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Identification of multiple-derived peptides produced by *Saccharomyces cerevisiae* involved in malolactic fermentation inhibition

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One sentence summary: New antimicrobial peptides produced by *Saccharomyces cerevisiae* are identified and found to be responsible for the inhibition of malolactic fermentation

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ABSTRACT

An oenological strain of *Saccharomyces cerevisiae* was previously shown to produce a 5–10 kDa peptidic fraction responsible for the inhibition of malolactic fermentation (MLF). In the present study, we aim to further purify the anti-MLF peptides of this fraction. The yeast fermented synthetic grape juice medium was fractionated by ammonium sulfate precipitation combined with ultrafiltration. The 5–10 kDa fraction recovered at a saturation degree of 60%–80% was the only fraction that inhibited both the bacterial growth and the malate consumption *in vivo*. It also inhibited the malolactic enzyme activity *in vitro* at a pH range between 3.5 and 6.7. Therefore, it was purified by both anion and cation exchange chromatography. The eluates that inhibited the malolactic enzyme activity *in vitro* were migrated on Tricine SDS-PAGE and the protein bands were excised and sequenced by LC-MS/MS. The sequencing revealed nine peptides originating from eight proteins of *S. cerevisiae*. Two GAPDH cationic fragments of 0.9 and 1.373 kDa having a p*I* of 10.5 and 11 respectively, Wtm2p and Utr2p anionic fragments of 2.42 kDa with a p*I* of 3.5 and 4 respectively were thought to contribute the most to the MLF inhibition.

Keywords: malolactic fermentation (MLF); anti-MLF yeast peptides; Wtm2p; Utr2p; glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

INTRODUCTION

Antimicrobial peptides (AMPs) play an important role in the innate immune system of eukaryotes against virulent bacteria, viruses and fungi (Wong, Xia and Ng 2007; Sang and Blecha 2009). The majority of AMPs are cationic in nature and kill microbes by interacting with the negatively charged components of the cell-wall and/or plasma membrane (Brogden 2005). However, many anionic AMPs have also been found to be involved in the defense mechanisms (Harris, *Dennison and Phoenix* 2009). The activity of anionic AMPs can be enhanced either by divalent metal

cations such as Fe^{++}, Mn^{++} and Mg^{++} that facilitate their binding to anionic membrane components (Dashper *et al.* 2005), or by the aggregation of several peptides (Nissen-Meyer *et al.* 1992). Some AMPs are able to translocate across the membrane of sensitive cells or increase the membrane permeability by forming pores (Harris, *Dennison and Phoenix* 2009), while others have been found to induce apoptosis in sensitive cells. For example, killer strains of *Saccharomyces cerevisiae* that produce killer toxins K1 and K28, induce apoptosis in sensitive yeast cells (Reiter *et al.* 2005). Besides, bacteriocins produced by some strains of Gram positive bacteria are either bacteriostatic or bactericidal, against a wide range of pathogenic and spoilage bacteria. Some of them like nisin are already used as biopreservatives in different food sectors such as the dairy industry; however, their use in the wine sector is still prohibited. Pediocin PD-1 produced by *Pediococcus damnosus* NCFB 1832, was able to kill *Oenococcus oeni* and outcompete nisin (Nel *et al.* 2002).

In winemaking, several AMPs were found to be responsible for the inhibition of malolactic fermentation (MLF). These AMPs, produced by different strains of *S. cerevisiae*, were partially purified, and assumptions about their mechanism of action were given but the results were not conclusive. Most of the time, the peptides were considered to target the plasma membrane of *O. oeni*, resulting in the inhibition of the bacterial growth and consequently the malate consumption (Comitini *et al.* 2005; Osborne and Edwards 2007). The AMPs found by Mendoza, *Manca de Nadra and Farias* (2010) inhibited the bacterial growth only. Branco *et al.* (2014) were the first to identify the nature of the AMPs produced by *S. cerevisiae* CCMI 885. These AMPs were active against several non-*Saccharomyces* yeasts and one strain of *O. oeni*. They corresponded to two fragments of the C-terminal amino acid sequence of the *S. cerevisiae* GAPDH isoenzymes (GAPDH 2/3 of 1.638 kDa and GAPDH1 of 1.622 kDa, p*I* of 4.37). Recently, Branco *et al.* (2017) found that these AMPs were also produced by other strains of *S. cerevisiae* but at different concentrations. The GAPDH peptidic fragments disturbed the plasma membranes of sensitive yeast cells and increased their permeability. They were able to enter the cytoplasm and trigger apoptosis. However, the experiments were only conducted on yeasts and not on *O. oeni*.

Nehme, *Mathieu and Taillandier* (2010) demonstrated that *S. cerevisiae* Uvaferm BDX released a 5–10 kDa peptidic fraction that strongly inhibited the growth and the malate consumption during the MLF performed by *O. oeni* Vitilactic F. Later on, Rizk *et al.* (2016) showed that this fraction was gradually released during alcoholic fermentation (AF) and reached its highest concentration at late stages of the stationary phase (120 h). It was able to directly inhibit the malolactic enzyme activity *in vitro* at a pH range between 3.5 and 6.7. Rizk *et al.* (2016) were the first researchers to demonstrate that AMPs can inhibit the malate consumption by directly inhibiting the malolactic reaction. These results suggest that *in vivo* the peptides would enter the cells by mechanisms yet to be identified in order to inhibit MLF. Since MLF is one of the main energy sources during winemaking, the inhibition of the bacterial growth can be a consequence of the MLF inhibition. Rizk *et al.* (2016) also showed that the same pairing (*S. cerevisiae* Uvaferm BDX/*O. oeni* Vitilactic F) maintained the MLF inhibition in Cabernet Sauvignon and Syrah wines.

In the present work, we intend to further purify the AMPs of the 5–10 kDa peptidic fraction in order to identify their nature and to better understand their mechanism of action. The synthetic grape juice (SGJ) medium fermented by *S. cerevisiae* Uvaferm BDX is fractionated by ammonium sulfate precipitation combined with ultrafiltration. Then, the peptidic fraction responsible for the MLF inhibition *in vivo* and *in vitro* experiments is purified by ion exchange chromatography (both anion and cation) followed by SDS-PAGE. Finally, the peptides of interest are sequenced by LC-MS/MS and characterized regarding their amino acid sequence, molecular weight, theoretical p*I*, the yeast proteins from which they derive and their putative mechanisms of action.

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). The Aurum ion exchange mini kits and columns and the chemicals used for SDS-PAGE were purchased from Bio-Rad (California, USA).

Strains and storage conditions

Saccharomyces cerevisiae Uvaferm BDX, Lalvin CY3079 and *O. oeni* Vitilactic F used in this work were kindly provided by Lallemand Inc. (Blagnac, France). Yeast stock cultures were kept at 4°C in YEPD (Yeast Extract Peptone Dextrose) agar slants composed of 10 g L^{-1} Yeast Extract, 20 g L^{-1} peptone, 20 g L^{-1} D-glucose and 20 g L^{-1} agar. The bacterial strain was kept frozen at –20°C in MRS (*De Man, Rogosa and Sharpe*) broth containing 20% glycerol (v/v).

Growth media

SGJ medium

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

Modified MRS medium

The MRS medium was supplemented with L-malic acid (3 g L^{-1}) 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.

Alcoholic fermentation

The SGJ media were inoculated either with *S. cerevisiae* Uvaferm BDX or with *S. cerevisiae* Lalvin CY3079 at an initial concentration of 3×10^6 cells mL^{-1} (Thoma counting chamber). The AF was followed until total or cessation of sugar consumption (<2 g L^{-1}). The yeast inoculum was beforehand prepared in two steps. First, a pre-culture of the yeast strain was obtained by reactivating the stock culture in YEPD broth for 24 h. Second, the pre-culture was used to inoculate a low sugar concentration SGJ medium: D-Glucose 50 g L^{-1} and absence of D-Fructose. This step was carried out for 24 h and provided the yeast inoculum. All fermentation steps were carried out at 22°C with stirring at 150 rpm in Erlenmeyer flasks.

AFs were performed in three independent replicates.

Fractionation of yeast fermented media by ammonium sulfate precipitation combined with ultrafiltration

After completion of the AF (120 h), yeast cells were removed by centrifugation (3500 rpm, 20 min at 4°C) and 500 mL of the supernatant were recuperated. The amounts of solid ammonium

sulfate added to the supernatant were calculated according to the program [www.encorbio.com/protocols/AM_SO4.htm] in order to reach the following saturation percentages: A: 0%–20%, B: 20%–40%, C: 40%–60%, D: 60%–80% and E: 80%–100%.

For each saturation level, the salt was added gradually to the supernatant for 1 h while shaking at 4°C. 90 min later, the medium was centrifuged at 5000 rpm for 30 min at 4°C. After centrifugation, the protein pellet was recovered and suspended in 10 mL of a phosphate buffer (0.1 M, pH 6.7). This resulted in samples that were 50 times more concentrated (50×). Afterwards, the sample was fractionated through a centrifugal filter unit presenting a cut-off of 10 kDa (Amicon® Ultra-15 with ultracel-10 membrane) at 3500 g for 45 min at 4°C. The filtrate with a MW ≤10 kDa was recovered and ultrafiltered again using a centrifugal filter unit of 5 kDa cut-off (Corning® Spin-X UF 20 Sigma-Aldrich). A desalted protein retentate of 0.2 mL with a MW between 5 and 10 kDa was then recovered. The ultrafiltration added a new concentration factor of 50×. The protein retentate (2500×) was added to 20 mL of a modified MRS medium in order to evaluate any potential effect on MLF. It was 100 times diluted and was finally 25 times concentrated in the MRS broth.

The remaining supernatant was used again to reach a new saturation level and the same procedure was applied. The impact of the five precipitated fractions (A, B, C, D and E) on MLF was evaluated separately in modified MRS media, inoculated with 2×10^6 cells mL^{-1} (Petroff-Hausser counting chamber) of *O. oeni* Vitilactic F corresponding to an initial bacterial biomass of 0.0016 g L^{-1}. The bacterial inoculum was beforehand prepared in two steps. A pre-culture was obtained by reactivating the stock culture in MRS broth, with 3% ethanol (v/v) added. After 24 h, the pre-culture was used to inoculate another MRS broth with 6% ethanol (v/v) added. The latter provided the bacterial inoculum after an incubation period of 24 h. All the fermentation steps were performed at 22°C with stirring at 150 rpm.

Both bacterial growth and malate consumption were followed by regular sampling until cessation of malate consumption and were compared to those of a modified MRS control.

All the experiments were performed in three independent replicates.

Protein purification by Aurum ion exchange mini kits and columns

Aurum anion exchange (AEX) and cation exchange (CEX) columns allowed rapid fractionation of complex protein mixtures in only a few steps using the common technique of ion exchange chromatography. Each Aurum AEX (732–6706) or CEX (732–6703) column contained 0.2 mL of UNOsphere Q [$-N^+(CH_3)_3$] or S [$-SO_3^-$] support. The buffers selected were the following:

- Binding buffers: 20 mM phosphate pH 6.7 for CEX and 20 mM Tris pH 8.3 for AEX
- Elution buffers: binding buffer +0.5 M NaCl or 1 M NaCl

For each analysis, 1 mL (5 × 0.2 mL) of the peptidic fraction (2500×), collected in its appropriate binding buffer, was filtered through a 0.45 μm syringe filter, loaded onto the column, and gravity filtered. The column placed in a collection tube, was then washed with 0.9 mL of the appropriate Aurum AEX or CEX binding buffer, and was centrifuged for 20 sec at 1000 g in a microcentrifuge. The aim of this step was to properly bind the sample. The eluate obtained, also called 'unbound fraction' was recovered. Next, the column was placed in a new 2 mL collection tube, and 0.3 mL of the appropriate Aurum AEX or CEX elution buffer containing 0.5 M NaCl were added to the top of the column before centrifugation at 1000 g for 10 sec. The elution step was repeated twice and the eluate was recovered (eluate 1). Finally, the column was placed in a new 2 mL collection tube and washed with 0.3 mL of the Aurum AEX or CEX elution buffer containing 1 M NaCl. It was then centrifuged for 10 sec at 1000 g and the eluate was recovered (eluate 2).

All the experiments were performed in three independent replicates.

Table 1. Reaction mixture for the monitoring of the L-malic acid consumption *in vitro* at different pH values with or without the peptidic fractions.

Aliquot 1 (Control)	Aliquot 2 (Test)	Amounts
Cell-free enzymatic extract	Cell-free enzymatic extract	1 mL
Mn^{++}	Mn^{++}	0.1 mg
NAD^+ (35 g L^{-1})	NAD^+ (35 g L^{-1})	0.1 mL
[a] buffer	[b] AMPs	0.14 mL
L-malic acid (1 g L^{-1})	L-malic acid (1 g L^{-1})	1 mg

[a] buffer: 0.1 M of citrate buffer pH 3.5, phosphate buffer pH 6 or phosphate buffer pH 6.7.
[b] AMPs: antimicrobial peptides of the peptidic fractions tested.

In vitro evaluation of the peptidic fractions effect on the malolactic enzyme activity

This method was implemented and fully described by Rizk *et al.* (2016). It consisted of preparing a cell-free enzymatic extract containing the malolactic enzyme of *O. oeni* Vitilactic F. The enzymatic extract was recovered in 2 mL of a buffer (0.1 M of citrate buffer pH 3.5, phosphate buffer pH 6 or phosphate buffer pH 6.7). 2 mL buffer was divided into two aliquots of 1 mL each. One was used as a control and the other as a test medium to which the peptidic fractions were added. The reaction mixture is given in Table 1. The reagents were added to each aliquot in the order listed in Table 1. The enzymatic reaction was performed at 30°C for 70 min at pH 3.5, 30 min at pH 6 and 40 min at pH 6.7.

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 effect of each peptidic fraction on the malolactic enzyme activity was tested in three independent replicates.

Protein analysis by Tricine SDS-PAGE

The peptidic fractions were analyzed by Tricine SDS-PAGE as described by Schägger and von Jagow (1987) and Schägger (2006), to separate proteins on the basis of their molecular sizes. A trichloroacetic acid solution or TCA solution (20% TCA, 0.2% DTT in ice-cold acetone −20 °C, Bio-Rad) was used for protein precipitation. For each sample of 1 mL, 250 μL of TCA solution were added and the samples were dried in a dry heat block (Major Science, USA) for 15 min. Proteins were then pelleted (14 000 g, 4°C, 15 min), washed twice in 200 μL of ice-cold acetone (14 000 g, 4°C, 5 min) and suspended in 100 μL of Triton X-100 buffer (2% Triton X-100, 1% DTT, Sigma-Aldrich). Electrophoresis was carried out using ready-to-use [8%–16%] Mini-PROTEAN® TGX precast gels composed of 12 wells in a Mini-PROTEAN® Tetracell (Bio-Rad). 20 μL of concentrated samples (10×) were run against 10 μL precision plus protein Dual-Xtra standard (2–250 kDa, criterion 10%–20% Tris-Tricine) composed of 12 recombinant proteins (Bio-Rad) at 100 V for 15 min. Gels were stained using either Bio-safe Coomassie G-250 stain or Silver stain kit (Bio-Rad). Imaging and documentation were done by using the GelDoc XR+ system, controlled by Image Lab software (Bio-Rad).

Peptides sequencing by LC-MS/MS (LC1D-nanoESI-LTQ-Velos Orbitrap)

The sequencing was contracted out to PAIB2 (Plate-forme d'Analyse Intégrative des Biomolécules Phénomiques des Animaux d'Intérêt Bio-agronomique), Laboratoire de Spectrométrie de Masse, Centre de Recherches INRA Val de Loire-Tours, France. The protein bands were first excised from SDS-PAGE and submitted to in-gel tryptic digestion. The peptidic extract was then dehydrated and analyzed by using high resolution mass spectrometry. The peptides derived from the digestion of the bands were analyzed by a nano UHPLC Ultimate 3000 RSLC system (Thermo Electron, Waltham-Massachusetts, USA) coupled with a high resolution mass spectrometer LTQ Orbitrap Velos ETD (Thermo Electron). The samples were desalted and concentrated in line by a precolumn Acclaim PepMap100 C18 trap (3 μm, 100 μm $\times$ 2 cm) (Dionex, California, USA), then separated on an analytical reverse phase column Acclaim PepMap 100 C18 (2 μm, 75μm $\times$ 50 cm) (Dionex) with a flow rate of 300 μL min^{-1}. The column was equilibrated with 96% of solvent A (0.1% formic acid, 2% acetonitrile, 97.9% water) and 4% of solvent B (0.1% formic acid, 80% acetonitrile, 19.9% water) with a gradient of 4% to 55% B for 90 min, followed by 99% B for 10 min.

The data acquisition was performed automatically between high resolution MS modes (R = 60 000) and low resolution fragmentation MS/MS. An enhanced centroid full scan (*m/z* 400–1800) was followed by MS/MS centroid scans of the 20 most intense peaks detected (q_z 0.25, activation time 10 ms, collision energy 35%). The dynamic exclusion was turned on for 30 sec with a repeat count of 1.

The data were converted into Mascot Generic file format (mgf) using Proteome discoverer 1.3 software. It was then compared to the database through Mascot/PD Deamon 2.3 software by taking into consideration the following criteria: NCBI nr database; trypsin and no enzyme; 2 miscleavages; 5 ppm mass accuracy for MS and 0.8 Da for MS/MS; peptide load 2 and 3+; carbamidomethylation of cysteine; N-terminal acetylation; oxidation of methionine; data format: mgf; decoy database; instrument: ESI-FTICR; taxonomy: *Saccharomyces cerevisiae*.

The peptides and proteins identified by the Mascot search engine were validated by the Peptide Prophet and Protein Prophet algorithms using Scaffold 4 software.

Determination of the protein concentration by the Lowry method

Protein concentration was determined using the Lowry method as described by Frolund, *Griebe and Nielsen* (1995). Results were determined according to a BSA standard curve and expressed in mg L^{-1}.

Determination of the L-malic acid concentration

The L-malic acid concentration was determined using an enzymatic assay (Boehringer Mannheim/R-Biopharm, kit. No 10 139 068 035, Darmstadt-Germany) and the results were expressed in g L^{-1}.

Determination of the L-lactic acid concentration

The L-lactic acid concentration was determined using an enzymatic assay (Boehringer Mannheim/R-Biopharm, kit. No 10 139 084 035, Darmstadt-Germany) and the results were expressed in g L^{-1}.

Determination of the bacterial biomass

The biomass (dry weight) was determined using a thermobalance or moisture analyzer (Ohaus-UK). A correlation between the bacterial cell concentration (cells mL^{-1}) and the bacterial biomass (g L^{-1}) was established using high bacterial concentrations in modified MRS media. The correlation obtained was the following:

$$y_{(gL^{-1})} = 8.10^{-10}\ x_{\ (cells\ mL^{-1})}.$$

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

Statistical analyses

Means and standard deviations of the assays were calculated using conventional statistical methods. Each experiment was performed in three replicates. The statistical analysis was carried out using XLSTAT 2014.5 for MS Excel.

RESULTS

Fractionation of the SGJ medium fermented by *S. cerevisiae* Uvaferm BDX by ammonium sulfate precipitation combined with ultrafiltration

The SGJ medium fermented by *S. cerevisiae* Uvaferm BDX was fractionated by ammonium sulfate precipitation combined with ultrafiltration. A 5–10 kDa peptidic fraction was recovered from each ammonium sulfate precipitate (A: 0%–20%, B: 20%–40%, C: 40%–60%, D: 60%–80% and E: 80%–100%). The effect of each peptidic fraction on MLF was evaluated separately in a modified MRS medium inoculated with *O. oeni* Vitilactic F. This choice was made because it was previously shown that *S. cerevisiae* Uvaferm BDX was able to produce and release a peptidic fraction with an apparent MW between 5 and 10 kDa that strongly inhibited *O. oeni* Vitilactic F (Nehme, *Mathieu and Taillandier* 2010; Rizk *et al.* 2016). In the previous works, the peptidic fraction was recovered by ultrafiltration whereas in this paper, the ammonium sulfate precipitation was combined with ultrafiltration in order to further reduce the amount of peptides and easily select the anti-MLF ones.

Fig. 1 shows that neither the growth nor the malate consumption were affected by the 80%–100% precipitate and the kinetics were similar to those of the control. However, the 0%–20%, 20%–40% and 40%–60% precipitates strongly inhibited the bacterial growth with no effect on the malate consumption. The 60%–80% precipitate was the only one that strongly inhibited both the bacterial growth and the malate consumption. Its protein concentration was the highest (650 mg L^{-1}); 2.54 times higher than that of the other precipitates (data not shown).

The same work was carried out with the SGJ medium fermented by *S. cerevisiae* Lalvin CY3079 that is a non-inhibitory strain (Nehme, *Mathieu and Taillandier* 2008). The results obtained with all the precipitates were similar to those of the modified MRS control, showing no inhibition, neither on the bacterial growth nor on the malate consumption (data not shown). Therefore, *S. cerevisiae* Lalvin CY3079 was used as a reference strain in this study.

In vitro evaluation of the malolactic enzyme inhibition by the 5–10 kDa peptidic fraction of the 60%–80% ammonium sulfate precipitate

In this experiment, the malolactic enzyme of the cell-free bacterial enzymatic extract was directly exposed to the peptidic fractions. The protein concentration of all the enzymatic extracts

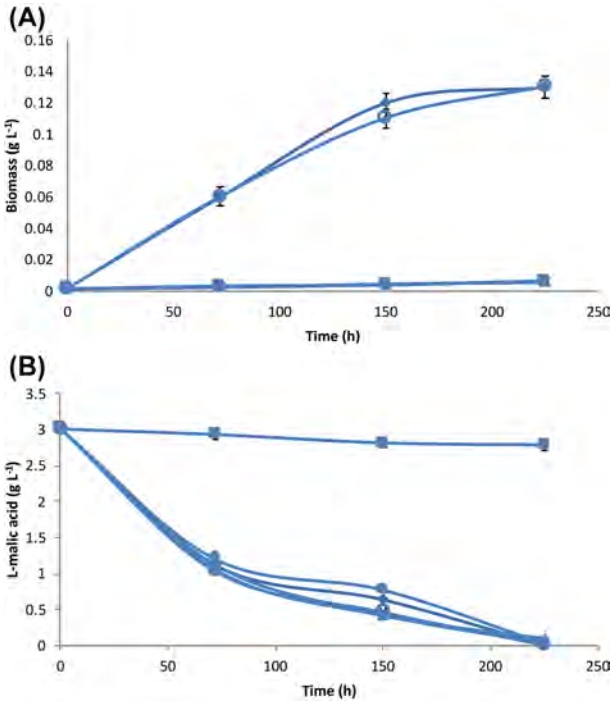

Figure 1. Growth (A) and malic acid consumption (B) kinetics of *O. oeni* Vitilactic F in the presence of the 5–10 kDa peptidic fractions of the ammonium sulfate precipitates obtained from the SGJ medium fermented by *S. cerevisiae* Uvaferm BDX. (♦) Control: modified MRS medium without peptidic fractions; modified MRS medium with 5–10 kDa fractions collected at different saturation degrees: (x) 0%–20%, (▲) 20%–40%, (•) 40%–60%, (■) 60%–80%, (○) 80%–100%. Each value is the mean of triplicate experiments ± SD.

prepared was measured before addition of the peptidic fractions and was approximately the same with an average value of 302.2 (± 20) mg L^{-1}. pH 3.5 was chosen because all the MLFs of this study were carried out at this pH value. pH 6 was used because it is the optimum pH for the malolactic enzyme activity of *O. oeni* and pH 6.7 is around the cytoplasmic pH of the majority of lactic acid bacteria. Table 2 shows that the 5–10 kDa peptidic fraction of the 60%–80% ammonium sulfate precipitate obtained from *S. cerevisiae* Uvaferm BDX was able to inhibit the malate consumption by directly targeting the malolactic enzyme activity at all the pH values tested. For instance, at pH 6.7, the malate consumption was reduced by 99% in comparison to the control. The 0%–20% precipitate was used as an additional control since it only inhibited the bacterial growth in the modified MRS medium and not the malate consumption (Fig. 1). Table 2 also shows that this precipitate did not affect the malolactic enzyme activity *in vitro*. Additionally, the 5–10 kDa fraction of the 60%–80% precipitate isolated from the SGJ medium fermented by *S. cerevisiae* Lalvin CY3079 (reference strain or non-inhibitory strain), was tested and gave results identical to the control. In terms of specific malolactic activities, the highest value (0.1 g L^{-1} of L-malic acid consumed/min/mg of proteins) was obtained at pH 6 in the absence of inhibition. It was 1.37 and 2.33 times higher than those obtained at pH 6.7 and 3.5 respectively. However, in the presence of the inhibitory fraction (5–10 kDa of the 60%–80% precipitate from Uvaferm BDX), the specific activities were almost null at all the pH values tested (Table 3).

The amounts of L-lactic acid produced were measured and found to be stoichiometrically equivalent to the amounts of L-malic acid consumed. This is an additional proof that the inhibitory peptidic fraction targeted specifically the malolactic enzyme of the enzymatic extract.

Altogether, it can be concluded that the inhibition of the malolactic enzyme by the 5–10 kDa peptidic fraction of the 60%–80% ammonium sulfate precipitate of *S. cerevisiae* Uvaferm BDX was highly specific. Therefore, it was retained for further purification.

Comparison of the protein profiles of *S. cerevisiae* Uvaferm BDX and Lalvin CY3079 by Tricine SDS-PAGE

The 5–10 kDa peptidic fractions of the 60%–80% ammonium sulfate precipitates obtained from the SGJ media fermented by both strains of *S. cerevisiae* were analyzed and compared by Tricine SDS-PAGE. The results are given in Fig. 2. Tricine SDS-PAGE analysis of the inhibitory fraction released by *S. cerevisiae* Uvaferm BDX, revealed three protein bands with an apparent MW of

Table 2. Consumed L-malic acid (g L^{-1}) and produced L-lactic acid (g L^{-1}) during the enzymatic reaction *in vitro* performed at different pH values in the presence of peptidic fractions isolated from the SGJ media fermented by *S. cerevisiae* Uvaferm BDX and Lalvin CY3079.

	pH = 6.7 [b]t = 40 min		pH = 6 [b]t = 30 min		pH = 3.5 [b]t = 70 min	
	Consumed L-malic acid (g L^{-1})	Produced L-lactic acid (g L^{-1})	Consumed L-malic acid (g L^{-1})	Produced L-lactic acid (g L^{-1})	Consumed L-malic acid (g L^{-1})	Produced L-lactic acid (g L^{-1})
Control (absence of [a]AMPs)	1.00 (±0.02)	0.670 (±0.013)	0.933 (±0.020)	0.620 (±0.001)	0.974 (±0.002)	0.65 (±0.01)
5–10 kDa fraction of the 60%–80% precipitate from Uvaferm BDX	0.0080 (±0.0002)	0.0053 (±0.0001)	0.0012 (±0)	0.0080 (±0.0002)	0.0090 (±0.0002)	0.0060 (±0.0001)
5–10 kDa fraction of the 0%–20% precipitate from Uvaferm BDX	0.90 (±0.02)	0.60 (±0.01)	0.90 (±0.02)	0.60 (±0.01)	1.00 (±0.02)	0.67 (±0.01)
5–10 kDa fraction of the 60%–80% precipitate from Lalvin CY3079	0.94 (±0.02)	0.620 (±0.001)	1.00 (±0.02)	0.620 (±0.001)	0.98 (±0.02)	0.650 (±0.001)

[a]AMPs: antimicrobial peptides of the peptidic fractions tested.
[b]t = 40 min, t = 30 min and t = 70 min: incubation time required for the total conversion of L-malic acid (1 g L^{-1}) into L-lactic acid and CO_2 during the malolactic reaction *in vitro* in the control aliquot at the different pH values tested.

Table 3. Specific malolactic activities of the enzymatic reactions *in vitro* calculated in the controls and in the presence of different yeast peptidic fractions.

	[b] Specific malolactic activities (g L^{-1} of L-malic acid consumed/min/mg of proteins)		
	pH 6.7	pH 6	pH 3.5
Control (absence of [a]AMPs)	0.08	0.1	0.05
5–10 kDa fraction of the 0%–20% precipitate from Uvaferm BDX	0.07	0.09	0.04
5–10 kDa fraction of the 60%–80% precipitate from Lalvin CY3079	0.07	0.1	0.04
Average specific malolactic activities in the absence of inhibition	0.073 (±0.006)	0.1 (±0.006)	0.043 (±0.006)
5–10 kDa fraction of the 60%–80% precipitate from Uvaferm BDX	0.0005	0.0001	0.0003

[a]AMPs: antimicrobial peptides of the peptidic fractions tested.
[b]Specific malolactic activities are calculated by dividing the amount of L-malic acid consumed (g L^{-1}) by the duration of the enzymatic reaction (min) and by the amount of proteins present in the enzymatic mixture (mg).
The different amounts of proteins are: 0.302 mg in the controls; 0.338 mg in the presence of the 0%–20% precipitate from Uvaferm BDX, 0.349 mg in the presence of the 60%–80% precipitate from Lalvin CY3079 and 0.393 mg in the presence of the 60%–80% precipitate from Uvaferm BDX.

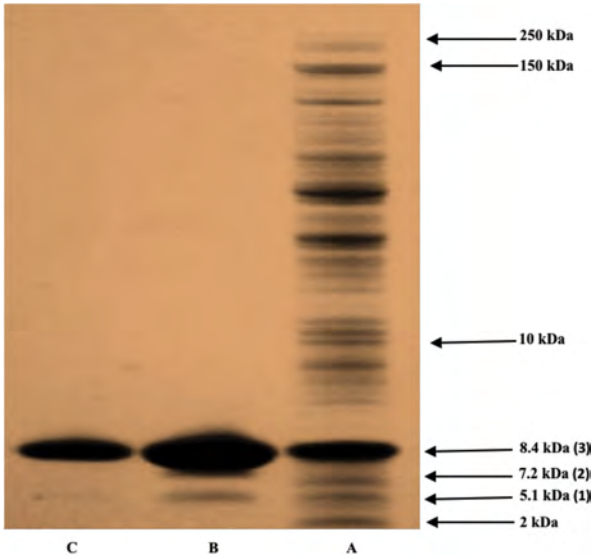

Figure 2. Silver stained Tricine SDS-PAGE gel. Lane A corresponds to the dual-xtra MW standard 2–250 kDa (Bio-Rad). Lane B corresponds to the 5–10 kDa fraction of the 60%–80% precipitate of *S. cerevisiae* Uvaferm BDX. Lane C corresponds to the 5–10 kDa fraction of the 60%–80% precipitate of *S. cerevisiae* Lalvin CY3079 (reference strain).

approximately 5.1 kDa (1), 7.2 kDa (2) and 8.4 kDa (3) as shown in lane B. Two of these protein bands, (1) and (2), were not present in the SGJ medium fermented by Lalvin CY3079 (lane C). Besides, band (3) of approximately 8.4 kDa was released by both strains, but was more concentrated in the SGJ medium fermented by Uvaferm BDX. Therefore, it is likely that the peptides of these bands were involved in the MLF inhibition. The use of Lalvin CY3079 strain as a reference strain was helpful since the comparison of the protein profiles proved that strain Uvaferm BDX was able to produce additional peptides.

Fractionation of the 5–10 kDa peptidic fractions of the 60%–80% ammonium sulfate precipitates by anion and cation exchange chromatography and evaluation of the inhibitory effect of the eluates on the malolactic enzyme activity *in vitro*

In order to further reduce the amount of peptides and specifically target the anti-MLF ones, the 5–10 kDa peptidic fraction of the 60%–80% ammonium sulfate precipitate of *S. cerevisiae* Uvaferm BDX was analyzed by ion exchange chromatography. The eluates from both anion and cation exchange chromatography (AEXC and CEXC) were tested *in vitro* in the presence of the malolactic enzyme of the cell-free enzymatic extract.

Table 4. Consumed L-malic acid (g L^{-1}) and produced L-lactic acid (g L^{-1}) in the controls and in the presence of the eluates obtained from AEXC and CEXC.

	pH = 6.7; t = 40 min	
	Consumed L-malic acid (g L^{-1})	Produced L-lactic acid (g L^{-1})
[a]Control 1	0.98 (±0.02)	0.66 (±0.01)
[b]Control 2	1.00 (±0.02)	0.68 (±0.01)
[c]Control 3	1.00 (±0.02)	0.68 (±0.01)
[d]Unbound fraction	0.96 (±0.02)	0.64 (±0.01)
Eluate 1 from AEXC (0.5 M NaCl)	0.002 (±0)	0.0013 (±0)
Eluate 1 from CEXC (0.5 M NaCl)	0.002 (±0)	0.0013 (±0)
Eluate 2 from AEXC (1 M NaCl)	0.96 (±0.02)	0.65 (±0.01)
Eluate 2 from CEXC (1 M NaCl)	0.97 (±0.02)	0.67 (±0.01)

[a]Control 1: 0.14 mL of phosphate buffer 0.1 M pH 6.7 were added instead of the peptidic eluate.
[b]Control 2: 0.14 mL of NaCl 0.5 M were added instead of the peptidic eluate.
[c]Control 3: 0.14 mL of NaCl 1 M were added instead of the peptidic eluate.
[d]Unbound fraction: 0.14 mL of the fraction that was not retained on the column after sample binding in AEXC or CEXC.

Control 1 in Table 4 is the usual control used in all the *in vitro* experiments. It enables us to monitor the malolactic enzyme activity *in vitro*. Controls 2 and 3 were added to this experiment to evaluate the effect of NaCl on the malolactic enzyme activity. In fact, the elution of peptides in both AEXC and CEXC was performed using increasing concentrations of NaCl (0.5 M then 1 M of NaCl) in the elution buffers. We wanted to make sure that the inhibition observed in the presence of the eluates was only due to the peptides and not to the NaCl. The results show that the presence of NaCl did not affect the malolactic enzyme activity since the 1 g L^{-1} of L-malic acid initially present in the control aliquots (controls 2 and 3) was totally consumed. The unbound fraction corresponds to the fraction that was not retained on the column after loading and binding the sample. It did not inhibit the malolactic enzyme activity as shown in Table 4. Therefore, it did not contain any inhibitory peptide. Its analysis underlines the effectiveness of this purification step.

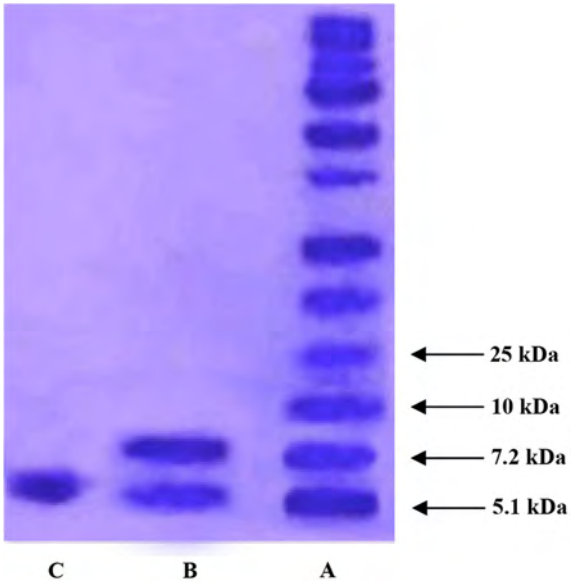

Figure 3. Coomassie G-250 stained Tricine SDS-PAGE gel. Lane A contains dual-xtra MW standard 2–250 kDa (Bio-Rad). Lane B corresponds to eluate one from CEXC. Lane C corresponds to eluate one from AEXC.

Eluates obtained after elution with 0.5 M NaCl from both AEXC and CEXC (eluates 1 of Table 4) strongly inhibited the malolactic enzyme activity since only 0.2% of the malate present were consumed. However, those eluted with 1M NaCl did not affect the enzyme's activity and the malate was entirely consumed. Therefore the peptides of interest were recuperated in eluates 1 of both AEXC and CEXC. The same procedure was performed with strain Lalvin CY3079 and none of the eluates showed any inhibition (data not shown).

Tricine SDS-PAGE analysis of the inhibitory eluates followed by sequence analysis of the protein bands by LC-MS/MS

Fig. 3 shows the results obtained after migration of the inhibitory eluates on Tricine SDS-PAGE (eluates one from both AEXC and CEXC). The cationic eluate gave two protein bands of approximately 5.1 and 7.2 kDa whereas the anionic eluate gave one protein band of 5.1 kDa. The three protein bands from both eluates were excised and sequenced in order to identify the nature of the putative antibacterial peptides responsible for the MLF inhibition.

Tables 5 and 6 give the nature of the peptides identified by LC-MS/MS (LC1D-nanoESI-LTQ-Orbitrap) as well as their amino acid sequence, molecular weight and theoretical isoelectric point (pI). The cationic eluate revealed 7 protein fragments corresponding to 6 proteins of *S. cerevisiae* whereas the anionic eluate revealed 2 protein fragments corresponding to 2 yeast proteins.

DISCUSSION

The current study aims to further purify and identify the putative anti-MLF peptides of the 5–10 kDa peptidic fraction produced by *S. cerevisiae* Uvaferm BDX that inhibited the growth of *O. oeni* Vitilactic F and its ability to consume L-malic acid during sequential fermentations (Nehme, *Mathieu and Taillandier* 2010; Rizk *et al.* 2016). First, the SGJ medium, fermented by *S. cerevisiae* Uvaferm BDX, was fractionated by ammonium sulfate precipitation combined with ultrafiltration. The results obtained suggested the presence of two categories of inhibitory proteinaceous compounds (Fig. 1). The first one inhibited the growth of *O. oeni* Vitilactic F only (5–10 kDa peptidic fractions of the 0%–60% precipitates), while the second one inhibited both the bacterial growth and the malate consumption (5–10 kDa peptidic fraction of the 60%–80% precipitate). With the latter, the inhibition of the bacterial growth could be a consequence of the malate consumption inhibition or it could be due to the presence of both categories of protein compounds. In winemaking conditions, MLF is one of the main energy sources for bacterial survival and growth (Cox and Henick-Kling 1995; Bouix and Ghorbal 2015), thus, an inhibition of the malate consumption can also cause an inhibition of the bacterial growth.

Second, the 5–10 kDa peptidic fraction of the 60%–80% ammonium sulfate precipitate was tested *in vitro* in a cell-free cytosolic extract of *O. oeni* Vitilactic F containing the malolactic enzyme. The results of Table 2 show that the peptidic fraction strongly inhibited the malolactic enzyme activity *in vitro* at a pH range between 3.5 and 6.7. Therefore, this fraction is believed to enter the bacterial cytoplasm *in vivo* by mechanisms yet to be identified in order to directly target and inhibit the malolactic enzyme. These results are in accordance with those obtained by Rizk *et al.* (2016) who were the first to show that yeast proteinaceous compounds can inhibit MLF by directly targeting the malolactic enzyme activity.

The 5–10 kDa peptidic fraction of the 60%–80% ammonium sulfate precipitate was further purified by ion exchange chromatography. The anionic and cationic eluates that inhibited the malolactic enzyme activity *in vitro* (eluates 1 of Table 4) were migrated by SDS-PAGE and the bands of interest were excised and sequenced by LC-MS/MS. The cationic eluate revealed 7 protein fragments corresponding to 6 proteins of *S. cerevisiae* whereas the anionic eluate revealed 2 protein fragments corresponding to 2 yeast proteins. The following identified proteins play diverse vital roles in *S. cerevisiae* cells: the short-chain dehydrogenase/reductase protein binds and stabilizes the pre-tRNAs and RNA polymerase III transcripts (Schenk *et al.* 2012). Yeast actin is a structural protein involved in cell polarization, endocytosis, cytoskeleton functions and histone acetyltransferase activity (Greeber and Schenkman 1982; Moseley and Goode 2006). YDL025C-like protein is a major protein kinase involved in protein phosphorylation (Ptacek *et al.* 2005). Petite colonies protein or Pet127p is a membrane-associated protein involved in stability and processing of *S. cerevisiae* mitochondrial RNAs (Wiesenberger and Fox 1997). Utr2p has a hydrolase activity, hydrolyzing O-glycosyl compounds (Novo *et al.* 2009). Wtm1p (WD repeat-containing transcriptional modulator) is a protein present in a large nuclear complex and presenting two homologs, Wtm2p and Wtm3p, which probably arose by gene duplications. These proteins are transcriptional modulators with roles in meiotic regulation and silencing and RNR genes expression. They are also involved in response to replication stress and are relocalized in the cytosol in response to hypoxia (Pemberton and Blobel 1997). Therefore, peptidic fragments of Wtmp are produced and released by yeasts in response to stress.

There are no previous studies that describe the antimicrobial activities of the above mentioned proteins and/or their corresponding fragments against bacteria or against fungi.

The sequencing also revealed two peptidic fractions of GAPDH in the cationic eluate, one having a MW of 0.9 kDa and a pI of 10.5 while the other presented a MW of 1.373 kDa and a pI of 11 (Table 5). Branco *et al.* (2014) previously demonstrated that GAPDH fragments were involved in the growth

Table 5. Sequence analysis by LC1D-nanoESI-LTQ-Orbitrap of the peptides corresponding to the 5.1 and 7.2 kDa protein bands obtained from the cationic eluate.

Cationic peptides/proteins							
Identified proteins	Accession number	Sequence of amino acids	Identification probability	Actual mass (Da)	Start	Stop	pI
Putative short-chain dehydrogenase/reductase [S. cerevisiae S288c]	gi—6 322 742 (+4)	(K)DmAVSYLSR(Y)	100%	1056.49	118	126	9
Actin [S. cerevisiae S288c]	gi—14 318 479 (+2)	(K)AGFAGDDAPR(A)	100%	975.4408	19	28	6.5
Conserved protein [S. cerevisiae YJM789]	gi—151 944 993 (+1)	(S)SAASAGVSR(I)	96%	804.4088	15	23	10.5
Glyceraldehyde-3-phosphate dehydrogenase [S. cerevisiae YJM789]	gi—151 943 468 (+4)	(K)KVVITAPSS(T)	99%	900.5276	116	124	10.5
Glyceraldehyde-3-phosphate dehydrogenase [S. cerevisiae YJM789]	gi—151 943 468 (+4)	(R)TASGNIIPSSTGAAK(A)	100%	1373.71	199	213	11
YDL025C-like protein [S. cerevisiae AWRI796]	gi—323 334 240 (+1)	(–)mEVVTNHTQR(Q)	97%	1271.60	1	10	7.4
Petite colonies protein [S. cerevisiae YJM789]	gi—151 944 772 (+1)	(K)TNGAASLDPTKER(K)	95%	1358.68	53	65	10.2

Table 6. Sequence analysis by LC1D-nanoESI-LTQ-Orbitrap of the peptides corresponding to the 5.1 kDa protein band obtained from the anionic eluate.

Anionic peptides/proteins							
Identified proteins	Accession number	Sequence of amino acids	Identification probability	Actual mass (Da)	Start	Stop	pI
Wtm2p [S. cerevisiae Lalvin QA23]	gi—323 346 419	(D)DDDDDNDDDDEEGNxKTKSAAT(P)	98%	2420.89	89	110	3.5
Utr2p [S. cerevisiae EC1118]	gi—259 145 860 (+3)	(F)CNATQACPEDKPCCSQYGEcGTG(Q)	97%	2420.89	27	49	4

inhibition of *O. oeni* and some non-*Saccharomyces* yeasts. However, the two GAPDH fragments identified by these authors were not the same as those found in the current study. They had different amino acid sequences that corresponded to two fragments of the C-terminal amino acid sequence of the GAPDH isoenzymes (GAPDH2/3 with a MW of 1.638 kDa and GAPDH1 with a MW of 1.622, pI of 4.37). Their antibacterial activity was tested only against the growth and not against the malate consumption. Branco *et al.* (2017) demonstrated that several strains of *S. cerevisiae* were able to release the same GAPDH fragments previously identified but at different concentrations. The GAPDH derived peptides were able to induce membrane permeabilization, enter the cytoplasm of sensitive yeast cells and trigger apoptosis. Their experiments were conducted on non-*Saccharomyces* strains and not on *O. oeni* strains.

In the present study, the cationic eluate containing the GAPDH fragments strongly inhibits the malate consumption *in vitro* at pH 6.7 (pH of the enzymatic reaction) (Table 4), which leads us to suggest that the GAPDH peptides may have inhibited the malolactic enzyme activity by competing for the binding site of NAD^+. In fact, NAD^+ is essential for the activation of the enzyme. By depriving the malolactic enzyme from NAD^+, it will become dysfunctional. Moreover, the GAPDH fragments may bind to other parts of the enzyme, thus modifying its conformation and affecting its function. *In vivo*, they should enter the bacterial cytoplasm by mechanisms yet to be identified in order to exert their inhibitory effect.

Although there is no previous study on the antimicrobial role of Wtm2p and Utr2p, their fragments seem to play a major role in the inhibition since the corresponding anionic eluate strongly inhibited the malolactic enzyme activity *in vitro*, as previously shown in Table 4. Giving their pI (3.5 and 4), the peptidic fragments are anionic at pH 6.7 that is the pH of this *in vitro* experiment. Therefore, they may deprive the malolactic enzyme of its Mn^{++} cofactors by chelating them or of NAD^+ by binding to it, thus inhibiting the malolactic enzyme activity and the malate consumption. Since pH 6.7 is around the cytoplasmic pH of most lactic acid bacteria, we can assume that when Wtm2p and Utr2p peptidic fragments enter the cytoplasm of *O. oeni* Vitilactic F *in vivo*, they will be able to work similarly, leading to an inhibition of the MLF.

Moreover, it was previously demonstrated that some AMPs are able to translocate across membranes and target cytoplasmic constituents without disrupting plasma membranes (Park *et al.* 2000; Powers and Hancock 2003; Brown and Hancock 2006). Therefore, it would be interesting to see if the peptides

obtained in the current study are able to disrupt the bacterial plasma membrane, or translocate inside the cell without membrane disruption. The entrance of anionic peptides like Wtm2p and Utr2p may require some divalent metal cations like Fe^{++}, Mn^{++}, Mg^{++}, or others. In fact, several studies have shown that the antimicrobial activity of anionic peptides is enhanced by metal cations that promote their binding to the negatively charged components of cell-walls and/or plasma membranes (Dashper *et al.* 2005; Dashper, *Liu and Reynolds* 2007). Some of them are able to translocate across membranes or increase membrane permeability by forming pores (Harris, *Dennison and Phoenix* 2009).

CONCLUSIONS

Saccharomyces cerevisiae Uvaferm BDX was able to release nine anti-MLF peptides with a MW range between 0.8 and 2.42 kDa. These peptides derived from eight proteins that play diverse vital roles in yeast cells. Two GAPDH fragments of 0.9 and 1.373 kDa having a pI of 10.5 and 11 respectively, and fragments of Wtm2p and Utr2p of 2.42 kDa with a pI of 3.5 and 4 respectively were thought to contribute the most to the MLF inhibition. However, it is likely that one or more of the nine peptides worked synergistically to inhibit MLF. The peptides were able to inhibit the malolactic enzyme activity *in vitro* at a pH range between 3.5 and 6.7. *In vivo*, they are supposed to enter the bacterial cytoplasm by mechanisms yet to be identified in order to reach the malolactic enzyme. It would be interesting for future studies to synthesize these peptides in order to test them individually *in vivo* and *in vitro* experiments and to evaluate the contribution of each in the MLF inhibition. It would be also important to evaluate their spectrum of activity against other *O. oeni* strains, wine spoilage and pathogenic microorganisms. Studying their interaction with the plasma membrane, the mechanisms by which they enter inside the bacterial cytoplasm and inhibit the malolactic enzyme activity would also be beneficial.

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Conflicts of interest. None declare.

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