### 2.6. Analytical methods

#### 2.6.1. L-malic acid consumption

The L-malic acid concentration was determined using an enzymatic assay (Boehringer Mannheim/R-Biopharm, kit. No 10139068035, Darmstadt-Germany) and the results were expressed in g/L.

**Table 1**
Reaction mixture for the monitoring of the L-malic acid consumption *in vitro* by the malolactic enzyme of the enzymatic extract isolated from *O. oeni* Vitilactic F at different pH values with or without the 5–10 kDa peptidic fractions.

| Aliquot 1 (control) | Aliquot 2 (test) | Amounts |
|---|---|---|
| Enzymatic extract | Enzymatic extract | 1 mL |
| $Mn^{2+}$ | $Mn^{2+}$ | 0.1 mg |
| $NAD^+$ (35 g/L) | $NAD^+$ (35 g/L) | 0.1 mL |
| Buffer[a] | ABP[b] (10.5×) | 0.14 mL |
| L-malic acid (1 g/L) | L-malic acid (1 g/L) | 1 mg |

[a] ABP (10.5×): antibacterial peptides of the 5–10 kDa fractions 10.5 times concentrated.
[b] Buffer: 0.1 mol/L of citrate buffer pH 3.5, phosphate buffer pH 6 or phosphate buffer pH 6.7.

#### 2.6.2. L-lactic acid production

The L-lactic acid concentration was determined using an enzymatic assay (Boehringer Mannheim/R-Biopharm, kit. No 10139084035, Darmstadt-Germany) and the results were expressed in g/L.

#### 2.6.3. Total polyphenols

Total polyphenols in Cabernet Sauvignon and Syrah wines were evaluated at the end of the AF carried out by both yeast strains. The method used was based on the reduction of the Folin-Ciocalteu reagent by polyphenols as described by Ribéreau-Gayon et al. (2006). The results were expressed in mg/L equivalent gallic acid.

#### 2.6.4. Dry weight of bacteria

The dry weight was determined using a thermobalance or moisture analyzer (Ohaus-UK). A correlation between the bacterial cell concentration (cells/mL) and the bacterial biomass (g/L) was established using high bacterial concentrations. The correlation obtained was the following:

$$y_{(g/L)} = 8.10^{-10} \times_{(cells/mL)}.$$

It was then used to determine the biomass of low concentrated samples.

### 2.7. Statistical analyses

Means and standard deviations of the assays were calculated using conventional statistical methods. Each experiment was performed in three replicates. Statistical analysis (ANOVA) was applied to the data to determine differences ($p < 0.05$). Means differences were made by using Tukey's HSD test. The statistical analysis was carried out using Statgraphics XV·I for windows.

## 3. Results and discussion

### 3.1. Impact of the 5–10 kDa peptidic fraction produced by S. cerevisiae BDX on MLF in natural winemaking conditions

It was previously shown that the growth of *O. oeni* Vitilactic F and its ability to consume L-malic acid were strongly inhibited by *S. cerevisiae* BDX during sequential fermentations performed in synthetic grape juice (SGJ) media. The MLF inhibition was mainly due to a peptidic fraction presenting an apparent MW of 5–10 kDa working synergically with ethanol (Nehme et al., 2010). The $SO_2$ and medium chain fatty acids concentrations produced by *S. cerevisiae* BDX were lower than the ones found in the literature to cease MLF and the nutrient depletion was excluded from inhibitory factors (Nehme et al., 2008, 2010). *S. cerevisiae* CY3079 produced similar amounts of ethanol, $SO_2$ and medium chain fatty acids as *S. cerevisiae* BDX and presented the same nutritional behavior. MLF carried out by *O. oeni* Vitilactic F in SGJ media fermented by CY3079 was totally accomplished (Nehme et al., 2008). Besides, 5–10 kDa peptidic fractions isolated from SGJ media fermented by CY3079 did not inhibit *O. oeni* Vitilactic F (data not published). Therefore CY3079 was chosen as a reference yeast strain for this study.

Table 2 shows the total phenolic content at the end of the AF carried out by *S. cerevisiae* BDX and *S. cerevisiae* CY3079 in Cabernet Sauvignon and Syrah wines. The corresponding grape musts were previously macerated at 10, 60, 70 and 80 °C for 48 h before AF. High phenolic contents were reached at maceration temperatures of 60, 70 and 80 °C because of a better phenolic extraction from the grape skin and seeds. Sequential fermentations were carried out in these wines by inoculating *O. oeni* Vitilactic F at the end of the AF. MLF was monitored for 3 months and was totally inhibited in wines fermented by *S. cerevisiae* BDX. Both the bacterial growth and the malate consumption were repressed regardless of the phenolic content (Tables 2 and 3). Despite high phenolic

**Table 2**
Total phenolic content (mg/L equivalent gallic acid) in Cabernet Sauvignon and Syrah wines at the end of the AF carried out by *S. cerevisiae* CY3079 and *S. cerevisiae* BDX.[a]

| Pre-fermentation maceration temperature (°C) | Cabernet Sauvignon wine | Syrah wine | Cabernet Sauvignon wine | Syrah wine |
|---|---|---|---|---|
| | Phenolic content at the end of the AF carried out by *S. cerevisiae* CY3079 (mg/L) | | Phenolic content at the end of the AF carried out by *S. cerevisiae* BDX (mg/L) | |
| 10 | 513 ± 18 | 365 ± 7 | 868 ± 18 | 378 ± 32 |
| 60 | 2635 ± 21 | 2425 ± 106 | 2925 ± 7 | 2718 ± 117 |
| 70 | 3725 ± 21 | 3043 ± 88 | 3978 ± 11 | 3645 ± 170 |
| 80 | 2985 ± 21 | 2295 ± 35 | 3078 ± 11 | 2585 ± 42 |

[a] Results are mean ± SD values of three replications.

contents, MLF conducted by *O. oeni* Vitilactic F in wines produced by *S. cerevisiae* CY3079 was totally completed. In comparison to the pre-fermentation maceration at 10 °C, the higher phenolic content obtained at 60, 70 and 80 °C reduced the bacterial biomass produced and slowed down the MLF but malate was totally consumed (Table 3).

Therefore, MLF results were reproducible in both synthetic and natural grape juice media with both yeasts/bacteria pairs tested.

The conservation of the inhibitory effect due to the 5–10 kDa yeast peptidic fraction in natural winemaking conditions despite the presence of phenolic compounds is an interesting finding. It is usually known that phenolic compounds are able to interact with proteins leading either to their precipitation or to changes of their bioactive properties (Sims et al., 1995; Yokotsuka and Singleton, 1995). Wine proteins derive mostly from grapes but also from yeasts during AF. Both reversible (van der Waals forces, hydrogen bonding and hydrophobic binding) and irreversible (covalent bonds) interactions are involved. Proline rich proteins like collagen, gelatin and casein, which are commonly used as protein fining agents in wine, and some salivary proteins reported to be involved in astringency perception, are particularly prone to interact with phenolic compounds. The precipitation depends on the phenols and proteins type and concentration (McRae and Kennedy, 2011; Ozdal et al., 2013).

Few studies investigated the possible interactions between peptides of low MW and phenolic compounds in wine. Besides, no previous studies dealt with peptides from wine origin. Yokotsuka and Singleton (1995) demonstrated that gelatin peptides (2, 5 and 10 kDa) and synthetic peptides of low MW (between 1.3 and 2.5 kDa) were very effective for fining wines, at least at the same levels as whole gelatin (70 kDa) with high affinity for phenols. The affinity depended on the pH and temperature. Osborne and Edwards (2007) found that *S. cerevisiae* RUBY. Ferm was able to produce a peptide of 5.9 kDa that inhibited *O. oeni* Viniflora oenos during sequential fermentations in synthetic grape juice media lacking phenols. However, Larsen et al. (2003) who previously used the same strains combination were able to successfully achieve MLF in Chardonnay wines. Therefore, it was suggested that the Chardonnay wine contained phenolic compounds that may have interacted with the 5.9 kDa peptide.

The 5–10 kDa peptides of this study may have not presented binding sites for phenolic compounds and thus were not removed. Regardless of the different types and concentrations of phenolic compounds, the inhibition caused by the 5–10 kDa peptidic fraction released by *S. cerevisiae* BDX was preserved in natural winemaking conditions.

### 3.2. Timing of the release of the 5–10 kDa anti-MLF peptidic fraction produced by S. cerevisiae BDX during AF

In order to assess the timing of the release of the 5–10 kDa peptidic fraction, the AF in the SGJ medium was followed until total sugar consumption. It lasted 120 h whereas the stationary growth phase started after 50 h. A peptidic fraction of 5–10 kDa was collected each 24 h of the AF. Five fractions were obtained and each one was tested in a modified MRS medium where it was finally 10.5 times concentrated. The results were compared with those obtained in a modified MRS control. Fig. 1a shows that the growth kinetic profiles of *O. oeni* Vitilactic F in the presence of the fractions collected after 24, 48 and 72 h were very similar. Although the average specific growth rates were identical to that of the control (0.009 $h^{-1}$), the final biomass reached (0.072 g/L) was 10% less than the one obtained in the control (0.08 g/L) (Table 4). When the fractions collected after 96 and 120 h were tested, the specific growth rates were slightly higher (0.013 $h^{-1}$, $p < 0.05$), but the maximum biomass reached (0.03 g/L) was reduced by 62.5% compared to the control (Table 4). Moreover, a decline phase was detected with these two fractions leading to the death of the whole population with the last fraction (120 h).

The malate was totally consumed in the presence of the fractions collected after 24 h (D1), 48 h (D2) and 72 h (D3) but with a gradual decrease of the consumption kinetics (Fig. 1b and Table 4). The average specific rate of malate consumption in the presence of the fraction D1 was similar to that of the control (0.37 g $g^{-1}$ $h^{-1}$, $p > 0.05$). In the presence of the fractions D2 and D3, the specific rates were 23% lower than in the control ($p < 0.05$). The fractions taken after 96 h (D4) and 120 h (D5) of AF were the most inhibiting ones as only 0.37 and 0.19 g/L of L-malic acid were consumed reducing the control specific rate of 57 and 77% respectively ($p < 0.05$).

Therefore, we can conclude that the 5–10 kDa peptidic fraction was gradually released during AF and reached its maximum inhibitory concentration at late stages of the yeast stationary phase (96 and 120 h). Its effect started to be detectable with the fraction D2 (48 h) which corresponded to the end of the AF exponential growth phase, reducing

**Table 3**
Growth of *O. oeni* Vitilactic F and malate consumption during MLF performed in the Syrah and Cabernet Sauvignon wines obtained after AF by *S. cerevisiae* CY3079 or *S. cerevisiae* BDX.[a]

| Pre-fermentation maceration temperature (°C) | AF carried out by *S. cerevisiae* CY3079 | | | | AF carried out by *S. cerevisiae* BDX | | | |
|---|---|---|---|---|---|---|---|---|
| | Cabernet Sauvignon wine | | Syrah wine | | Cabernet Sauvignon wine | | Syrah wine | |
| | 10 | 60, 70 and 80 | 10 | 60, 70 and 80 | 10 | 60, 70 and 80 | 10 | 60, 70 and 80 |
| Bacterial biomass produced (g/L) | 0.120 ± 0.005 | 0.080 ± 0.004 | 0.120 ± 0.006 | 0.080 ± 0.004 | 0.0024 ± 0.0002 | 0.0004 ± 0 | 0.0044 ± 0.0002 | 0.0004 ± 0 |
| Consumed L-malic acid (g/L) | 3.00 ± 0.06 | 3.00 ± 0.06 | 3.00 ± 0.06 | 3.00 ± 0.06 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| Experiment duration (days)[b] | 30 | 45 | 30 | 45 | 90 | 90 | 90 | 90 |

[a] The experiment was followed until total consumption of L-malic acid (30 and 45 days) in wines produced by *S. cerevisiae* CY3079 (non-inhibitory strain). Although malate was not consumed, it was followed for 90 days in wines produced by *S. cerevisiae* BDX (inhibitory strain).
[b] Results are mean ± SD values of three replications.

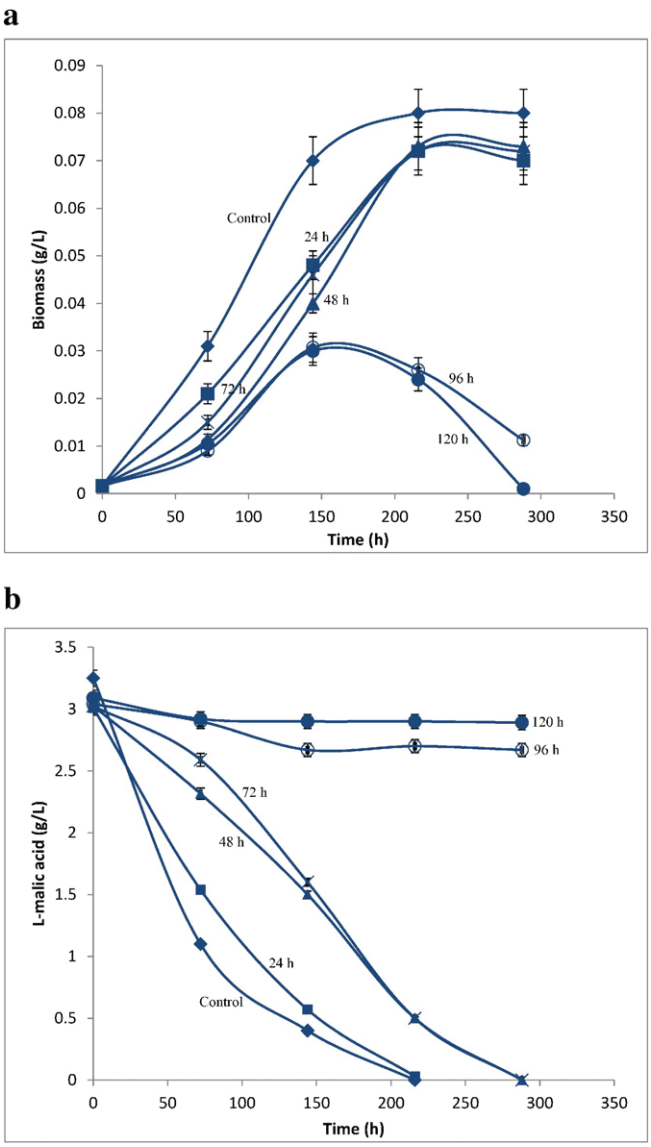


**Fig. 1.** Growth (a) and malic acid consumption (b) kinetics of *O. oeni* Vitilactic F in the control and in the presence of the 5–10 kDa inhibitory fractions collected at different intervals of the AF. (♦) Control: modified MRS medium, (■) 5–10 kDa collected after 24 h of the AF (D1), (▲) 5–10 kDa collected after 48 h of the AF (D2), (×) 5–10 kDa collected after 72 h of the AF (D3), (○) 5–10 kDa collected after 96 h of the AF (D4), (●) 5–10 kDa collected after 120 h of the AF (D5). Each value is the mean of triplicate experiments ± SD.

mainly the rate of malate consumption. The same was noticed with the fraction D3 (72 h). The strong inhibition of malate consumption with the fractions D4 (96 h) and D5 (120 h) can somewhat explain the weak final biomasses formed. It is known that MLF produces ATP through a chemiosmotic mechanism thus enhancing the bacterial growth (Bouix and Ghorbal, 2015; Cox and Henick-Kling, 1995). The MLF is one of the main sources of energy for bacteria in winemaking conditions. Therefore, an inhibition of malate consumption can also affect the bacterial growth.

Information about the timing of the release of antibacterial peptides/proteins is scarce and to our best knowledge no previous work has described simultaneously the kinetics of their production and the kinetics of their anti-MLF activity. Albergaria et al. (2010) showed that *S. cerevisiae* CCMI 885 started to secrete three antifungal peptides of about 6, 4.5 and 4 kDa at the end of the AF exponential growth phase (day 2) with a gradual increase of their concentration during the stationary growth phase (days 4 and 7). Later on, Branco et al. (2014) who used the same yeast strain demonstrated that these peptides were also active against the growth of *O. oeni* and corresponded to GAPDH-derived peptides of 1.6 kDa. They suggested that the peptides were released by apoptotic yeast cells during the stationary phase. In fact, Silva et al. (2011) showed that GAPDH is presumably a substrate of metacaspases during apoptosis. Our findings present some similarities with these conclusions since the most important antibacterial effect was detected with the fractions obtained from late stationary phase. However, further investigation must be carried out to see if any of the 5–10 kDa peptides is possibly a GAPDH fragment.

The timing results are in accordance with the co-culture results published by Nehme et al. (2010) who demonstrated that the co-culture strategy improved the MLF output with the same pair (*S. cerevisiae* BDX/*O. oeni* Vitilactic F). During co-cultures, AF and MLF were conducted simultaneously by inoculating yeasts and bacteria at the same time from the beginning in a membrane bioreactor using the same experimental conditions. The bacterial growth was twice better and 74% of the initial malate were successfully consumed in 3 weeks without any risk of increased volatile acidity or off-flavors. Although the 5–10 kDa peptides gradually appeared during the AF as demonstrated by the present study, the MLF was improved. More recently, co-inoculation was suggested as a worthwhile alternative for winemaking both for better malate consumption and sensorial characteristics when compared with the traditional sequential inoculation (Cañas et al. 2015; Versari et al., 2015; Tristezza et al., 2016).

Information about the timing of the release of the inhibitory peptides is very useful for the determination of the best moment for bacterial inoculation and for an optimum extraction of the targeted molecules.

### *3.3. In vitro evaluation of the malolactic enzyme inhibition by the 5–10 kDa peptidic fractions*

In this experiment, the malolactic enzyme of the cell-free bacterial enzymatic extract was directly exposed to the inhibitory peptides. The five fractions of 5–10 kDa collected each 24 h of the AF (D1 to D5) were tested as described in Table 1. Table 5 shows the amounts of L-malic acid consumed, L-lactic acid produced and the inhibition percentage of malate consumption for each 5–10 kDa fraction tested at pH 6.7. For pH 6 and 3.5, only the values of the inhibition percentage of malate consumption are given.

The first set of experiments was conducted at pH 6.7 which is around the cytoplasmic pH of the majority of lactic acid bacteria. First, the results clearly show that the 5–10 kDa fractions were able to reduce the amount of L-malic acid consumed of 28% (D1) to 91% (D5) when compared to the control, thus exhibiting a direct inhibitory effect on the malolactic enzyme activity ($p < 0.05$). The ethanol concentration in the five fractions was evaluated and a residual amount of 0.02 g/L was found in the 5–10 kDa fraction corresponding to D5. Therefore, all the fractions were ethanol free and the inhibition observed was only due to the presence of the peptides. Second, the kinetic of the inhibition was in agreement with the results of the timing experiment (Fig. 1b) proving once again that the 5–10 kDa peptidic fraction was gradually released during the AF with the highest concentration reached at the end of the stationary phase. Third, it is known that during the malolactic fermentation, each mole of L-malic acid decarboxylated by the malolactic enzyme releases one mole of L-lactic acid and one mole of $CO_2$. Therefore, the measurement of the lactate produced was an additional indicator of the malolactic enzyme activity. The amounts of L-lactic acid produced were measured and found to be stoichiometrically equivalent to the amounts of L-malic acid consumed. As an example, in the presence of the fraction D1, 0.72 g/L of L-malic acid equivalent to 5.4 mmol/L were consumed and gave 0.48 g/L of L-lactic acid which corresponded to 5.3 mmol/L. Consequently, the gradual decrease in the amount of L-lactic acid produced proved that the 5–10 kDa peptidic fractions targeted

**Table 4**
Determination of the average specific growth rates and the average specific rates of malate consumption during the MLF carried out by *O. oeni* Vitilactic F in the modified MRS media containing the 5–10 kDa fractions collected each 24 h of the AF as well as in the control.[1]

| | Control: modified MRS medium | Modified MRS medium + D1[2] fraction | Modified MRS medium + D2[2] fraction | Modified MRS medium + D3[2] fraction | Modified MRS medium + D4[2] fraction | Modified MRS medium + D5[2] fraction |
|---|---|---|---|---|---|---|
| $X_0$: Initial biomass (g/L) | 0.0016 ± 0.0002 | 0.0016 ± 0.0002 | 0.0016 ± 0.0002 | 0.0016 ± 0.0002 | 0.0016 ± 0.0002 | 0.0016 ± 0.0002 |
| $X_f$: Final biomass (g/L) | 0.080 ± 0.006 | 0.072 ± 0.006 | 0.073 ± 0.005 | 0.072 ± 0.006 | 0.031 ± 0.002 | 0.030 ± 0.002 |
| $dt_1$: Growth duration (h) | 216 | 216 | 216 | 216 | 144 | 144 |
| $\mu$[3]: average specific growth rate ($h^{-1}$) | 0.009 ± 0.001[a] | 0.009 ± 0.001[a] | 0.009 ± 0.001[a] | 0.009 ± 0.001[a] | 0.013 ± 0.002[b] | 0.013 ± 0.001[b] |
| $S_0$: Initial L-malic acid (g/L) | 3.25 ± 0.16 | 3.01 ± 0.16 | 3.03 ± 0.20 | 3.03 ± 0.15 | 3.04 ± 0.15 | 3.09 ± 0.12 |
| $S_f$: Final L-malic acid (g/L) | 0 ± 0 | 0.03 ± 0 | 0 ± 0 | 0 ± 0 | 2.67 ± 0.12 | 2.90 ± 0.16 |
| $dt_2$: Duration of MLF (h) | 216 | 216 | 288 | 288 | 144 | 144 |
| $Q_s$[4]: average specific rate of malate consumption ($g\,g^{-1}\,h^{-1}$) | 0.37 ± 0.02[a] | 0.375 ± 0.020[a] | 0.28 ± 0.02[b] | 0.290 ± 0.015[b] | 0.16 ± 0.01[c] | 0.084 ± 0.006[d] |

[1] Results are mean ± SD values of three replications. In comparison to the control, values of average specific rates (growth or malate consumption) within the same row followed by different letters are significantly different ($p < 0.05$) according to Tukey's HSD test.
[2] $\mu\,(h^{-1}) = (dX / dt_1).1/X$ with $dX = (X_f - X_0)$ and $X = (X_f + X_0) / 2$.
[3] $Q_s\,(g\,g^{-1}\,h^{-1}) = (dS / dt_2).1/X$ with $dS = (S_0 - S_f)$ and $X = (X_f + X_0) / 2$.
[4] D1 to D5 (day 1 to day 5): 5–10 kDa fractions collected at 24, 48, 72, 96 and 120 h of the AF performed by *S. cerevisiae* BDX.

specifically the malolactic enzyme and not any other enzyme of the cytosolic extract.

5–10 kDa fractions isolated from the SGJ media fermented by *S. cerevisiae* CY3079 (reference strain or non-inhibitory strain) were also tested and gave results similar to the control (data not shown). In fact, the 1 g/L of L-malic acid were totally decarboxylated by the malolactic enzyme in the presence of these fractions showing no inhibition. Therefore, the inhibition of the malolactic enzyme by the 5–10 kDa peptides of *S. cerevisiae* BDX was highly specific.

The same experiment was repeated by changing the pH (Table 5). The pH 6 was chosen because it is the optimum pH for activity of the malolactic enzyme of *O. oeni* (Schümann et al., 2013). The inhibition of malate consumption gradually increased in the presence of the 5–10 kDa fractions leading to the same conclusions obtained at pH 6.7. However, the reaction was faster. 30 min instead of 40 min were required to entirely decarboxylate 1 g/L of L-malic acid into L-lactic acid and $CO_2$ in the control. The pH 3.5 was also tested because it represents the pH of wine at the beginning of the MLF in this study. At this extreme pH, similar results were also obtained. However, the reaction was the slowest and took 70 min in the control. Interestingly, Bouix and Ghorbal (2015) demonstrated that at low extracellular pH, *O. oeni* cells were able to drop their intracellular pH to values as low as 3.5 (equal to the extracellular pH). The MLF was then initiated indicating that the malolactic enzyme was active at this low pH. During MLF, the intracellular pH increased again due to the proton extrusion that accompanied the release of lactate and $CO_2$. It reached 6 at the end of the MLF and dropped again to 3.5 when the malate was totally consumed. The results of the *in vitro* experiment performed at 3.5 showed that the enzyme was indeed active at this low pH although the enzymatic reaction was 2.3 times slower than at pH 6 (Table 5).

From the previous results, it can be concluded that the peptides were able to exert their inhibitory effect in a pH range between 3.5 and 6.7.

The results also suggest that *in vivo*, the peptides of the 5–10 kDa fraction released by *S. cerevisiae* BDX would enter the bacterial cells by mechanisms yet to be identified and directly inhibit the malolactic enzyme. The presence of cell receptors to these peptides could be suggested.

No previous works have shown the involvement of yeast proteinaceous metabolites in the direct inhibition of the malolactic enzyme activity. Few have attempted to explain the mechanism of action of the yeast antibacterial peptides/proteins and evaluated mainly their impact on the bacterial growth. The proteinaceous compound ≥10 kDa found by Comitini et al. (2005), was dose dependent and was able to reduce the bacterial growth with a typical saturation kinetic thus suggesting the presence of a receptor on the bacterial cell. Therefore its bacteriostatic or bactericidal effect depended on its concentration and the MLF inhibition was correlated to its bactericidal effect. The 3–10 kDa peptidic fraction found by Mendoza et al. (2010) inhibited the bacterial growth but not the malate consumption with a typical saturation kinetic similar to that suggested by Comitini et al. (2005). Osborne and Edwards (2007) suggested that the antibacterial peptide of 5.9 kDa worked synergically with $SO_2$. The mechanism proposed was that of bacteriocins

**Table 5**
Amount of L-malic acid consumed (g/L) and L-lactic acid produced (g/L) during the enzymatic reaction *in vitro* performed at different pH in the presence of the 5–10 kDa fractions (ABP) collected each 24 h of the AF.[1]

| Sampling time of the 5–10 kDa fractions during the AF | pH = 6.7 $t$ = 40 min | | | pH = 6 $t$ = 30 min | pH = 3.5 $t$ = 70 min |
|---|---|---|---|---|---|
| | Consumed L-malic acid (g/L) | Produced L-lactic acid (g/L) | Inhibition % of malate consumption[2] | Inhibition % of malate consumption | Inhibition % of malate consumption |
| Control [3](absence of ABP) | 1.00 ± 0.02[a] | 0.670 ± 0.013[a] | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| After 24 h of the AF (D1) | 0.720 ± 0.015[b] | 0.48 ± 0.01[b] | 28.0 ± 0.6 | 8.00 ± 0.16 | 7.30 ± 0.15 |
| After 48 h of the AF (D2) | 0.630 ± 0.013[c] | 0.420 ± 0.009[c] | 37.0 ± 0.8 | 15.0 ± 0.3 | 14.6 ± 0.3 |
| After 72 h of the AF (D3) | 0.560 ± 0.011[d] | 0.380 ± 0.008[d] | 44 ± 1 | 30.0 ± 0.6 | 32.30 ± 0.65 |
| After 96 h of the AF (D4) | 0.100 ± 0.002[e] | 0.070 ± 0.002[e] | 90 ± 2 | 90.4 ± 2.0 | 94 ± 2 |
| After 120 h of the AF (D5) | 0.090 ± 0.002[f] | 0.060 ± 0.002[f] | 91 ± 2 | 94 ± 2 | 100 ± 2 |

[1] Results are mean ± SD values of three replications. In comparison to the control, values of malate consumed and lactate produced followed by different letters within the same column are significantly different ($p < 0.05$) according to Tukey's HSD test.
[2] Inhibition % of malate consumption = (consumed L-malic acid in the control − consumed L-malic acid in the presence of ABP) × 100 / (consumed L-malic acid in the control).
[3] Absence of ABP: absence of antibacterial peptides.

forming membrane pores and facilitating the entry of $SO_2$ inside the cells thus leading to the bacterial death and arrest of MLF. The mechanism of action of the GAPDH-derived peptides identified by Branco et al. (2014) and that inhibited the growth of *O. oeni* was not elucidated. In addition no data concerning the malate consumption was shown.

In conclusion, the results of the present work revealed that the anti-MLF peptidic fraction of 5–10 kDa produced by *S. cerevisiae* BDX was gradually released during AF with an increasing inhibitory effect mainly detected between the beginning and the late stages of the stationary phase. The MLF inhibition due to the peptidic fraction was maintained in natural winemaking conditions. It was shown for the first time that yeast proteinaceous compounds were able to inhibit the L-malic acid consumption by directly targeting the malolactic enzyme activity. Current work is carried out in order to purify and sequence the putative bioactive peptides of the 5–10 kDa fraction. Future work should investigate their antimicrobial range of action, their biochemical and inhibitory properties and the possibility of using them as natural alternative biopreservatives in food products.

## Acknowledgments

The authors would like to thank LARI (Lebanese Agricultural Research Institute) for their financial support and Lallemand Inc. for providing the strains.

## References

Albergaria, H., Francisco, D., Gori, K., Arneborg, N., Girio, F., 2010. *Saccharomyces cerevisiae* CCMI 885 secretes peptides that inhibit the growth of some non-*Saccharomyces* wine-related strains. Appl. Microbiol. Biotechnol. 86, 965–972.

Bartowsky, E., Costello, P., Henschke, P., 2002. Management of malolactic fermentation-wine flavor manipulation. Aust. N. Z. Grapegrow. Winemak 461a (7–8), 10–12.

Bouix, M., Ghorbal, S., 2015. Rapid assessment of *Oenococcus oeni* activity by measuring intracellular pH and membrane potential by flow cytometry and its application to the more effective control of malolactic fermentation. Int. J. Food Microbiol. 193, 139–146.

Branco, P., Francisco, D., Chambon, C., Hebraud, M., Arneborg, N., Almeida, M.G., Caldeira, J., Albergaria, H., 2014. Identification of novel GAPDH-derived antimicrobial peptides secreted by *Saccharomyces cerevisiae* and involved in wine microbial interactions. Appl. Microbiol. Biotechnol. http://dx.doi.org/10.1007/s00253-013-5411-y.

Britz, T.J., Tracey, R.P., 1990. The combination effect of pH, $SO_2$, ethanol and temperature on the growth of *Leuconostoc oenos*. J. Appl Bacteriol. 68, 23–31.

Cañas, P.M., Romero, E.G., Pérez-Martín, F., Seseña, S., Palop, M.L., 2015. Sequential inoculation versus co-inoculation in cabernet franc wine fermentation. Food Sci. Technol. Int. 21 (3), 203–212.

Capucho, I., San Romao, M.V., 1994. Effect of ethanol and fatty acids on malolactic activity of *Leuconostoc oenos*. Appl. Microbiol. Biotechnol. 42, 391–395.

Carreté, R., Vidal, M.T., Bordons, A., Constanti, M., 2002. Inhibitory effect of sulphur dioxide and other stress compounds in wine on the ATPase activity of *Oenococcus oeni*. FEMS Microbiol. Lett. 211, 155–159.

Comitini, F., Ferreti, R., Clementi, R., Mannazzu, I., Ciani, M., 2005. Interactions between *Saccharomyces cerevisiae* and malolactic bacteria: preliminary characterization of a yeast proteinaceous compound(s) active against *Oenococcus oeni*. J. Appl. Microbiol. 99, 105–111.

Cox, D.J., Henick-Kling, T., 1995. Protonmotive force and ATP generation during malolactic fermentation. Am. J. Enol. Vitic. 46, 319–323.

Dick, K.J., Molan, P.C., Eschenbruch, R., 1992. The isolation from *Saccharomyces cerevisiae* of two antibacterial cationic proteins that inhibit malolactic bacteria. Vitis 31, 105–116.

Edwards, C.G., Beelman, R.B., 1987. Inhibition of the malolactic bacterium, *Leuconostoc oenos* (PSU-1), by decanoic acid and subsequent removal of the inhibition by yeast ghosts. Am. J. Enol. Vitic. 38, 239–242.

Henick-Kling, T., Park, Y.H., 1994. Considerations for the use of yeast and bacterial starter cultures: $SO_2$ and timing of inoculation. Am. J. Enol. Vitic. 45, 464–469.

Larsen, J.T., Nielsen, J.C., Kramp, B., Richelieu, M., Riisager, M.J., Arneborg, N., Edwards, C.G., 2003. Impact of different strains of *Saccharomyces cerevisiae* on malolactic fermentation by *Oenococcus oeni*. Am. J. Enol. Vitic. 54, 246–251.

Larsen, N., Boye, M., Siegumfeldt, H., Jakobsen, M., 2006. Differential expression of proteins and genes in the lag phase of *Lactococcus lactis* subsp. *lactis* grown in synthetic medium and reconstituted skim milk. Appl. Environ. Microbiol. 72, 1173–1179.

Lonvaud Funel, A., Joyeux, A., Desens, C., 1988. Inhibition of malolactic fermentation of wines by products of yeast metabolism. J. Sci. Food Agric. 44, 183–191.

Lonvaud-Funel, A., 1999. Lactic acid bacteria in the quality improvement and depreciation of wine. Ant. Van. Leeuw. 76, 317–331.

Lonvaud-Funel, A., 2002. Lactic acid bacteria—a survey. In: Verlag, T.S. (Ed.), Proceedings of the 13th International Oenology Symposium; 9–12 June 2002. International Association of Oenology, Management and Wine Marketing, Montpellier-France, pp. 59–74.

McRae, J.M., Kennedy, J.A., 2011. Wine and grape tannin interactions with salivary proteins and their impact on astringency: a review of current research. Molecules 16, 2348–2364.

Mendoza, L., Manca de Nadra, M., Farias, M., 2010. Antagonistic interaction between yeasts and lactic acid bacteria of oenological relevance. Partial characterization of inhibitory compounds produced by yeasts. Food Res. Int. 43, 1990–1998.

Nehme, N., Mathieu, F., Taillandier, P., 2008. Quantitative study of interactions between *Saccharomyces cerevisiae* and *Oenococcus oeni* strains. J Ind. Microbiol. Biotechnol. http://dx.doi.org/10.1007/s10295-008-0328-7.

Nehme, N., Mathieu, F., Taillandier, P., 2010. Impact of the co-culture of *Saccharomyces cerevisiae-Oenococcus oeni* on malolactic fermentation and partial characterization of a yeast-derived inhibitory peptidic fraction. Food Microbiol. 27, 150–157.

Osborne, J.P., Edwards, C.G., 2006. Inhibition of malolactic fermentation by *Saccharomyces cerevisiae* during the alcoholic fermentation under low and high nitrogen conditions: a study in synthetic media. Aust. J. Grape Wine Res. 12, 69–78.

Osborne, J.P., Edwards, C.G., 2007. Inhibition of malolactic fermentation by a peptide produced by *Saccharomyces cerevisiae* during alcoholic fermentation. Int. J. Food Microbiol. 118, 27–34.

Ozdal, T., Capanoglu, E., Altay, F., 2013. A review on protein-phenolic interactions and associated changes. Food Res. Int. 51, 954–970.

Patynowski, R.J., Jiranek, V., Markides, A.J., 2002. Yeast viability during fermentation and sur lie ageing of a defined medium and subsequent growth of *Oenococcus oeni*. Aust. J. Grape Wine Res. 8, 62–69.

Reguant, C., Bordons, A., Arola, L., Rozes, N., 2000. Influence of phenolic compounds on the physiology of *Oenococcus oeni* from wine. J. Appl. Microbiol. 88, 1065–1071.

Remize, F., Gaudin, A., Kong, Y., Guzzo, J., Alexandre, H., Krieger, S., Guilloux-Benatier, M., 2006. *Oenococcus oeni* preference for peptides: qualitative and quantitative analysis of nitrogen assimilation. Arch. Microbiol. 185, 459–469.

Ribéreau-Gayon, P., Glories, Y., Maujean, A., Dubourdieu, D., 2006. Handbook of Enology Volume 2: The Chemistry of Wine, Stabilization and Treatments. second ed. Dunod, Paris.

Saguir, F.M., Manca de Nadra, M.C., 2002. Effect of L-malic and citric acids metabolism on the essential amino acid requirements for *Oenococcus oeni* growth. J. Appl. Microbiol. 93, 295–301.

Schümann, C., Michlmayr, H., del Hierro, A.M., Kulbe, K.D., Jiranek, V., Eder, R., Nguyen, T., 2013. Malolactic enzyme from *Oenococcus oeni*. Heterologous expression in *Escherichia coli* and biochemical characterization. Bioengineered. 4 (3), 147–152.

Silva, A., Almeida, B., Sampaio-Marques, B., Reis, M.I.R., Ohlmeier, S., Rodrigues, F., Do Vale, A., Ludovico, P., 2011. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a specific substrate of yeast metacaspase. Biochim. Biophys. Acta 1813, 2044–2049.

Sims, C.A., Eastridge, J.S., Bates, R.P., 1995. Changes in phenols, color, and sensory characteristics of Muscadine wines by pre- and post-fermentation additions of PVPP, casein, and gelatin. Am. J. Enol. Vitic. 46, 155–158.

Terrade, N., Mira de Orduna, R., 2009. Determination of the essentiel nutrient requirements of wine-related bacteria from the genera *Oenococcus* and *Lactobacillus*. Int. J. Food Microbiol. 133, 8–13.

Tristezza, M., di Feo, L., Tufariello, M., Grieco, F., Capozzi, V., Spano, G., Mita, G., Grieco, F., 2016. Simultaneous inoculation of yeasts and lactic acid bacteria: Effects on fermentation dynamics and chemical composition of Negroamaro wine. LWT Food Sci. Technol. 66, 406–412.

Vaillant, H., Formysin, P., Gerbaux, V., 1995. Malolactic fermentation of wine: study of the influence of some physico-chemical factors by experimental design assays. J. Appl Bacteriol. 79, 64–650.

Versari, A., Patrizi, C., Parpinello, G.P., Mattioli, A.U., Pasini, L., Meglioli, M., Longhini, G., 2015. Effect of co-inoculation with yeast and bacteria on chemical and sensory characteristics of commercial cabernet franc red wine from Switzerland. J. Chem. Technol. Biotechnol. http://dx.doi.org/10.1002/jctb.4652.

Yokotsuka, K., Singleton, V.L., 1995. Interactive precipitation between phenolic fractions and peptides in wine-like model solutions: turbidity, particle size, and residual content as influenced by pH, temperature and peptide concentration. Am. J. Enol. Vitic. 46, 329–338.