LWT - Food Science and Technology 108 (2019) 353–360

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Survival and implantation of indigenous psychrotrophic *Oenococcus oeni* strains during malolactic fermentation in a Patagonian Pinot noir wine

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ARTICLE INFO

Keywords:
Psychrotrophic capacity
Oenococcus oeni
Patagonian wine
Malolactic fermentation
Wine implantation

ABSTRACT

Spontaneous malolactic fermentation (MLF) in the North Patagonian region of Argentina may have to bear low environmental temperatures, an important stress factor for the lactic acid bacteria (LAB) that conduct this process. Thus, inducing MLF by inoculation of a native starter culture able to lead this biological process at low temperatures is encouraged because it would allow preserving the regional character of the wine. The goal of this work was to obtain autochthonous *Oenococcus oeni* strains able to consume L-malic acid when MLF takes place at temperatures lower than 15 °C. Among the psychrotrophic isolates obtained from a Pinot noir wine, sixteen *O. oeni* strains were identified by sequencing the *16S rRNA* gene and typing by RAPD PCR, allowing the differentiation of five genotypic groups. After the evaluation of the viability and metabolic performance of the strains in sterile wine at low temperatures, strains UNQOe4 and UNQOe19 were selected to evaluate their behavior in a non-sterile wine at 4 and 10 °C. The strains selected showed good implantation capacity and were able to successfully conduct MLF, suggesting that their use as starter cultures is suitable to improve this biological process in a wine region where environmental temperatures can be low.

1. Introduction

Winemaking is a complex process frequently involving two successive fermentations: an alcoholic fermentation (AF) conducted by yeasts and a subsequent malolactic fermentation (MLF) carried out by lactic acid bacteria (LAB) species like *Lactobacillus plantarum* and *Oenococcus oeni*, which are found in wines during spontaneous MLF (Berbegal et al., 2016; Bravo-Ferrada et al., 2013; Lucio, Pardo, Heras, Krieger-Weber, & Ferrer, 2017; Valdés La Hens, Bravo-Ferrada, Delfederico, Caballero, & Semorile, 2015). However, *O. oeni* strains are still referred to as the main responsible for MLF, due to their wide range of physiological characteristics and their ability to cope with several environmental stresses (Miranda-Castilleja et al., 2016; Nisiotou et al., 2015; Spano & Massa, 2006).

The MLF is responsible for the conversion of L-malic acid (MA) to L-lactic acid and CO_2, which causes a decrease in the total acidity of wine and a small increase in pH. MLF also leads to enhanced microbial stability and improves the complexity of the wine flavor and can vary according to the LAB strains used to induce the MLF (Brizuela, Bravo-Ferrada, Pozo-Bayón, Semorile, & Tymczyszyn, 2018; Cappello, Zapparoli, Logrieco, & Bartowsky, 2017).

Although MLF can occur spontaneously, by action of native LAB

Abbreviations: LAB, Lactic Acid Bacteria; MLF, Malolactic fermentation; MLO, Medium for *Leuconostoc oenos*; MAC, Malic acid consumption; MA, Malic acid; AF, Alcoholic fermentation; UPGMA, Unweighted Pair Group Method using Arithmetic Averages; RAPD-PCR, Random Amplification of Polymorphic DNA-Polymerase chain reaction; *hdc*, Histidine decarboxylase; *ptc*A, Putrescine carbamoyl transferase

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https://doi.org/10.1016/j.lwt.2019.02.063
Received 24 August 2018; Received in revised form 8 February 2019; Accepted 18 February 2019
Available online 22 February 2019
0023-6438/

from grapes and cellar, it implies risks such as an increase in the volatile acidity, consumption of residual sugars, and formation of undesirable metabolites such as biogenic amines (Marcobal, Martín-Álvarez, Polo, Muñoz, & Moreno-Arribas, 2006; Mira de Orduña, Liu, Patchett, & Pilone, 2000). To prevent production losses, the control of MLF is mandatory, and the main strategy is the inoculation of MLF starter cultures. Although there are commercial MLF starter cultures, the use of autochthonous selected LAB could be preferable to preserve the regional character of wines.

The North Patagonian region of Argentina, which is one of the southernmost winegrowing regions of the world, has optimal agro-ecological conditions for high-quality viticulture, in which the Pinot noir varietal has found the optimal conditions to express its full oenological potential (Crisóstomo, 2007). In this region, spontaneous MLF takes place during April and May, when environmental temperatures average 14 to 10 °C, eventually reaching 4-2 °C, quite below the optimal temperature required by LAB to develop their metabolic activities. Local wineries usually solve this problem by heating the fermentation tanks or the cellar environment, with a significant increase in the production costs. When LAB are exposed to cold environments, they may suffer important physiological changes, which result in a reduced efficiency of DNA translation, transcription and replication (Serrazanetti, Ndagijimana, Miserocchi, Perillo, & Guerzoni, 2013). However, different studies have revealed that *O. oeni* has developed cellular mechanisms that make it more resistant to adverse conditions than other LAB species (Beltramo, Desroche, Tourdot-Maréchal, Grandvalet, & Guzzo, 2006; Bourdineaud, Nehme, Tesse, & Lonvaud-Funel, 2003; Olguín, Bordons, & Reguant, 2009, 2010).

A previous study (Bravo-Ferrada et al., 2016) has shown that, at laboratory scale and optimal growth temperatures for LAB (28 °C), *O. oeni* strains from the Patagonian region are able to consume MA and tolerate stress conditions in a wine-like medium (low pH and/or high ethanol, SO_2, and lysozyme). However, these conditions not necessarily reflect the actual environmental conditions to which the LABs responsible for conducting MLF are subjected in the North Patagonian region.

The aim of this study was to obtain a collection of native Patagonian psychrotrophic *O. oeni* strains, able to remain viable and to perform MLF at low winemaking temperatures. With this purpose, we first studied their behavior in sterile wine incubated at low temperatures by evaluating their survival and ability to consume MA. The strains with the best combination of both parameters were then inoculated into non-sterile wine at low temperatures and cell viability, MA consumption, and implantation ability were analyzed.

2. Materials and methods

2.1. Wine sample

A sample of Pinot noir wine (vintage 2014), at the end of AF (14.3% (v/v) ethanol, pH 3.7, 2.3 g L^{-1} MA), was aseptically collected at a 109-year-old commercial cellar, in General Roca, North Patagonia, Argentina, which had never used commercial malolactic starter cultures. In this cellar, the winemaking process begins with a cold pre-fermentation for 48–72 h, followed by AF in concrete tanks of 9000 L for 20 days; afterwards the MLF for 25–40 days and finally, the wine undergoes aging in French oak barrels for seven or more months.

2.2. Isolation of psychrotrophic LAB strains

To obtain psychrotrophic LAB isolates, 200 mL of wine was kept at ~ 8–10 °C for 30 days until MA was exhausted (0 g L^{-1}). After that, two strategies were applied: 1- aliquots of 100 μL were directly plated into medium for *Leuconostoc oenos* (MLO) agar (Maicas, González-Cabo, Ferrer, & Pardo, 1999) supplemented with 15% (v/v) tomato juice, cysteine 0.5 g L^{-1}, L-MA 4.5 g L^{-1}, cycloheximide 0.01% (w/v) (Sigma, USA), nystatin 20 mg mL^{-1} and sodium azide 0.01% (w/v), at pH 4.8, and incubated at ~ 8–10 °C, for 30 days under microaerophilic conditions; 2- aliquots of 2 mL were subjected to previous enrichment in 45 mL of MLO broth and incubated under microaerobiosis conditions, taking samples at 0, 5, 10, 15, 20, and 30 days, which were then spread in MLO agar and incubated at ~ 8–10 °C. Recovered colonies were subjected to Gram staining and catalase activity test.

2.3. Identification of psychrotrophic isolates

DNA was extracted according to the method previously described in Bravo-Ferrada et al. (2011). DNA samples were quantified with a Nanodrop spectrophotometer (Thermo Scientific, 1000) and visualized on a 1.0% (w/v) agarose gel. For identification of the isolates, the *16S rRNA* gene was amplified (Rodas, Ferrer, & Pardo, 2003) using the primers pA-16SF (5′-AGAGTTTGATCCTGGCTCAG-3′) and pH-16SR (5′-AAGGAGGTGATCCAGCCGCA-3′), and then sequenced (Macrogen Corp., Korea). Sequences were analyzed by comparison with sequences deposited at GenBank (NCBI, NIH).

2.4. Typing by random amplification of polymorphic DNA-Polymerase chain reaction (RAPD-PCR)

Strains were analyzed by RAPD-PCR by using the M13 primer (5′-GAGGGTGGCGGTTCT-3′) (Stendid, Karlsson, & Hogberg, 1994). The PCR products were resolved by electrophoresis in a 2% (w/v) agarose gel. Fragment lengths were estimated by comparison to a 100-bp ladder marker as a size standard (Productos Bio-Lógicos, Universidad Nacional de Quilmes, Buenos Aires, Argentina). High-resolution photographs of RAPD-PCR gels were obtained by the Kodak Electrophoresis Documentation and Analysis System 120, and subsequently analyzed using Kodak Digital Science V.3.0.2 (Eastman Kodak Company, Rochester, NY, USA). Patterns were evaluated by calculating the genetic similarity index by using a simple matching coefficient (Apostol, Black, Miller, Reiter, & Beaty, 1993). The unweighted pair group method using arithmetic averages (UPGMA) cluster analysis was carried out using PAUP* 4.0b10 (Sinauer Associates, Sunderland, MA, USA).

2.5. Cell culture acclimation

Bacterial cells in the early stationary phase (~10^9 cfu mL^{-1}) were harvested by centrifugation at 5000 × *g* for 10 min and suspended in the same volume of a modified acclimation medium (50 g L^{-1} MRS, 40 g L^{-1} D (−) fructose, 20 g L^{-1} D (−) glucose, 4 g L^{-1} L-malate, 1 g L^{-1} Tween 80, 0.1 mg L^{-1} pyridoxine, pH 4.6) supplemented with 6% v/v ethanol (Bravo-Ferrada, Tymczyszyn, Gómez-Zavaglia, & Semorile, 2014). Cultures were incubated at 4, 10 and 21 °C for 48 h, without shaking.

2.6. Fermentation assays in sterile wine

Samples of Pinot noir wine, at the final stage of AF, were sterilized by filtration through 0.2 μm pore size (Sartorius Stedim Biotech Gmbh, Göttingen, Germany). Acclimated cells of each strain were harvested by centrifugation and suspended in 10 mL of sterile wine at a final concentration of 2 × 10^7 cfu mL^{-1}. Incubations were performed at 4, 10, and 21 °C (control), for 20 days, taking samples at 0, 5, 10, 15, and 20 days for evaluation of the number of viable cells and the remaining concentration of MA.

2.7. L-malic acid consumption (MAC)

The remaining MA was measured with an enzymatic kit (L-Malic Acid Enology Enzymatic kit, BioSystems SA, Barcelona, Spain). An exponential one-phase decay equation model was used to fit the MAC kinetics of the different strains tested. The equation for this model was

obtained by the Graph Pad Prism® software and is:

$$[MA_t] = ([MA_0] - [Ma_i])\, e^{-kt} + [Ma_i] \qquad \text{Eq 1}$$

Where [MA*t*] is the MA concentration at time = t, [MA_0] is the initial concentration of MA (2.3 g L^{-1} in the wine used), [MA*i*] is the MA concentration at infinite time and K is the rate constant. Also, the percentage of MA consumed (MAC %) after 20 days of wine incubation was calculated following the equation:

$$MAC\% = 100 - ([MA_f]\ 100\ /\ [MA_0]) \qquad (2)$$

Where [MA_0] is the initial concentration of MA in the wine sample and [MA_f] is the final MA concentration measured after 20 days of wine incubation (Brizuela et al., 2017).

2.8. Fermentation at low temperatures in non-sterile wine

The psychrotrophic *O. oeni* strains selected were acclimated (6% ethanol v/v, 21 °C) and then, 1 mL was inoculated in 10 mL of non-sterile Pinot noir wine at ~2×10^7 cfu mL^{-1} cell density, which was incubated at 4, 10, and 21 °C (control) for 20 days. Samples were taken at 0, 5, 10, 15, and 20 days to determine the remaining MA concentration and cell survival. Non-inoculated wine samples were also used as a control of fermentation.

2.9. Implantation capacity of the strains

The implantation capacity of the strains in non-sterile wine was determined by RAPD-PCR using the primer M13 (Stendid et al., 1994). Ten colonies from each sample were randomly taken from MLO plates before and after the MLF and inoculated in MLO broth to obtain DNA from each culture. PCR products were separated by 2.0% (w/v) agarose electrophoresis gel using a 100-bp ladder PB-L (Productos Bio-Lógicos, Universidad Nacional de Quilmes, Buenos Aires, Argentina). The implantation capacity was evaluated by comparing the RAPD profiles obtained from colonies with the profiles of the inoculated strains.

2.10. Screening of the hdc and ptcA genes

As a first approach to study the ability of the *O. oeni* strains to produce biogenic amines, the presence of the histidine decarboxylase (*hdc*) and putrescine carbamoyl transferase (*ptcA*) genes was screened in the selected psychrotrophic strains, as previously described in Bravo-Ferrada et al. (2013). These genes are involved in the synthesis of the biogenic amines histamine and putrescine, respectively. *Lactobacillus buchneri* ST2A and *Lactobacillus hilgardii* X1B were used as positive controls for the *hdc* and *ptcA* genes, respectively.

2.11. Reproducibility assay

All determinations were the average of three independent replicate assays. Data are shown as mean values. The statistical analyses were carried out using Graph Pad Prism 6.01 software (Graph Pad Software Inc., San Diego, CA, USA, 2007). Means were compared by one-way ANOVA, and if $P < 0.05$, the difference was considered statistically significant.

3. Results

3.1. Isolation of psychrotrophic O. oeni strains

A total of 20 psychrotrophic isolates were obtained previous incubation for 30 days at low temperature (Manera et al., 2017). Sixteen potential *O. oeni* isolates showed small white colonies (diameter less than 1 mm), developed on MLO agar after 5–7 days of incubation at low temperatures. These were selected and classified as putative LAB by positive Gram staining, catalase negative activity, and light microscopy. The microscopic evaluation showed ellipsoidal to spherical cocci grouped in pairs or chains (Bravo-Ferrada et al., 2018; Garvie, 1967).

3.2. Identification of presumptive O. oeni strains

The identification of the sixteen LAB isolates presumptively classified as *O. oeni* was confirmed by sequence analysis of the *16S rRNA* gene. Sequence data were analyzed using BLAST and MEGA 4 programs and sequences were deposited at GenBank. The sequences obtained were multiply aligned with the corresponding sequences deposited at GenBank, using the Clustal X program, and obtained similarity percentages varying between 96 and 99%. The GenBank Accession Numbers are: UNQOe1 (KY561608), UNQOe2 (KY561602), UNQOe4 (KY561600), UNQOe5 (KY561599), UNQOe6 (KY561598), UNQOe7 (KY561597), UNQOe9 (KY561596), UNQOe12 (KY561609), UNQOe13 (KY561607), UNQOe14 (KY561606), UNQOe15 (MG808379), UNQOe16 (MG808380), UNQOe17 (KY561605), UNQOe18 (KY561604), UNQOe19 (KY561603) and UNQOe20 (KY561601).

3.3. Typing of O. oeni strains

The genetic heterogeneity of the sixteen *O. oeni* strains was determined by numerical analysis of the DNA profiles obtained by RAPD PCR with the M13 primer (Fig. 1), which allowed grouping the strains into five clusters, defined at a minimum similarity level of 85%, and showing 12 different genotypes. Cluster I contains only one strain (UNQOe14), which appears as the most genetically distant. Cluster II is composed of five members, showing UNQOe1 and UNQOe2 100% of similarity, while UNQOe4, UNQOe5 and UNQOe6 exhibit genetic heterogeneity. Cluster III contains three strains, UNQOe19, UNQOe15, and UNQOe16, showing these two last 100% of similarity. Cluster IV is composed of strains UNQOe18 and UNQOe20, with 100% of similarity. Finally, Cluster V contains five strains, of which UNQOe7 and UNQOe9 share 100% of similarity.

3.4. Evaluation of MAC and cell survival in sterile wine

The sixteen *O. oeni* strains were subjected to comparative performance assays in sterile wine incubated at low temperatures. The strains were previously adapted to improve cell survival by incubating cultures

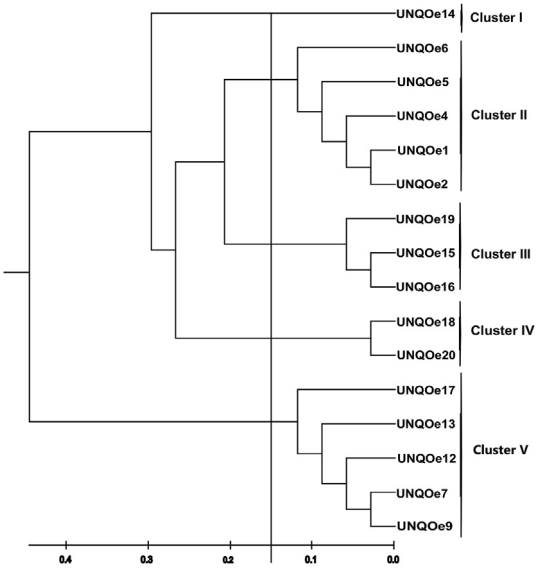

Fig. 1. Dendrogram based on the Unweighted Pair Group Method using Arithmetic Averages (UPGMA) clustering of RAPD M13 patterns corresponding to sixteen *O. oeni* strains.

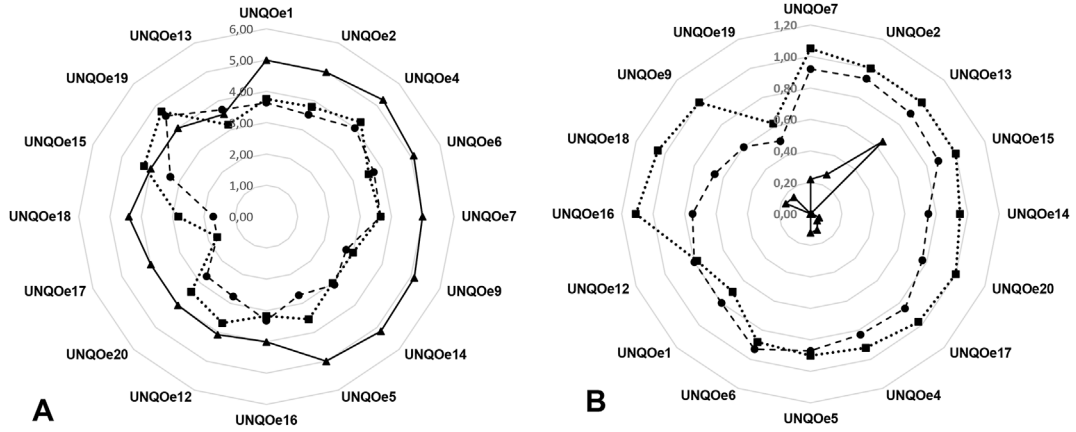

Fig. 2. Cell survival (log cfu mL^{-1}) **(A)** and L-malic acid residual concentration (g L^{-1}) **(B)** after inoculation of *O. oeni* strains in sterile Pinot noir wine incubated at 4 (●), 10 (■) and 21 °C (▲), for 20 days. Three independent replicates were carried out.

in an acclimation medium containing a sub-lethal ethanol concentration (6% v/v). When a second stress factor (incubation at 4 and 10 °C) was added in the acclimation process, none of the strains inoculated into sterile wine was able to remain viable. Only cell cultures acclimated in 6% ethanol v/v, incubated at 21 °C, and inoculated at a cell density of $\sim 2 \times 10^7$ cfu mL^{-1} were able to survive in sterile wine.

The values of cell survival and residual MA exhibited by the different *O. oeni* strains after 20 days of incubation at low (4 and 10 °C) and control temperatures (21 °C) are displayed in Fig. 2. At 20 days of incubation, thirteen strains (UNQOe1, UNQOe2, UNQOe4, UNQOe5, UNQOe6, UNQOe7, UNQOe9, UNQOe12, UNQOe14, UNQOe16, UNQOe17, UNQOe18, and UNQOe20) showed higher viability at 21 °C, while only one (UNQOe19) showed higher viability at low temperatures. UNQOe13 showed similar values at the three incubation temperatures and UNQOe15 displayed similar values at 10 and 21 °C. On the other hand, UNQOe1, UNQOe2, UNQOe4, UNQOe6, UNQOe7, UNQOe9, UNQOe13, UNQOe14, UNQOe16, and UNQOe17 showed similar viability values at 4 °C and 10 °C, whereas UNQOe5, UNQOe12, UNQOe20, UNQOe18 and UNQOe15 showed higher survival values at 10 °C than at 4 °C. UNQOe17 and UNQOe18 were not able to remain viable at 4 °C, with viable count values varying between 10^1 and 10^2 cfu mL^{-1} (Fig. 2 A).

Regarding the remaining MA, all the strains showed capacity to consume MA at the three incubation temperatures assayed, although their behavior was heterogeneous (Fig. 2B). At 21 °C, UNQOe1, UNQOe6, UNQOe12, UNQOe14, UNQOe15, UNQOe16 and UNQOe19 were able to exhaust (0 g L^{-1}) the MA content of wine, whereas six of them achieved this at 15 days of incubation and UNQe14 at 20 days. On the other hand, UNQOe7, UNQOe4, UNQOe5, UNQOe9, UNQOe17, and UNQOe18 showed similar residual MA values, in a range from 0.1 to 0.2 g L^{-1}, being UNQOe2 and UNQOe13 the strains that exhibited the lowest MAC.

At 4 °C, UNQOe9, UNQOe18, and UNQOe19 exhibited the lowest values of residual MA, in a range of 0.5–0.7 g L^{-1}, being UNQOe19 the best consumer. UNQOe1, UNQOe2, UNQOe4, UNQOe5, UNQOe6, UNQOe7, UNQOe12, UNQOe13, UNQOe14, UNQOe15, UNQOe16, UNQOe17, and UNQOe20 showed residual MA values in a range of 0.8–0.9 g L^{-1} (Fig. 2B).

At 10 °C, UNQOe1, UNQOe12, and UNQOe19 showed residual MA values in a range of 0.6–0.8 g L^{-1}, being UNQOe19 the best consumer, whereas UNQOe7 and UNQOe18 exhibited residual MA values of 1.1 g L^{-1}, and UNQOe16 exhibited the lowest MAC (Fig. 2B).

UNQOe2, UNQOe4, UNQOe9, UNQOe13, UNQOe14, UNQOe15, UNQOe16, UNQOe17, UNQOe18, UNQOe19 and UNQOe20 showed better MA consumption at 4 °C than at 10 °C (Fig. 2B).

Another important observation was the relation between the phenotypic characteristics and clustering analysis of strains (Fig. 1). Although UNQOe5, UNQOe6, UNQOe4, UNQOe1 and UNQOe2 were included in Cluster II and UNQOe1 and UNQOe2 showed 100% of similarity, they showed differences in cell viability and MAC in sterile wine (Fig. 2). The same observation was valid for the strains included in Clusters III, IV and V.

According to their genetic diversity, capacity of viability and MAC at different temperatures, UNQOe1, UNQOe4, UNQOe9, and UNQOe19 were selected to analyze their MAC kinetics in sterile wine incubated at 4, 10 and 21 °C (Fig. 3). Table 1 shows the values of viable cell count (cfu mL^{-1}) and kinetic parameters of MAC (Eq. 1) after incubation in sterile Pinot noir wine for 20 days. The MAC kinetics was faster at 21 °C than at 4 and 10 °C (Fig. 3), highlighting the behavior of UNQOe1 and UNQOe19, which showed values of 100% MAC between 15 and 20 days of incubation (Table 1 and Fig. 3). UNQOe9 and UNQOe19 showed faster MAC kinetics at 4 °C than at 10 °C, while at these temperatures UNQOe1 and UNQOe4 showed similar MAC kinetics. UNQOe19 and UNQOe4 showed a good combination of cell survival and MAC values, at low temperatures (Table 1), and were thus selected for fermentation assays in non-sterile wine.

3.5. Fermentation assays in non-sterile wine incubated at low temperatures

The purpose of these assays was to evaluate the performance of UNQOe19 and UNQOe4 in non-sterile wine incubated at low temperatures, in the presence of the native microbial consortium (Fig. 4). The wine sample inoculated with UNQOe4 showed similar viable cell count values at 4, 10 and 21 °C, while the sample inoculated with UNQOe19 showed higher viable cell count values at 4 °C and similar values at 10 and at 21 °C. On day 20, the wine sample inoculated with UNQOe4 showed higher viable cell count values at 4, 10 and 21 °C than the wine sample inoculated with UNQOe19. On the other hand, the wine samples inoculated with UNQOe4 and UNQOe19 exhibited higher viable cell count values than non-inoculated wines, at both 4 and 10 °C. However, at 21 °C, the non-inoculated wines showed higher viable cell count values.

Regarding MAC values, at 20 days of incubation, these were lower than 50% in the wine sample inoculated with UNQOe4, at the three temperatures. In contrast, in the wine sample inoculated with UNQOe19, MAC values were 100% at 4 °C (at 15 days), 96% at 10 °C (at 20 days), and 91% at 21 °C (at 20 days) (Fig. 4). The MAC values in non-inoculated wine samples were lower than in wine samples inoculated with UNQOe19 or UNQOe4.

RAPD-PCR analysis with the M13 primer allowed evaluating the implantation capacity of strains in non-sterile wine samples. UNQQOe4 and UNQOe19 were able to successfully implant in wine at low temperatures and at 21 °C, according to the similar RAPD-PCR profiles marked with a black asterisk in Fig. 5. In the non-inoculated wine

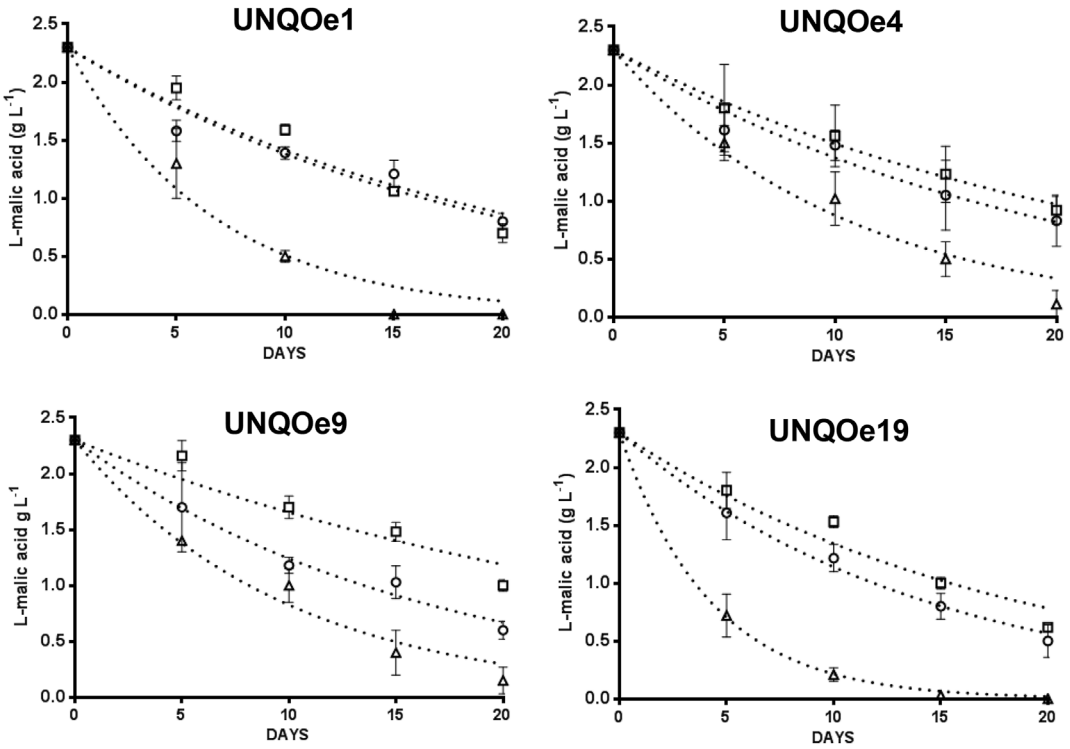

Fig. 3. L-malic acid consumption (MAC) kinetics exhibited by the *O. oeni* strains UNQOe1, UNQOe4, UNQOe9 and UNQOe19, incubated in sterile Pinot noir wine at 4, 10, and 21 °C. The dashed line represents MAC kinetics (g L^{-1}). Temperature symbols: 4. (fx2), 10 (fx3), and 21 °C (fx4). Three independent replicates were carried out.

samples, no profiles corresponding to UNQOe4 and UNQOe19 were observed (Fig. 5). It is important to note that, as the incubation temperature increased, the number of RAPD-PCR profiles corresponding to inoculated strains was higher (Fig. 5).

3.6. Screening of the hdc and ptcA genes

The *hdc* and *ptcA* genes, involved in the synthesis of the biogenic amines histamine and putrescine, respectively, were not detected in UNQOe4 or UNQOe19 (Fig. 6).

4. Discussion

In the North-Patagonia wine region, spontaneous MLF of red wines may occur at low environmental temperatures. Thus, the selection of native *O. oeni* strains able to perform this biological transformation at low temperatures becomes relevant. Vigentini, Picozzi, Tirelli, Giugni, and Foschino (2009) and Vigentini et al. (2016) showed that psychrotrophy is a phenotypic trait present in native *O. oeni* strains and that it may be possible to select some of these strains to manage MLF in cold climate territories, such as Aosta Valley in Italy, where this process is very difficult to control. Considering the problem of low environmental temperatures that can affect Patagonian cellars, we obtained and evaluated psychrotrophic *O. oeni* native strains from a Pinot noir wine. In contrast to that found by Vigentini et al. (2009), the *O. oeni* strain collection from Patagonian wines was obtained applying a different isolation strategy. It is possible that previous enrichment of the wine sample in addition to selection pressure of low incubation temperature

Table 1
Viable cell counts (cfu mL^{-1}) and kinetic parameters of MAC (Eq. 1) (obtained from Fig. 3) after 20 days incubation of UNQOe1, UNQOe4, UNQOe9, and UNQOe19 *O. oeni* strains in sterile Pinot noir wine.

Strains	AN	Temp (°C)	MAC (%)	N_0 (cfu ml^{-1})	Log N/N_0	K	R^2	[MA*i*]
UNQOe1	KY561608	4	65.22 ± 3.04 [a]	3.1E+07	−3.69	0.08 ± 0.02	0.9283	0.56
		10	69.56 ± 3.48[a]	3.1E+07	−3.69	0.05 ± 0.01	0.7916	0.00
		21	100[a]	3.2E+07	−2.34	0.15 ± 0.03	0.9544	0.00
UNQOe4	KY561600	4	63.91 ± 9.6[a]	1.3E+07	−3.36	0.07 ± 0.03	0.8794	0.34
		10	60 ± 5.20[a]	2.4E+07	−3.12	0.04 ± 0.03	0.8476	0.01
		21	95.22 ± 5,21[a]	1.7E+07	−2.18	0.10 ± 0.02	0.9491	0.01
UNQOe9	KY561596	4	73.91 ± 3.48[b]	5.0E+07	−4.54	0.06 ± 0.01	0.9183	0.00
		10	56.52 ± 2.17[a]	2.1E+07	−4.32	0.03 ± 0.01	0.8843	0.00
		21	93.48 ± 5.22[ab]	3.0E+07	−2.21	0.10 ± 0.02	0.9504	0.00
UNQOe19	KY561603	4	78.26 ± 6.09[a]	6.3E+07	−2.82	0.07 ± 0.02	0.9626	0.00
		10	78.26 ± 6.09[a]	2.1E+07	−2.62	0.05 ± 0.01	0.9519	0.00
		21	100[a]	5.1E+07	−3.40	0.24 ± 0.03	0.9930	0.09

AN: GenBank accession number; **MAC (%)**: Percentage of L-malic acid consumed after 20 days of incubation.; **N_0**: Number of viable cells at time = 0; **Log N/N_0**: Change in number of viable cells after 20 days of incubation.; **K**: Constant of first order exponential decay.; **R_2**: Coefficient of determination.; **[MA*i*]** : Minimum L-malic acid concentration (time = infinite).
Letters **a** and **b** denote statistically significant difference ($P < 0.05$).

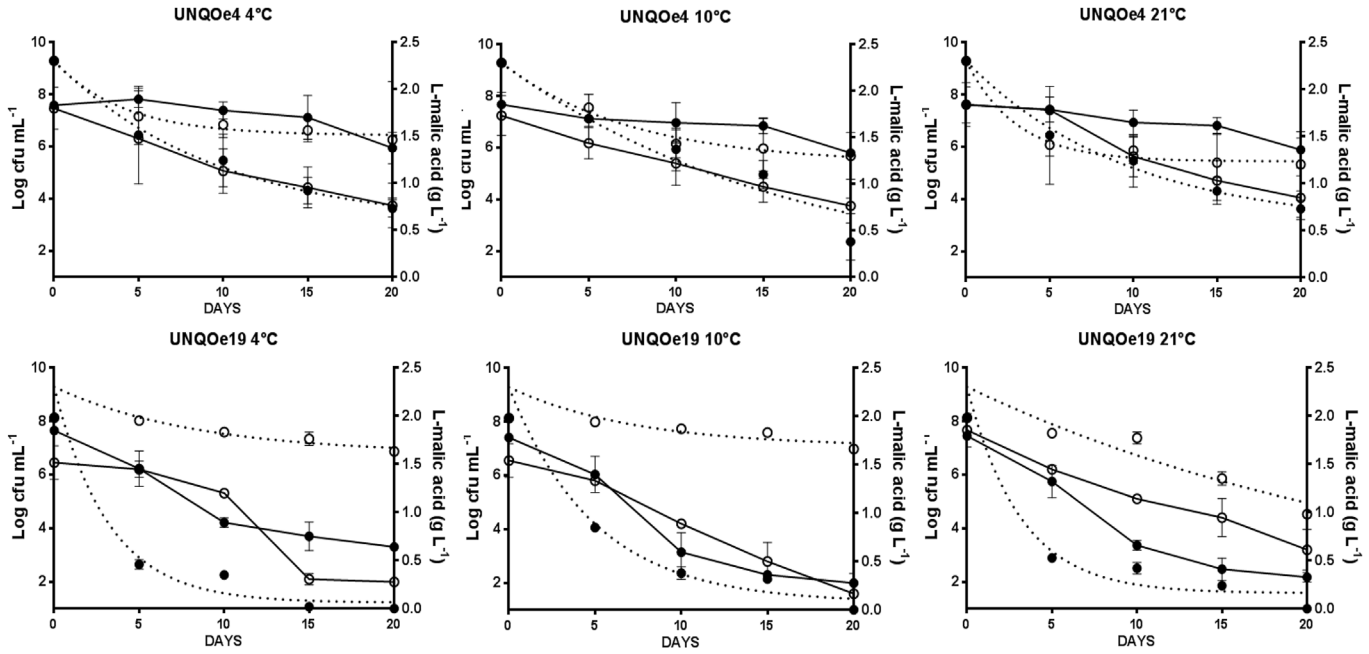

Fig. 4. Analysis of cell survival and MAC by UNQOe4 and UNQOe19 in non-sterile Pinot noir wine. The solid line denotes log cfu mL^{-1}, whereas the dashed line represents MAC kinetics (g L^{-1}). Full circles correspond to inoculated wine, whereas empty circles correspond to non-inoculated wine used as control. Three independent replicates were carried out.

allowed a substantial increase in the frequency of isolation of psychrotrophic LAB, suggesting that the conditions selected favored the development of this particular LAB population within the native wine microbiota. This phenomenon could be a consequence of the expression of genes related to environmental adaptation (Olguín, Valdes La Hens, Delfederico, & Semorile, 2018). The fully assembled genome of UNQOe19 (GenBank Accession Number CP027431) was obtained and stress response genes were detected (Iglesias et al., 2018). Further evaluation will help to elucidate the molecular and physiological bases of the stress response in UNQOe19.

Renouf, Vayssieres, Claisse, and Lonvaud-Funel (2009) confirmed that the genetic variability in the evolution of *O. oeni* populations during MLF can be affected by stress factors such as the ones occurring in oenological conditions. Although Olguín et al. (2018) have already obtained some promising results, currently in progress, the response to cold stress of Patagonian oenological LAB strains requires further studies on the molecular mechanisms involved.

We have previously performed a first analysis of five Patagonian *O. oeni* strains, demonstrating low tolerance to the wine environment (Bravo-Ferrada et al., 2016). These preliminary tests were carried out under optimal conditions (synthetic wine and 28 °C) and do not reflect similarity with the winery environment of the Patagonian region. In the present work, a collection of native Patagonian psychrotrophic *O. oeni* strains was obtained, and a technological and enological evaluation of them was carried out.

As a first criterion of selection of psychrotrophic *O. oeni* strains, cultures previously adapted to stress conditions were inoculated in sterile wine and incubated at low temperatures. The acclimation

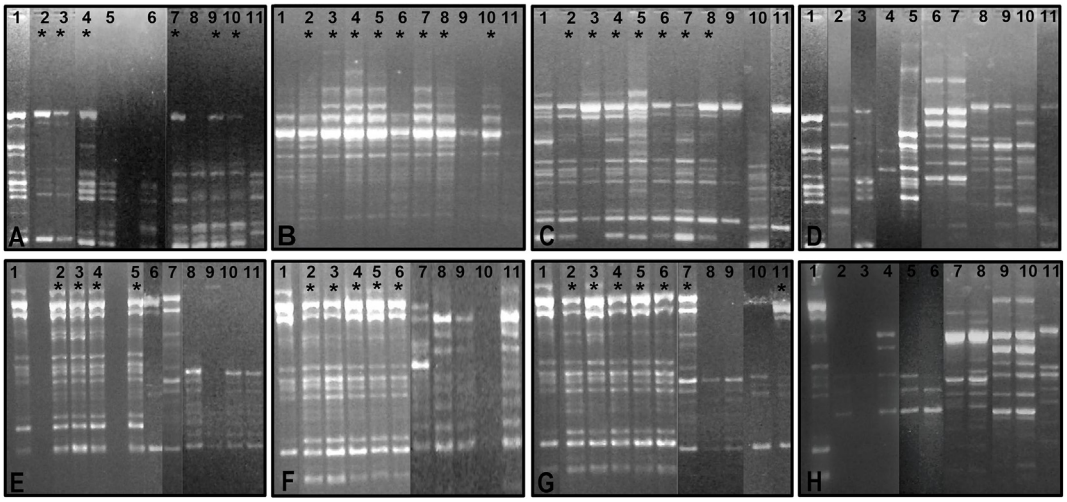

Fig. 5. Implantation capacity analysis of UNQOe4 and UNQOe19 in non-sterile Pinot noir wine. **A-H** depict RAPD-PCR profiles of the control strain in well 1 (UNQOe4: **A-D** and UNQOe19: **E-G**) and 10 colonies taken from wine (wells 2–11). **A**- Wine inoculated with UNQOe4, incubated at 4 °C. **B-** Wine inoculated with UNQOe4 incubated at 10 °C. **C-** Wine inoculated with UNQOe4 incubated at 21 °C. **D-** Non-inoculated wine incubated at 21 °C. **E-** Wine inoculated with UNQOe19 incubated at 4 °C. **F-** Wine inoculated with UNQOe19 incubated at 10 °C. **G-** Wine inoculated with UNQOe19 incubated at 21 °C. **H-** Non-inoculated wine incubated at 21 °C. Three independent replicates were carried out.

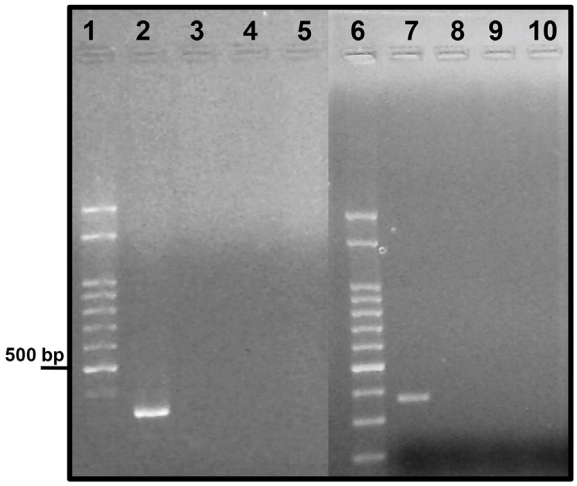

Fig. 6. Polymerase chain reaction gel electrophoresis to evaluate the presence of the *ptcA* and *hdc* genes in UNQOe4 and UNQOe19. **1** and **6**- 100-bp ladder, **2**-*Lactobacillus hilgardii* X1B strains as positive control for the *ptcA* (350 bp) gene fragment, **3-** UNQOe4, **4-** UNQOe19, **5-** Negative control (H_2O), **7**-*Lactobacillus buchneri* ST2A as positive control for the *hdc* (375 bp) gene fragment. **8-** UNQOe4, **9-** UNQOe19, **10-** Negative control (H_2O).

treatment was effective when only one sub-lethal factor was applied (6% ethanol v/v, 21 °C). When low temperature (6% ethanol v/v, 4 or 10 °C) was added, cells were not able to survive in the sterile wine. New acclimation treatments at sub-lethal low temperatures (15–18 °C) should be analyzed since it has been extensively demonstrated that the adaptation of *O. oeni* strains improves their cell survival and metabolic performances (Bravo-Ferrada et al., 2016; Bravo-Ferrada et al., 2014; Brizuela et al., 2017; Maicas, Pardo, & Ferrer, 2000).

Data of cell survival and MAC kinetics in sterile wine allowed selecting two strains, UNQOe4 and UNQOe19, to further study their performance in non-sterile wine. In sterile wine samples, both strains showed a good combination of cell viability and capacity of MAC. No correlation between phenotypic characteristics and genotypic clustering of *O. oeni* strains was found, suggesting that the strain collection obtained could be even more heterogeneous than that indicated by RAPD-PCR analysis (Vigentini et al., 2009) using the M13 primer. Also, Cappello, Stefani, Grieco, Logrieco, and Zapparoli (2008) and Solieri, Génova, De Paola, and Giudici (2010) found only a partial correspondence when considering genotypic data of *O. oeni* strains obtained by pulsed field gel electrophoresis, amplified fragment length polymorphism, and RAPD-PCR with the M13 and OPA2 primers, respectively) and metabolic performances.

In non-sterile wine samples inoculated with UNQOe19, and incubated at low temperatures, MAC values were higher than in wine samples inoculated with UNQOe4. Nonetheless, the number of viable cells was higher in the latter. The better performance of UNQOe19 suggests a good adaptation capacity to the wine conditions and a positive interaction with the native microbiota. Also, it would be interesting to evaluate whether this strain is also present in other grape varieties in the same cellar and if so, analyze its behavior in wines produced from it. The implantation capacity analysis in non-sterile wine showed that both UNQOe19 and UNQOe4 were able to survive in the presence of the native microbial consortium at the low temperatures assayed. The considerable thermal amplitude of the Patagonian region could favor the adaptation of mesophilic LAB in terms of their ability to consume MA at low temperatures, which would explain the better performance of those bacteria at 21 °C. This behavior was observed for all strains in sterile wine but only for UNQOe4 and UNQOe19 in the presence of native wine microbiota. Further studies are thus necessary to better interpret these results.

On the other hand, the absence of RAPD-PCR profiles corresponding to UNQOe4 and UNQOe19 in the non-inoculated wine samples suggests that these strains did not constitute a majority fraction of the native population. Vigentini et al. (2009) and Vigentini et al. (2016) reported that psychrotrophic *O. oeni* strains isolated from Aosta Valley wines were able to remain viable and consume MA in heat-treated wines, whereas, in the present study, the implantation and oenological properties of Patagonian strains were evaluated in non-sterile wine.

We consider that the novelty of this work lies on the combination of the isolation strategy used to obtain psychrotrophic LAB and the analysis of implantation capacity in the presence of the wine native microbiota. The results obtained allowed selecting two *O. oeni* strains with potential to be use as malolactic starter cultures in the Patagonian wine region.

Several *O. oeni* strains carry the *hdc* and *ptcA* genes, which are involved in the synthesis of histamine and putrescine, the biogenic amines present at highest concentrations in wines, being putrescine the most effective potentiator of the histamine toxicity to humans (Soufleros, Barrios, & Bertrand, 1998; Taylor & Eitenmiller, 1986). The results obtained in this work suggest that these *O. oeni* strains are not able to produce histamine and putrescine, and agree with the analysis of other Patagonian LAB strains in which genes related to the synthesis of biogenic amines were not detected (Bravo-Ferrada et al., 2016; Brizuela et al., 2017). However, further studies should be performed to search for genes involved in the synthesis of other biogenic amines, such as tyramine and cadaverine.

5. Conclusion

In this work, sixteen psychrotrophic *O. oeni* strains, which were able to remain viable and consume MA at low temperatures in sterile wine, were obtained from a Patagonian Pinot noir wine. No correlation between phenotypic characteristics and genotypic clustering was found, suggesting a high level of polymorphism in the *O. oeni* population.

Two strains of this collection (UNQOe19 and UNQOe4) were then selected to analyze their performance in non-sterile wine, in the presence of native biota. This analysis highlighted the behavior of UNQOe19 at low temperatures. Additional studies of strain adaptation should be carried out in the presence of combined sub-lethal stress factors (ethanol and temperature), as well as winemaking assays at pilot scale. A good management of MLF at low environmental temperatures, without the need to heat the fermentation tanks or cellar ambient, would be an innovative oenological practice in the North-Patagonia wine region.

Declarations of interest

None.

Acknowledgements

This work was funded by grants from Universidad Nacional de Quilmes, Argentina (Programa Microbiología Molecular Básica y Aplicaciones Biotecnológicas 2015–2019), Comisión de Investigaciones Científicas – Provincia de Buenos Aires, Argentina (CIC-PBA), Consejo Interuniversitario Nacional–Consejo Nacional de Investigaciones Científicas y Técnicas (CIN – CONICET) (PDTS CIN CONICET 2014 Nº 173), and Agencia Nacional de Promoción Científica y Técnica (ANPCyT), Argentina (PICT SU 2012 Nº 2804, PICT 2013 Nº 1481, PICT 2014 N° 1395). CM is a fellow of CIC-BA, Argentina. LS and DVH are members of the Research Career of CIC-BA; BMBF, NO and ET are members of the Research Career of CONICET.

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