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Prevalence of *Lentilacobacillus hilgardii* over *Lactiplantibacillus plantarum* in Low-Temperature Spontaneous Malolactic Fermentation of a Patagonian Pinot Noir

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Abstract: The spontaneous malolactic fermentation (MLF) in a centenary winery from Patagonia, Argentina, is conducted by predominantly mesophilic *Oenococcus oeni* and *Lactiplantibacillus plantarum*. In this region, MLF takes place from 14 to 4 °C, leading to heat cellars incurring in higher costs and non-sustainable practices. Previously, psychrotrophic strains of *O. oeni* had been obtained from a Patagonian wine. The goal of this work was to identify the Lactobacillaceae microbiota related to low-temperature MLF and assess their contribution. Nine sychrotrophicc *Lentilactibacillus hilgardii* strains were identified by sequencing the 16S rRNA gene, and the strains typified by RAPD-PCR. All strains consumed L-malic acid at 4 and 10 °C in sterile wine. The selected UNQLh1.1 strain revealed implantation capacity and L-malic acid consumption at 4 and 10 °C in the presence of the native microbial consortium. Furthermore, the histidine decarboxylase (*hdc*) gene was not detected in any of the *Len. hilgardii* strains. The prevalence of *Len. hilgardii* under low-temperature conditions represents a novelty compared to previous findings of LAB diversity in the MLF of Patagonian wines. The native Patagonian psychrotrophic *Len. hilgardii* strains are a new player in fermentations conducted at low temperatures with the potential to be used as a sustainable MLF starter.

Keywords: *Lentilactobacillus hilgardii*; psychotropic strains; malolactic fermentation; wine implantation; starter cultures; Patagonian wine

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1. Introduction

Winemaking involves complex microbial bioconversions in which malolactic fermentation (MLF) can occur during or after alcoholic fermentation (AF) [1,2]. MLF can occur spontaneously, through indigenous LAB metabolic activity, or can be induced by a commercial starter culture due to the unpredictability of the process. In addition to L-malic acid (MA), many other compounds present in wine are metabolized by the best-adapted LAB, guaranteeing their viability. As a result, metabolic products are species- and strain-dependent, which could positively or negatively impact the sensory qualities of wine [1,3,4]. Although the diversity of LAB during MLF is wide, *Oenococcus oeni* has

been the main LAB isolated from winemaking due to its tolerance to hostile wine conditions [5–7]. Also, it has been the most widely used microbial resource to develop malolactic starters to guarantee wine quality and safety [8–10]. However, the availability of next-generation sequencing technologies has made it possible to detect new and long-known LAB species, with potential applications as specific starters for each terroir [11–14]. The heterofermentative capacity of mainly *O. oeni*, *Levilactobacillus brevis*, *Lactiplantibacillus plantarum*, and *Lentilactobacillus hilgardii* to catabolize residual wine nutrients positions them as the main dominant species of MLF [15–17]. Accordingly, some strains of *Lpb. plantarum* and *Len. hilgardii*, although less popular but oenologically promising, have attracted attention [15,18–22]. Recently, a native strain, *Len. hilgardii* Q19 from China, was used to carry out MLF at 15–18 °C in Cabernet Sauvignon wine [23]. Moreover, autochthonous strains of *Lacticaseibacillus paracasei* and *Len. hilgardii* from Albariño wines have been suggested as good candidates for malolactic starter culture [24].

In Northern Patagonia, Argentina, spontaneous MLF takes place at environmental temperatures that can eventually descend to 4 °C. Regional wineries counteract this by keeping LAB growth at 20–22 °C, which involves heating the cellar environment and, in turn, raising the production costs. At 20–22 °C, the dynamics and diversity of LAB associated with the spontaneous MLF of Pinot noir and Merlot Patagonian wines have shown numerous LAB species detected during MLF; however, *Lpb. plantarum* and *O. oeni* prevail as the dominant species [19,25–27]. The Pinot noir and Merlot varietals develop well in this cold climate region, displaying a balanced alcohol content (12 and 13%) with a very marked fruit intensity [28]. Contrary to other cool-climate countries, the acidity of grape must often be significantly enhanced, and the concentration of aroma compounds is considerably low [29]. The growing interest in the management of MLF in cool-climate regions encouraged the isolation of a native psychrotrophic *O. oeni* strains collection from a Patagonian Pinot noir wine [30], and the evaluation of the relative expression of stress genes [31] has revealed differences from the diversity of LAB previously described for Patagonian MLF developed at 20–22 °C [19,20,26–28,32,33]. Thus, the aim of this work was to isolate and evaluate the oenological properties of the Lactobacillaceae species involved in the low-temperature MLF and to assess their contribution to this process. Furthermore, we aimed to evaluate the impact of low temperatures on the viability and MA consumption of the previously reported mesophilic Patagonian *Lpb. plantarum* native collection strains.

2. Materials and Methods

2.1. Wine Samples

Wine samples of Pinot noir (14.3% (*v*/*v*) ethanol, pH 3.7, 2.3 g/L MA) and Malbec (12.4 % (*v*/*v*) ethanol, pH 3.6, 2.8 g/L), vintage 2014, were aseptically collected at the end of spontaneous AF in General Roca, Argentinean Northern Patagonian. In the cellar studied, the winemaking process began with a cold pre-fermentation for 48–72 h, followed by AF in 9000 L concrete tanks for 20 days and MLF for 25–40 days afterward. Finally, the wine underwent aging in French oak barrels for seven months.

2.2. Lactiplantibacillus p*lantarum Strains Collection*

To evaluate the effect of low temperatures on the *Lpb. plantarum* strains previously studied in the Patagonian region, the following strains were used for comparison purposes: *Lpb. plantarum* strains UNQLp11, complete genome GenBank Accession Numbers CP031140 [34], UNQLp22, UNQLp27, and UNQLp12.a (GenBank Accession Numbers KU693340, KU693341, KU985242, and KU985241, respectively) obtained from Pinot noir wine samples vintage 2012 [26]. Also, *Lpb. plantarum* strain UNQLp65.3, UNQLp133, and UNQLp155 (GenBank Accession Numbers KC670960, KC679064, and KC679067, respectively) obtained from Pinot noir wine samples, vintage 2008, were used [19].

2.3. Isolation of Psychrotrophic Lactobacillaceae Strains

To obtain psychrotrophic Lactobacilaceae isolates, 200 mL of wine samples were incubated at 8–10 °C for 30 days until MA was exhausted (0 g/L). Two isolation strategies were used: (1) Aliquots of 100 μL were directly plated into MRS agar (Biokar, Allonne, France) [35] 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 MRS broth (Biokar, France) and incubated under microaerophilic conditions. Samples of wine incubated at 8–10 °C were taken at 0, 5, 10, 15, 20, and 30 days, then spread in MRS agar and incubated at 8–10 °C. Isolates were evaluated by a Gram-stained catalase test and were stored at −80 °C in MRS broth with glycerol 30 % (*v*/*v*) (Biopack Bs. As., Buenos Aires, Argentina). In both isolation strategies, the MRS used were supplemented with 15% (*v*/*v*) tomato juice, cysteine 0.5 g/L, MA 4.5 g/L, cycloheximide 0.01% (*w*/*v*) (Sigma, St. Louis, MI, USA), nystatin 20 mg/mL, and sodium azide 0.01% (*w*/*v*) at pH 4.8.

2.4. Identification of Psychrotrophic Isolates

Genomic DNA was extracted according to [25]. DNA samples were quantified with a Nanodrop spectrophotometer (Thermo Scientific, 1000, Waltham, MA, USA) and visualized on a 1.0% (*w*/*v*) agarose gel. For isolate identification, the 16S rRNA gene was amplified using the primers pA-16SF (5′-AGAGTTTGATCCTGGCTCAG-3′) and pH-16SR (5′-AAGGAGGTGATCCAGCCGCA-3′) [36]. Gene products were sequenced by Macrogen (Seoul, South Korea) and analyzed by comparison with sequences deposited at GenBank (NCBI, NIH). All sequences obtained were submitted to GenBank (www.ncbi.nlm.nih.gov/genbank (accessed on 12/03/2017)).

2.5. Typification by Random Amplification of Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR)

The DNA of LAB isolates was analyzed by RAPD-PCR using the single-primer M13 (5′-GAGGGTGGCGGTTCT-3′) [37]. The estimation of fragment lengths was resolved by comparison to a 100 bp ladder marker as a size standard (Productos Bio-Lógicos, Universidad Nacional de Quilmes, Bernal, Buenos Aires, Argentina). The amplification products were resolved by electrophoresis on a 2% (*w*/*v*) agarose gel. RAPD-PCR pattern images, obtained with Kodak Digital Science V.3.0.2 (Eastman Kodak Company, Rochester, NY, USA), were evaluated by calculating the genetic similarity index using a simple matching coefficient [38]. The cluster analysis through the unweighted pair group method using arithmetic averages (UPGMA) was carried out with PAUP* software 4.0b10 (Sinauer Associates, Sunderland, MA, USA).

2.6. Cell Culture Acclimation

Cultures of psychrotrophic isolates and *Lpb. plantarum* strains were harvested at the early stationary phase (10^9 CFU/mL) by centrifugation at 5000× *g* for 10 min and suspended in the same volume of a modified acclimation medium (50 g/L MRS, 40 g/L D (−) fructose, 20 g/L D (−) glucose, 4 g/L L-malate, 1 g/L Tween 80, 0.1 mg/L pyridoxine, pH 4.6) supplemented with 6% *v*/*v* ethanol; culture incubations were carried out at 21 °C for 48 h [39].

2.7. Fermentation at Low Temperatures in Sterile Wine at a Laboratory Scale

As a first screening of the oenological aptitudes at low temperatures of *Lpb. plantarum* strains and psychrotrophic isolates obtained in this work, the viability and L-malic acid consumption (MAC) were evaluated in sterile wine. The acclimated cells of the strains obtained in this work, as well as the control *Lpb. plantarum* strains obtained previously, were harvested by centrifugation. Cells were suspended in 10 mL of sterilized Pinot noir wine at the final stage of AF using 0.2 μm pore size filtration (Sartorius Stedim Biotech Gmbh, Göttingen, Germany) to obtain a final concentration of ~ 2×10^7 CFU/mL. The cell

viability was monitored by plate count in an MRS agar at 4, 10, and 21 °C (control), for 20 days, taking samples at 0, 5, 10, 15, and 20 days.

2.8. Fermentation and Implantation at Low Temperatures in Non-Sterile Wine

Psychrotrophic LAB strains were previously acclimated (6% ethanol *v*/*v*, 21 °C), and 1 mL was inoculated in 10 mL of non-sterile Pinot noir wine at ~ 2 × 10^7 CFU/mL cell density and incubated at 4, 10, and 21 °C as a control. Cell survival was analyzed every 5 days until the 20th day by plate count on MRS plates [35]. Non-inoculated wine samples were also used as a control of fermentation.

The implantation ability of the selected strain was determined by comparing the RAPD-PCR profiles of 10 colonies randomly taken from MRS plates inoculated with each wine sample, which had been previously inoculated in non-sterile Pinot noir wine and incubated for 20 days at 4, 10, and 21 °C [30].

2.9. L-Malic Acid Consumption (MAC)

The remaining MA was measured during the fermentation assay in sterile and non-sterile wine (L-Malic Acid Oenology Enzymatic kit, BioSystems SA, Barcelona, Spain). An exponential one-phase decay equation model obtained through the Graph Pad Prism® software 8.0.1 version was used to fit the MAC kinetics of the different strains tested [26,30].

The percentage of MA consumed (MAC %) after 20 days of incubation was calculated according to the equation:

$$\%MAC = 100 - (100 * \frac{[MAf]}{[MA0]})$$

where [*MA0*] is the initial concentration of MA in the wine sample, and [*MAf*] is the final MA concentration measured after 20 days of wine incubation [26].

2.10. Screening of the hdc Gene

We screened for the presence of the histidine decarboxylase (*hdc*) gene using the primers JV16Hc and JV17Hc [40] in the Lactobacillaceae isolates to amplify a fragment of 375 bp. The *Lentilactobacillus buchneri* ST2A strain was used as a positive control.

2.11. Reproducibility Assay

All the determinations were made on 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 by the Low-Temperature Selection Method

To obtain oenological Lactobacillaceae isolates able to grow at low temperatures, two isolation strategies were employed (see Section 2.3). Using strategy (1), no LAB isolates were obtained from Malbec or Pinot noir wines. On the other hand, although strategy (2) yielded no LAB isolates from Malbec wine, 15 psychrotrophics were isolated from Pinot noir wine on MRS agar incubated at 8 to 10 °C after 20 days of MLF being underway. Nine isolates from Pinot noir wine samples were presumptively identified as potential Lactobacillaceae species. Two colony morphologies were detected: the first had a diameter of 4–5 mm, a light brown color, irregular margins, a raised center, and dry texture, whereas the second morphology had a size of 2–3 mm in diameter, a cream to golden color, with entire margins, a concave surface, and smooth texture. The ropy phenotype was not detected in any of the isolates. The isolates were described as presumptive

members of the Lactobacillaceae family due to their rod-shaped morphology, Gram-positive staining, non-sporulation, and catalase-negative reaction. The low-temperature range (8–10 °C) for LAB isolation was decided according to the climatic temperatures that Patagonian LAB must frequently withstand during the harvest and winemaking process, and 21 °C has been the temperature used for acclimation in previous work [39].

3.2. Identification of Psychrotrophic Isolates

The nine Lactobacillaceae isolates were identified by multiple sequence analysis of the 16S rRNA gene with sequences deposited at GenBank. Using the BLAST match of 96 to 99% of genetic similarity between the nine isolates, they were classified as *Len. hilgardii*. The isolates were named UNQLh4.2, UNQLh4.1, UNQLh3.2, UNQLh3.1, UNQLh2.3, UNQLh2.1, UNQLh1.2, UNQLh1.1, and UNQLh1.3 (GenBank Accession Numbers, KY561610, KY561611, KY561612, KY561613, KY561614, KY561615, KY561617, KY561618, and KY561616, respectively).

3.3. Typification by RAPD-PCR Analysis

The genetic heterogeneity between *Len. hilgardii* strains were assessed by numerical analysis of the DNA electrophoretic profiles performed by RAPD-PCR using the M13 primer (Figure 1). Considering an arbitrary percentage similarity of 86.5% *Len. hilgardii*, isolates were differentiated into five biotypes and grouped into four clusters. Cluster I, II, and IV contained two isolates each, with 100% similarity to each other. Cluster I was composed of the UNQLh1.1 and UNQLh2.1 strains, and cluster II was composed of the UNQLh3.1 and UNQLh2.3 strains. Cluster III included three strains, of which UNQLh4.1 and UNQLh1.2 showed 100% similarity, while the UNQLh3.2 strain showed genetic variability. Finally, Cluster IV grouped the closely related UNQLh4.2 and UNQLh1.3 strains.

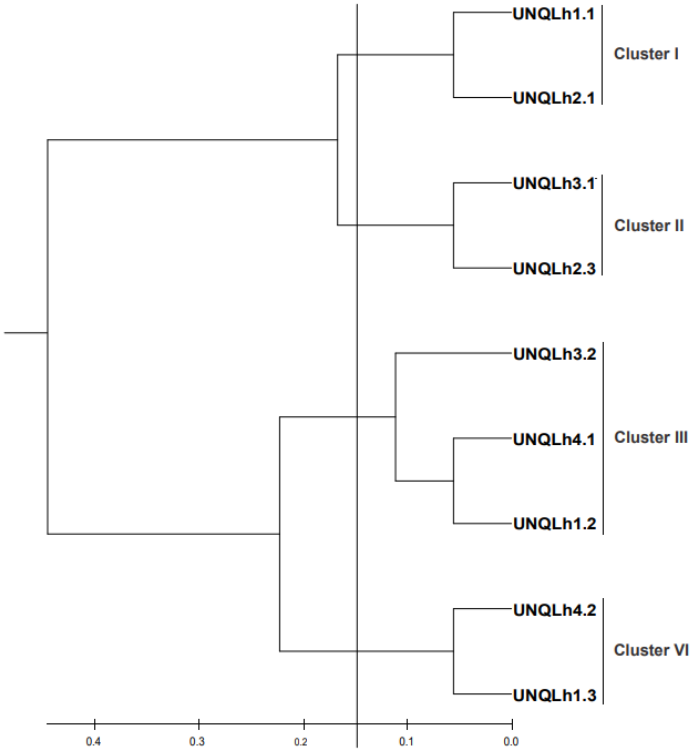

Figure 1. Dendrogram based on the Unweighted Pair Group Method using Arithmetic Averages (UPGMA) clustering of RAPD M13 patterns corresponding to nine *Len. hilgardii* strains. The vertical line indicates 86.5% of similarity.

3.4. Fermentation Assays in Sterile Pinot Noir Wine at Laboratory Scale

Fermentation assays at a laboratory scale were performed at 4, 10, and 21 °C in sterile Pinot noir wine, aiming to evaluate the viability and MAC values of *Len. hilgardii* strains using the *Lpb. plantarum* mesophilic strains as control.

Cultures of *Len. hilgardii* and *Lpb. plantarum* strains were successfully acclimated at a sub-lethal concentration of 6% (*v*/*v*) ethanol and incubated at 21 °C for 48 h to improve cell survival before being inoculated in sterile Pinot noir wine. The number of viable cells inoculated at a concentration of ~ 2×10^7 CFU/mL and residual MA values exhibited by *Len. hilgardii* and *Lpb. plantarum* strains at 20 days of incubation at 4, 10, and 21 °C in sterile Pinot noir wine are displayed in Figure 2A,B.

The viability values of *Len. hilgardii* strains showed relatively homogeneous results at all tested temperatures in the range of ~10^5–10^6 CFU/mL; however, the *Lpb. plantarum* strains showed heterogeneous viability values at the temperatures tested. The higher viability values at 4 and 10 °C were detected for the *Len. hilgardii* strains, while at 21 °C, higher viability values were detected for some of the *Lpb. plantarum* strains. The cell survival of *Len. hilgardii* viability was favored at low temperatures, while the *Lpb. plantarum* was negatively affected by the low incubation temperatures (Figure 2A).

The strains UNQLh1.1, UNQLh2.3, UNQLh4.1, and UNQLh4.2 showed similar viability performances (3×10^5–7×10^5 CFU/mL) at 4 and 10 °C. The strain UNQLh2.1 showed the highest viability value (5×10^5 CFU/mL) at 4 °C, and UNQLh4.1, UNQLh3.2, UNQLh1.3, and UNQLh2.3 displayed their best viability values (5×10^5–7×10^5 CFU/mL) at 10 °C. The strain UNQLh1.2 showed major viability at 21 °C, and no difference in viability at all tested temperatures was observed for UNQLh3.1.

Residual MA values for *Len. hilgardii* and *Lpb. plantarum* after 20 days at 4, 10, and 21 °C in sterile Pinot noir wine (MA initial content of 2.3 g/L) are exhibited in Figure 2B. Residual MA detected at the three temperatures for *Len. hilgardii* strains were highly heterogeneous. The strains of *Len. hilgardii* and *Lpb. plantarum* exhibited the highest MAC at 21 °C; however, at this temperature, the strains of *Len. hilgardii* exceeded ranging from 0.11 to 0.55 g/L for the values of MAC detected for the strains of *Lpb. plantarum*.

At 10 °C, residual MA values of *Len. hilgardii* were detected in a range from 0.45 to 0.95 g/L. The strains UNQLh2.3 and UNQLh4.1 showed the same values (0.54 g/L), UNQLh2.1 and UNQLh1.2 also recorded equal residual MA (0.65 g/L) values, and the lowest (0.95 g/L) MAC was recorded for UNQLh4.2. Moreover, at 4 °C, MAC was detected in a range of 0.15–0.66 g/L. The strain UNQLh3.1 exhibited the greatest MAC capacity, exhibiting values of residual MA of 0.15 g/L. On the other hand, UNQLh2.3, UNQLh3.2, and UNQLh1.2 displayed equal residual MA (0.52 g/L), whereas UNQLh4.1 showed the lowest (0.65 g/L) MAC.

In contrast, the decreased efficiency in the MA performance of *Lpb. plantarum* strains was directly related to the decrease in fermentation temperatures. At 21 °C, the residual MA was detected in a range of 0.24–0.88 g/L, with UNQLp11 showing the highest MAC and UNQLp65.3 the lowest. Residual MA at 10 °C was detected in a range of 0.78–0.98 g/L, where UNQLp133 exhibited the best MAC and UNQLp65.3 had the lowest values. Moreover, residual MA at 4 °C was detected in a range of 0.84–1.09 g/L, in which UNQLp22, UNQLp27, and UNQLp65.3 exhibited the highest MAC and UNQLp155 had the lowest values.

There was no correlation between the cell viability of *Len. hilgardii* strains and MAC, as the best cell survival record was found at low temperatures; however, the best MAC was detected at 21 °C. On the contrary, there was a correlation for the strains of *Lpb. plantarum* between viability and MAC at 21 °C, but this correlation did not hold at low temperatures. The strains UNQLp11 and UNQLp12a showed greater viability at 4 °C than at 10 °C, whereas MAC was higher at 10 °C than at 4 °C, revealing a strong strain-dependent behavior.

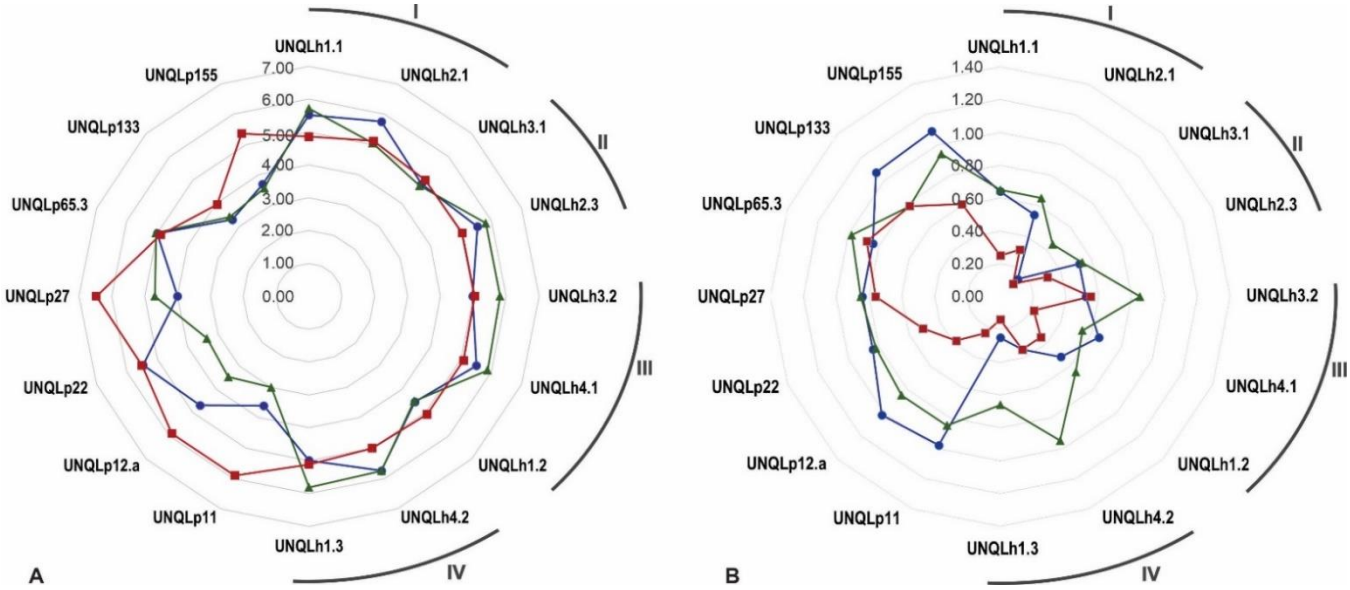

Figure 2. Cell survival (log CFU/mL) (**A**) and L-malic acid residual concentration (g/L) (**B**) after inoculation of *Len. hilgardii* and *Lpb. plantarum* strains in sterile Pinot noir wine incubated at 4 °C (Blue ●), 10 °C (Green ▲), and 21 °C (red ■), for 20 days. Roman numerals represent the distribution of strains according to (UPGMA) clustering of RAPD M13 patterns corresponding to *Len. hilgardii* strains.

3.5. Cell Viability and MAC Kinetics Analysis

The evolution of cell viability and MAC kinetics of the *Len. hilgardii* and *Lpb. plantarum* strains were studied. Figure 3 shows the evolution of cell viability and MAC of the *Len. hilgardii* strains incubated for 20 days at 4, 10, and 21 °C. The number of viable cells remained relatively constant at all temperatures tested during the first 5 days, except for the UNQLh1.2 strain, which decreased by 1 log on day 5 of incubation, suggesting low adaptive capacity.

At 21 °C, the viability of UNQLh1.1, UNQLh2.1 UNQLh2.3, and UNQLh4.1 remained constant until day 10, when a decrease was observed. However, the viability at 21 °C decreased slowly for the rest of the strains from day 5.

At 4 and 10 °C, UNQLh1.1, UNQLh2.3, UNQLh4.2, and UNQLh4.1 showed a slow decrease in viability until day 10 and then remained constant. The rest of the strains incubated at these temperatures showed a slow decrease in viability until day 15 and then remained constant. This suggests a slow adaptation to the wine environment at low incubation temperatures, so acclimation conditions before wine inoculation should be studied in more detail. However, at low temperatures, the viability values at day 20 of all *Len. hilgardii* strains, except UNQLh1.2, equaled or exceeded those recorded for the strains incubated at 21°C.

The MAC kinetics of the *Len. hilgardii* strains were fit for the one-phase exponential decay model (Figure 3). All strains of *Len. hilgardii* incubated at 21 °C except UNQLh3.2 and UNQLh4.2 consumed almost all the MA during the first 5 days, after which it remained constant. On the other hand, at 4 and 10 °C, the MAC occurred slowly and progressively, reaching the highest values of consumption at day 20 and with a tendency to continue the MAC (Figure 3). Therefore, in future MAC evaluations at low temperatures, sampling should be carried out beyond day 20, and other acclimatization conditions should be tested.

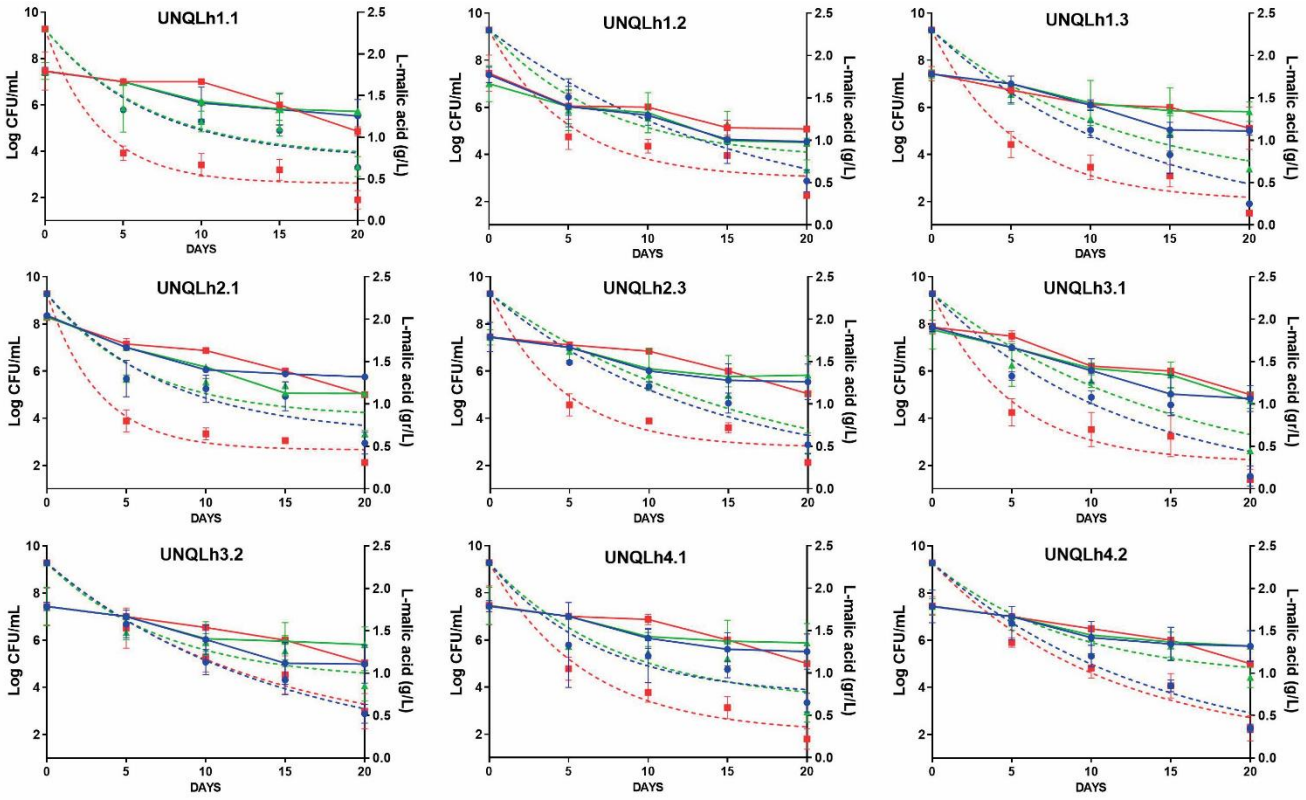

Figure 3. Cell survival (log CFU/mL) (solid line) and MAC (g/L) kinetics (dashed line), exhibited by *Len. hilgardii* strains incubated in sterile Pinot noir wine at 4 °C (blue ●), 10 °C (green ▲), and 21 °C (red ■).

The highest values of MAC % after 20 days were recorded at 21 °C and were greater than ~85% for all strains of *Len. hilgardii*, with UNQLh3.1 reaching a maximum MAC % of 95%.

Interestingly, at 4 °C, the MAC % values were higher than ~72%, with the UNQLh3.1 strain reaching a remarkable maximum of ~93%. On the other hand, MAC % at 10 °C was higher than 59% for all strains, with the UNQLh3.1 strain once again being the most efficient MA consumer, reaching a maximum MAC % value of 80% (Table 1).

Table 1. Kinetics parameter of malic acid consumption of *Len. hilgardii* strains evaluated with an exponential one-phase decay method at 20 days of inoculation in Pinot noir sterile wine.

	UNQLh1.1			**UNQLh1.2**			**UNQLh1.3**		
	4 °C	**10 °C**	**21 °C**	**4 °C**	**10 °C**	**21 °C**	**4 °C**	**10 °C**	**21 °C**
N_0 (CFU/mL)	2.8×10^7	2.8×10^7	3.0×10^7	2.4×10^7	2.6×10^7	2.8×10^7	2.6×10^7	2.0×10^7	2.7×10^7
MAC (%)	72.17 ± 1.30 ^{c}	71.30 ± 5.21 ^{b}	89.13 ± 5.22 ^{cb}	79.17 ± 5.65	71.30 ± 5.22 ^{d}	87.13 ± 1.30 ^{d}	89.13 ± 6.09	71.30 ± 6.09 ^{a}	93.91 ± 0.87 ^{a}
K	0.15 ± 0.06	0.15 ± 0.06	0.29 ± 0.09	0.15 ± 0.06	0.15 ± 0.07	0.29 ± 0.09	0.08 ± 0.02	0.08 ± 0.03	0.19 ± 0.06
R^2	0.937	0.9364	0.9682	0.937	0.9364	0.9682	0.9693	0.9724	0.9666
[MAi]	0.73	0.76	0.45	0.73	0.76	0.45	0.00	0.41	0.28

	UNQLh2.1			**UNQLh2.3**			**UNQLh3.1**		
	4 °C	**10 °C**	**21 °C**	**4 °C**	**10 °C**	**21 °C**	**4 °C**	**10 °C**	**21 °C**
N_0 (CFU/mL)	2.7×10^7	2.70×10^7	2.6×10^7	3.0×10^7	2.7×10^7	2.8×10^7	2.8×10^7	2.8×10^7	3.0×10^7
MAC (%)	76.52 ± 5.65	71.74 ± 1.74 ^{c}	86.52 ± 0.43 ^{c}	77.82 ± 4.87	76.52 ± 5.22	86.52 ± 0.87	93.48 ± 5.21	80.43 ± 1.30 ^{c}	95.22 ± 5.22 ^{c}
K	0.13 ± 0.07	0.17 ± 0.10	0.31 ± 0.08	0.07 ± 0.03	0.06 ± 0.01	0.22 ± 0.07	0.08 ± 0.02	0.06 ± 0.03	0.20 ± 0.07
R^2	0.9117	0.8751	0.9814	0.9649	0.9626	0.9606	0.9029	0.915	0.95
[MAi]	0.63	0.85	0.46	0.11	0.00	0.48	0.00	0.00	0.31

N_0: Initial number of viable cells at time = 0. **MAC (%):** Percentage of L-malic acid consumed after 20 days of incubation. **K:** Constant of first exponential decay. **R^2:** Coefficient of determination. **[Mai]:** Minimum L-malic acid concentration (time = infinity). Different letters (a, b, and c) denote statistically significant differences between the treatments at 4 °C and 10 °C compared to the control at 21 °C with a value of $p < 0.05$ (a), $p < 0.01$ (b), $p < 0.001$ (c), and $p < 0.0001$(d), 2-way ANOVA.

Conversely, the MAC kinetics of the *Lpb. plantarum* strains did not fit the one-phase exponential decay model (Figure 4). Viability at 21 °C for UNQLp11, UNQLp27, UNQLp22, UNQLp12, and UNQLp155 remained constant until day 15, after which it slowly decreased, while the viability of UNQLp133 decreased gradually until reaching three orders of magnitude.

At low temperatures, the kinetics of viability showed a gradual decrease in several orders of magnitude except for the strain UNQLp65.3, which gradually decreased one order of magnitude. The strains UNQLp11, UNQLp22, and UNQLp12a showed better adaptation at 4 °C than at 10 °C. Unlike what was observed for *Len. hilgardii*, at low temperatures, the viability of the *Lpb. plantarum* strains decreased progressively during at least the first 10–15 days of incubation. However, unlike *Len. hilgardii*, the MAC at low temperatures by *Lpb. plantarum* strains started slowly after the first 5 days of incubation, which suggests a low capacity for adaptation to low temperatures (Figure 4).

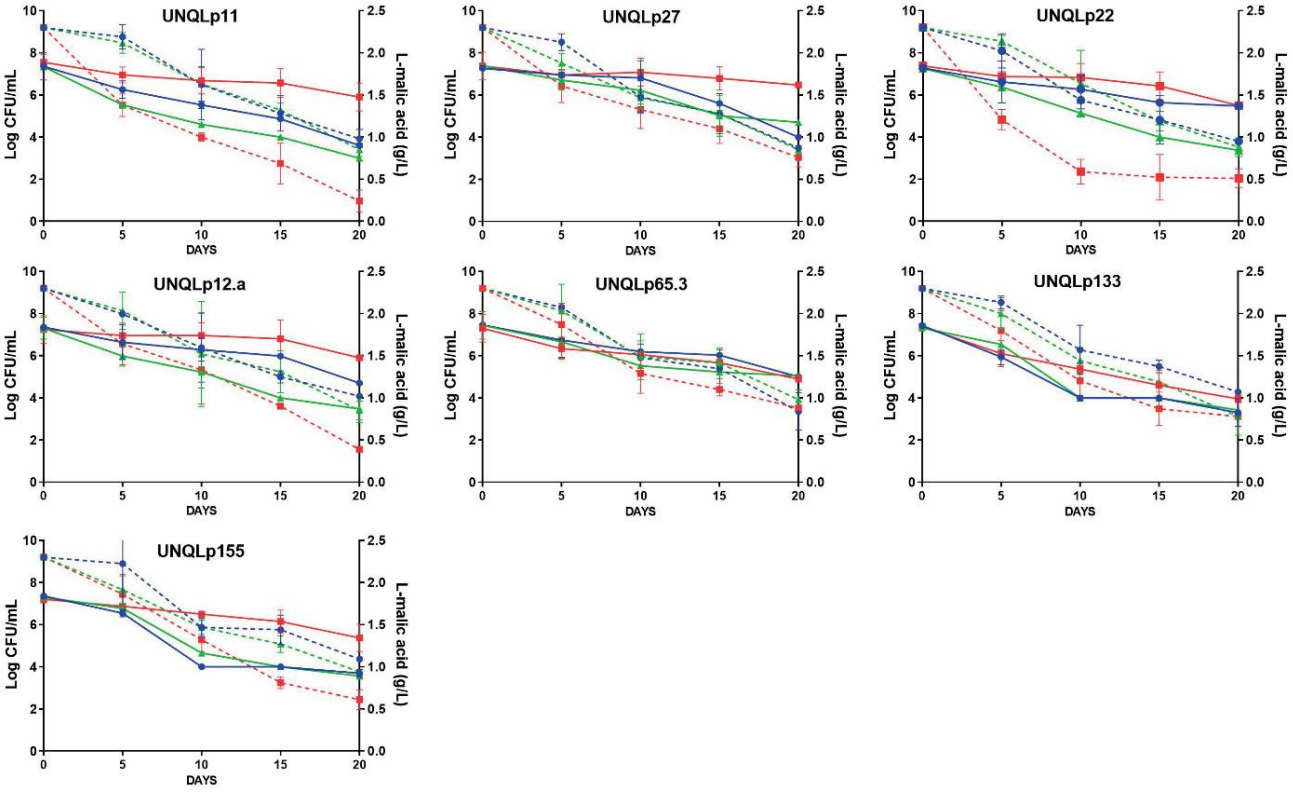

Figure 4. Cell survival (log CFU/mL) (solid line) and MAC (g/L) kinetics (dashed line) exhibited by *Lpb. plantarum* strains incubated in sterile Pinot noir wine at 4 °C (blue ●), 10 °C (green ▲), and 21 °C (red ■).

3.6. Implantation Capacity at Low Temperatures in Non-Sterile Wine

The strain UNQLh1.1 was selected to evaluate the implantation capacity in non-sterile wine because UNQLh1.1 showed similar values of viability and MAC at 4 and 10 °C, and viability values at lower temperatures significantly exceeded those registered at 21 °C.

The inoculation in non-sterile Pinot noir wine aimed to evaluate the implantation capacity of a psychrotrophic native strain in the presence of the wine microbial consortium by analyzing the viability and consumption of MA at low temperatures (Figure 5).

The wines inoculated and incubated at 4 and 10 °C showed similar viability kinetics as well as similar viability values of ~10^5 CFU/mL at day 20. However, the viability at 21 °C of the inoculated wine decreased to ~10^4 CFU/mL at day 20. In the control uninoculated wine incubated at 4 and 10 °C, the viable count of the native LAB decreased to 10^3–10^4 CFU/mL. Meanwhile, at 21 °C, the viability values exhibited a slow decrease until reaching values of ~10^6 CFU/mL.

At 4 °C, the kinetics of MAC of the inoculated wines showed that the MA decreased rapidly during the first 5 days until 0.5 g/L residual MA remained; then, almost all the MA was consumed at day 20. In the wines incubated at 10 and 21 °C, we observed that the MA was fully consumed at 20 days of incubation. At 4 and 10 °C, the consumption of MA in uninoculated wines was affected by low temperatures, reaching residual MA values of 1.5 g/L. The consumption kinetics at 21 °C showed a slow decrease, reaching residual MA values of 1.3 g/L at 20 days of incubation. These results suggest that both the viability and MAC in wines inoculated and incubated at low temperatures may have been due to the successful implantation of the selected strain UNQLh1.1 together with the native biota.

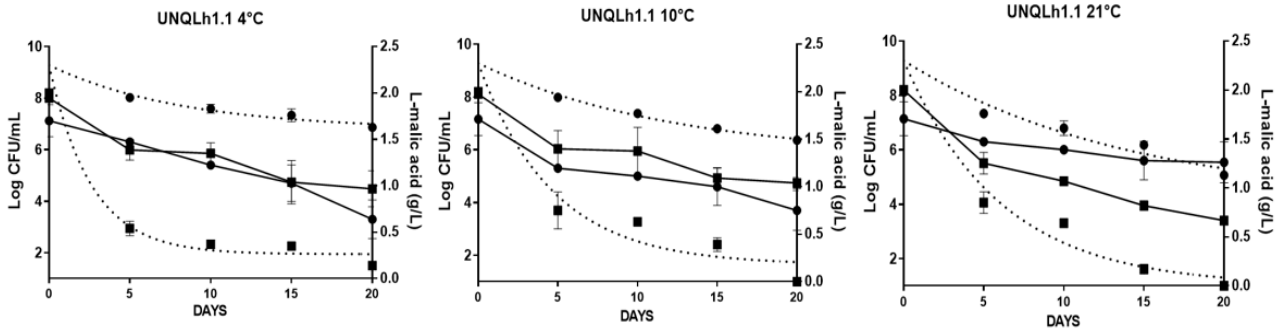

Figure 5. *Len. hilgardii* UNQLh1.1 strain analysis of cell survival (log CFU/ mL) (solid line) and MAC (g/L) (dashed line) in non-sterile Pinot noir wine. Circles (●) correspond to non-inoculated wine used as control and squares (■) to inoculated wine.

3.7. Implantation Capacity by RAPD-PCR

RAPD-PCR analysis with the M13 primer allowed the evaluation of the implantation capacity of UNQLh1.1 strains in non-sterile wine samples. Figure 6 shows that the UNQLh1.1 strain was able to successfully implant at all temperatures tested: 100% implantation was observed at 4 and 10 °C (Figure 6A,B), respectively, and 75% implantation was observed at 21 °C (Figure 6C). On the other hand, we could not detect RAPD-PCR profiles similar to those of the UNQLh1.1 strain in non-inoculated wine incubated at 21 °C (Figure 6D), suggesting that its predominance in the native population is low.

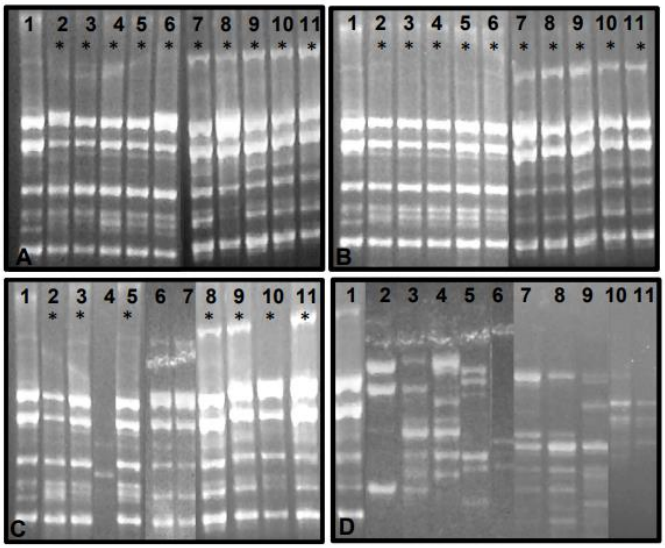

Figure 6. Implantation capacity of the UNQLh1.1 *Len. hilgardii* strain in non-sterile Pinot noir wine. Wells 1 (**A**–**D**) depicts RAPD-PCR profiles of UNQLh1.1 control strain. Wells 2–11 (**A**–**D**) show 10 colonies taken from wines: (**A**) Wine inoculated with UNQLh1.1, incubated at 4 °C. (**B**) Wine inoculated with UNQLh1.1, incubated at 10 °C. (**C**) Wine inoculated with UNQLh1.1, incubated at 21 °C. (**D**) Non-inoculated wine, incubated at 21 °C. * RAPD-PCR profiles that matched with the UNQLh1.1 control strain profile.

3.8. Screening of the hdc Gene

The histidine decarboxylase *hdc* gene, involved in the synthesis of the biogenic amine histamine, was not detected in any of the *Len. hilgardii* strains (Figure 7).

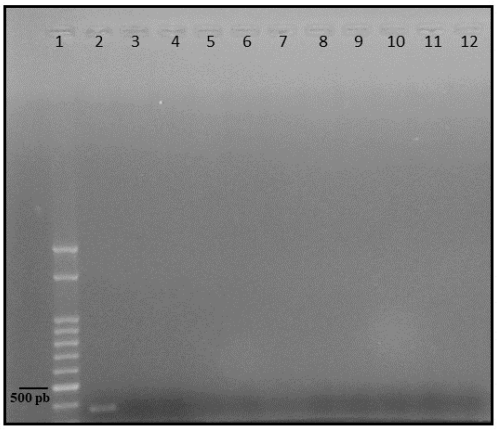

Figure 7. Polymerase chain reaction gel electrophoresis to evaluate the presence of the *hdc* gene in the *Len. hilgardii* strains. **1**—100 bp ladder, **2**—*Lactobacillus buchneri* ST2A strains as a positive control for the *hdc* (375 bp) gene fragment, **3**—UNQLh1.1, **4**—UNQLh1.2, **5**—UNQLh1.3, **6**—UNQLh2.1, **7**—UNQLh2.3, **8**—UNQLh3.1, **9**—UNQLh3.2, **10**—UNQLh4.1, **11**—UNQLh4.2, and **12**—negative control (H_2O).

4. Discussion

The diversity of LAB species associated with spontaneous MLF carried out at 21 °C of Patagonian wines samples, vintages 2008, 2010, 2012, and 2014, have been examined before. The predominant species found by culture-dependent and independent methods have been *O. oeni* and *Lpb. plantarum* [19,26,27]. Several species in the Lactobacillaceae family have shown the capacity to induce MLF and modify wine aromas [15,18–20,22,41]. Patagonia, one of the southernmost wine regions in the world, offers favorable

agroecological factors and grape varieties that define the adaptive ability of the autochthonous LAB strains, resulting in wines with a unique *terroir*. It is interesting to note that *Len. hilgardii* has never been isolated or detected by culture-independent methods in the previous diversity analysis of MLF Patagonian wines performed at 21 °C [19,25–27]. We found a change in the predominance of Lactobacillaceae species diversity usually described in Patagonian red wines. In this new low-temperature MLF scenario, *Len. hilgardii* and *O. oeni* [30] arose as the dominant species, whereas an unexpected absence of *Lpb. plantarum* was detected. This could suggest that the use of low temperatures during MLF favored the development of psychrotrophic *Len. hilgardii* that could be less represented in the microbiota associated with MLF carried out at 21 °C. In other wine regions, *Lpb. plantarum* [42], *Len. hilgardii,* and *Lcb. paracasei* [24,43,44] were found as the predominant LAB species in MLF carried out from 20 to 24 °C. The diversity of psychrotrophic LAB detected in spontaneous Patagonian Pinot noir MLF carried out at low temperatures consists of sixteen strains of *O. oeni* previously reported by Manera et al. [30] and nine psychrotrophic strains of *Len. hilgardii* reported in this work. This is the first report of Patagonian psychrotrophic *Len. hilgardii* strains capable of conducting MLF at 4 and 10 °C in wine in the presence of native microbiota. Although the number of psychrotrophic *Len. hilgardii* strains isolated was low, the typification analysis showed high genetic heterogeneity among the strains. Furthermore, between the *Len. hilgardii* strains that constituted each cluster, heterogeneity was observed in terms of the viability, consumption of MA, and colony morphology in MRS agar. The analysis of the complete genome sequence of *Len. hilgardii* LMG 7934 revealed a remarkable genome reorganization offering a variety of metabolic adaptation skills [45]. The fully assembled genomes of two Lactobacillaceae [34,46] and one *Oenococcus* [47] strains from Patagonian wines have been previously reported; in the future, it would be interesting to analyze the complete genome of selected Patagonian psychrotrophic *Len. hilgardii* strains.

This study assessed the viability and MAC of psychrotrophic strains of *Len. hilgardii* and *Lpb. plantarum* to begin to understand the predominance and role of *Len. hilgardii* in MLF carried out at low temperatures. In general, the viability of the *Lpb. plantarum* strains exceeded that of the *Len. hilgardii* strains incubated in sterile wine at 21 °C by one logarithmic order**.** The growth ability of *Lpb. plantarum* at 21 °C could result in low nutrient availability for the rest of the microbiota in these winemaking conditions and could be one reason why no strains of *Len. hilgardii* have previously been detected in MLF of Patagonian wines carried out at 21 °C. However, the *Len. hilgardii* strains exhibited better performance regarding MA consumption than the *Lpb. plantarum* strains at 21 °C, a metabolic trait that may allow them to compensate for their diminished viability. In spontaneous MLF conducted at low temperatures, the *Len. hilgardii* strains coexist with the psychrotrophic strains of *O. oeni* reported by Manera et al. [30], which makes them the dominant cultivable species. In the future, it would be interesting to evaluate the co-cultivation of *O. oeni* and *Len. hilgardii* psychrotrophic strains.

The implantation of commercial starters depends on the used strains and several factors that impact the adaptation to wine-specific conditions in each wine-growing area [48–50]. The implantation of the UNQLh1.1 *Len. hilgardii* strain was assessed, taking into account the possible interactions, both positive and negative, operating within the microbial consortium of wine. The strain showed successful viability, MAC, and implantation of 100% at 4 and 10 °C, which decreased when increasing the incubation temperature to 21 °C. This result does not agree with the implantation of the psychrotrophic Patagonian strains of *O. oeni* UNQOe4 and UNQOe19 described by [30], which showed a higher percentage of implantation as the incubation temperature increased. This could suggest that the strains of *Len. hilgardii* associated with Patagonian Pinot noir wine, even in small numbers, exhibited better adaptive attributes for implantation in wine at lower temperatures. The resilience of *Len. hilgardii* to stressful wine conditions has been also described [24,51] for Australian and Albariño wines.

The safety of the LAB strains used as an MLF starter culture is an essential criterion [52,53]. *Len. hilgardii* has been reported as one of the main LAB causing wine deterioration because it produces biogenic amines, especially histamine [54]. In addition, it depends strongly on the strain and has the capacity to degrade biogenic amines [55]. Figure 7 shows the absence of *hdc* genes, encoding histamine, in all the *Len. hilgardii* strains obtained in this work; this is in agreement with previous findings for other Patagonian strains [26,30,32]. However, further studies should be performed to evaluate genes involved in the synthesis of other biogenic amines.

The novelty of this work lies in the isolation of psychrotrophic *Len. hilgardii* strains from spontaneous MLF conducted at low temperatures, which were able to predominate over the usual Lactobacillaceae strains reported in Patagonian wine. Of similar significance was the ability to implant in the presence of the native wine microbiota and consume malic acid successfully, suggesting that the potential of this species as a next-generation MLF starter culture warrants further investigation.

5. Conclusions

Nine sychrotrophic *Len. hilgardii* strains that were able to remain viable and consume MA at low temperatures in sterile wine were isolated from an MLF of Patagonian Pinot noir wine carried out at 4 and 10 °C. Low temperatures are not lethal but decrease the enzymatic activity; in this new scenario, *Len. hilgardii* resulted as the dominant specie over the strains of *Lpb. plantarum* usually reported when MLF is carried out at 21 °C in this region. Typification clustering analysis suggested a high level of polymorphism between strains. The performance in non-sterile wine at low temperatures, in the presence of native biota of the selected UNQLh1.1 strain, showed the capacity for implantation and efficient consumption MA, suggesting a positive interaction with the autochthonous microbiota. Additional co-inoculation studies should be carried out with Patagonian psychrotrophic *Len. hilgardii* and *O. oeni* in sterile wine at low temperatures. Managing MLF at low environmental temperatures with autochthonous psychrotrophic strains, especially reconsidering the role of *Len. hilgardii* in winemaking, would be a sustainable, innovative oenological practice in the North Patagonia wine region.

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