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Preservation of Malolactic Starters of *Lactiplantibacillus plantarum* Strains Obtained by Solid-State Fermentation on Apple Pomace

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Abstract: Malolactic fermentation (MLF) is a biological process of deacidification of wines that may also help to increase the microbiological stability of the product and to enhance its sensorial attributes, in particular its aromatic profile. MLF can occur spontaneously due to the presence of indigenous bacteria in the environment of fermented beverages such as wines and ciders. However, the inoculation of lactic acid bacteria (LAB) starter cultures could prevent potential losses and standardize the process. The industrial-scale production of these starter cultures requires cost-effective, sustainable, and feasible approaches. A possible approach to acquire bacterial biomass, while addressing waste disposal concerns, is the use of by-products from the apple juice industry, such as apple pomace. Moreover, to ensure the long-term viability and activity of starter cultures, appropriate preservation methodologies need to be developed. Thus, the aim of the present study was to evaluate the growth of two *Lactiplantibacillus plantarum* strains, UNQLp11 and UNQLp155, in supplemented apple pomace and to optimize the preservation conditions. Cultures were either frozen or freeze dried (trehalose (20% m/v) as a protective agent, potassium phosphate buffer (pH 7), and a combination of trehalose and buffer), and then cell viability and malolactic activity were analyzed under wine stress conditions. Notably, the combination of phosphate buffer at pH 7.0 and trehalose emerged as the most effective preservation strategy for freeze drying, exhibiting enhanced viability and malic acid consumption for the two strains evaluated. These findings underscore the inexpensive and sustainable viability of using a semi-solid medium formulated with apple pomace for LAB biomass production and subsequent preservation.

Keywords: apple pomace; solid fermentation; *Lactiplantibacillus plantarum*; malolactic fermentation; alcoholic beverages

Citation: Navarro, M.E.; Brizuela, N.S.; Flores, N.E.; Morales, M.; Semorile, L.C.; Valdes La Hens, D.; Caballero, A.C.; Bravo-Ferrada, B.M.; Tymczyszyn, E.E. Preservation of Malolactic Starters of *Lactiplantibacillus plantarum* Strains Obtained by Solid-State Fermentation on Apple Pomace. *Beverages* **2024**, *10*, 52. https://doi.org/10.3390/beverages10030052

Academic Editor: Antonio Morata

Received: 20 May 2024
Revised: 23 June 2024
Accepted: 26 June 2024
Published: 28 June 2024

1. Introduction

Malolactic fermentation (MLF), which is the process of malic acid decarboxylation to lactic acid, reduces the acidity and can modify the taste of alcoholic beverages, mainly in wine [1] but also in ciders [2] and other alcoholic beverages from fruit fermentation [3]. MLF can occur either during or after alcoholic fermentation by the activity of lactic acid bacteria (LAB). This can occur spontaneously by the action of native LAB but can lead to undesirable results, such as an increase in the volatile acidity and the formation of biogenic

amines. In contrast, malolactic starter cultures could be inoculated during the fermentation process to guarantee the success of MLF when it is required [4].

In this context, the bacterium *Lactiplantibacillus plantarum* has shown to be tolerant of the harsh conditions of alcoholic beverages, and, since it has a great number of genes that code for enzymes associated with the production of aromatic active compounds, it can induce a range of compositional changes, which may positively affect the quality of the final product [5].

Two *L. plantarum* strains, UNQLp11 and UNQLp155, were previously isolated from a Patagonian Pinot noir wine [6,7] and selected for their ability to survive during the winemaking processes and successfully carry out MLF [6,7]. Both strains were capable of positively modifying the flavor of wine, changing the profile of volatile compounds and its sensory perception [8].

Scaling up biomass production of LAB starters requires the development of processes that are industrially applicable, economically viable, and environmentally sustainable. In this context, the recycling of by-products from the food industry constitutes an inexpensive and sustainable alternative to obtain microbial biomass, in line with the concept of the circular economy, and aims to prolong the useful life of products, materials, and resources, reducing the generation of waste to a minimum [9]. However, LAB are considered fastidious microorganisms, and they need a rich medium for growth. For this reason, by-products must be supplemented with peptides, yeast extract, magnesium, manganese, and tween. Yeast extract provides vitamins, aminoacids, purines, and pyrimidines; Mg^{2+} and Mn^{2+} are essential for nucleic acids, phospholipids, and ATP synthesis; and tween provides essential fatty acid [10].

Apple pomace (AP), which consists of pulp, skin, seeds, and stalks from the fruit, is a semi-solid by-product from apple juice production. Several strategies for the valuation of AP, including its direct use for animal feed, organic acids, enzymes, bioethanol, and biogas production by microbial fermentation, or the development of new materials as part of biocomposites, have been proposed [11]. Since AP can also be used as a carbon source, in a previous work, the growth of *L. plantarum* strains was optimized in the supernatant of AP. The supplementation of AP with yeast extract, salts, and tween significantly increased the biomass, nearly to values obtained with the commercial medium MRS (86% for UNQLp155 and 94% for UNQLp11 compared with MRS). For AP without supplementation, it was lower than 30%, and AP supplemented only with yeast extract showed results of 54–56% compared to MRS [12]. However, the production of a supernatant of AP at large scale by centrifugation is hardly applicable due to the high costs and long times. For this reason, the production of LAB biomass directly in solid-state AP is an area of interest.

The use of LAB as starter cultures in the food industry also depends on the preservation technologies used, which are required to guarantee the stability of cultures in terms of their long-term viability and functionality [13]. Thus, the design of a process that allows for obtaining concentrates of viable bacteria, with an activity similar to that of fresh cultures, is required. The most used methods for this are freezing and freeze drying, which reduce water activity, inhibiting deterioration reactions. However, a problem associated with these treatments is the loss of cell viability generated by damage to the plasma membrane and macromolecules (membrane lipids, DNA, and proteins) due to the loss of hydration, producing structural damage [14,15]. To reduce this damage, protective agents are added to the dehydration medium. These agents can be polyhydroxylated compounds such as sugars and amino acids, which are capable of replacing water molecules by forming hydrogen bonds with macromolecules or by forming a glassy state. The success of the preservation process is related to the correct choice of the protective agent to be used, the dehydration conditions, the moisture remaining in the dehydrated culture, the storage temperature, and the intrinsic conditions of the microorganism [16].

Based on the above, the aim of this work was to obtain biomass of LAB (*L. plantarum* strains UNQLp11 and UNQLp155) on supplemented AP as a solid waste from the apple juice industry. With the possibility of marketing these strains as malolactic starters, cell

survival was analyzed after freezing and freeze drying as a conservation process. Finally, their technological and enological capabilities, such as the ability to consume malic acid and survival in synthetic wine, were evaluated.

2. Materials and Methods

2.1. Strains

Lactiplantibacillus plantarum strains UNQLp155 (Gene Bank Accession Numbers rpoB gene KC679067 and 16S rRNA gene KC652904) and UNQLp11 (Gene Bank Accession Number CP031140) were isolated from Patagonian Pinot noir red wine [6,7]. Cells were grown in 10 mL of MRS broth (Biokar Diagnostics, Beauvais, France) [17] at 28 °C and pH 6.5 for 48 h in aerobic conditions.

2.2. Growth Conditions

Apple pomace (AP) was obtained from the production of natural juices squeezed from apples, free of additives (kindly donated by "Jugos Villa Regina®", Province of Río Negro, Argentina). Table 1 shows the physicochemical composition of AP (previously to supplementation). AP was mixed with water at 50% w/v; supplemented with yeast extract 1% w/v (Britania, Buenos Aires, Argentina), Tween 80 1 mL/L (Biopack, Buenos Aires, Argentina), and salts (cysteine HCl 0.5 g/L, ammonium citrate 2 g/L, K_2HPO_4 2 g/L, $MgSO_4$ 0.1 g/L, and $MnSO_4$ 0.05 g/L, all was purchased from Anedra (Buenos Aires, Argentina)) [12]; brought to a pH of 4.5; and sterilized in an autoclave at 120 °C for 15 min. Both strains were inoculated at 1% v/v (0.1 mL of culture in stationary phase in 10 g of fresh medium) in supplemented AP (sAP). MRS broth at pH 6.5 was used as the control medium. The growth kinetics were evaluated at 28 °C for 7 days.

Table 1. Physicochemical composition of apple pomace.

Parameter	Value
Humidity (%)	79.77 ± 1.04
Total Solids (% m/m)	20.23 ± 1.20
pH	4.51 ± 0.07
Reducing sugars (% m/m)	9.57 ± 1.19
Ammonium (mg/kg)	0.13 ± 0.10
Total polyphenols (g/kg)	1.70 ± 0.34
Assimilable Nitrogen (mg/kg)	131.3 ± 7.3

Results are presented as mean ± standard deviation from three different samples of AP (obtained from different lots of apple juice processing).

2.3. Freeze-Drying Conditions

Samples of 1 g of culture grown in sAP for 48 h were supplemented with (a) 1 mL of 20% w/v trehalose (Anedra, Buenos Aires, Argentina), (b) 1 mL of potassium phosphate buffer 1M (pH 7.0) (Anedra, Buenos Aires, Argentina), (c) 0.5 mL of trehalose and 0.5 mL of buffer phosphate, and (d) 1 mL of 0.9% sodium chloride solution (negative control) to protect the sample from the freezing and subsequent freeze-drying process. All samples were frozen for at least 1 week at −20 °C in glass vials, and then 50% of the samples from each condition were dried by freeze drying (BIOBASE freeze dryer system/BK-FD10P, Shandong, China) for 24 h (condenser temperature: −60 °C; chamber pressure: 10 Pa), and the other 50% of the samples were maintained frozen as a control. After freeze drying, sample were vacuum sealed at 4 °C for 24–48 h until their use.

Cultures grown in MRS and acclimated [6] were also assayed as a positive control. Samples of 1 mL of cultures in a stationary state were centrifugated for 10 min at 10,000× *g*, washed twice with 0.9% sodium chloride solution, resuspended in trehalose 20% w/v, and freeze dried in the same manner as the sAP cultures.

2.4. Inoculation of Dried Cultures in a Wine-like Medium

Fresh, frozen, and freeze-dried samples were inoculated in a wine-like medium at 21 °C for 14 days. The frozen samples were thawed, and the dried samples were hydrated in a 0.9% sodium chloride solution for 15 min at 21 °C. The volume of rehydration was calculated by the difference in weight before and after drying, which was approximately 0.9 mL from the sodium chloride solution added in each condition.

Cultures grown in MRS and acclimated (positive control) were rehydrated in 0.1 mL of MRS previous to wine inoculation [18].

The wine-like medium composition was 5 g/L tartaric acid, 4.5 g/L L-malic acid, 0.6 g/L acetic acid, 2 g/L glucose, 2 g/L fructose, and 12% (v/v) ethanol, pH 3.8.

The cultures were inoculated at 1% (1 mL of sample in 100 mL of wine) to obtain an initial cell count of ~7.5 Log CFU/mL. Cell viability and L-malic acid consumption were analyzed after fermentation.

2.5. Cell Viability and Survival

The number of viable cells during growth in sAP and in MRS, during preservation, and after inoculation in wine was determined by bacterial colony count on MRS agar plates incubated at 28 °C for 72 h. Serial dilutions (1/10) with a 0.9% sodium chloride solution were obtained, and 0.1 mL of each dilution was plated on an MRS agar. The plates were incubated at 28 °C for 72 h in aerobiosis, and the results are expressed as Log_{10} of colony-forming units (Log CFU) per mL or g accordingly.

2.6. Malolactic Activity

The remaining L-malic acid was measured with an L-malic acid enzymatic kit (L-Malic Acid Enology enzymatic kit, Bio Systems SA, Barcelona, Spain). The percentage of L-malic acid consumption (%MAC) was obtained using the following equation:

$$\%MAC = 100 - ([MA_t]\ 100/[MA_0]) \quad (1)$$

where $[MA_t]$ is the L-malic acid concentration at time = t and $[MA_0]$ is the initial concentration of L-malic acid.

At the same time, an exponential one-phase decay equation model was used to fit the MAC kinetics performed by the different strains tested in sterile wine. Equation (2) for this model was obtained by the GraphPadPrism® 6.01 software (Graph Pad Software Inc., San Diego, CA, USA, 2007) and is:

$$[MA_t] = ([MA_0] - [MA_i])e^{-Kt} + [MA_i] \quad (2)$$

where $[MA_t]$ is the L-malic acid concentration at time = t, $[MA_0]$ is the initial concentration of L-malic acid, $[MA_i]$ is the L-malic acid concentration at infinite time, and K is the rate constant.

2.7. Reproducibility of Results and Statistical Analysis

All experiments were carried out using three independent bacterial cultures. Cell viability is expressed as Log_{10} CFU/g or mL of sample, and results correspond to the median ± standard deviation. The relative differences were reproducible independently of the culture used. Analysis of variance (ANOVA) was carried out using the statistical program STATISTIX 8 Software (Analytical Software, Tallahassee, FL, USA). Means were compared by Tukey's test for multiple comparisons, and the difference was considered significant when $p < 0.05$.

3. Results

Figure 1 shows the growth kinetics of the two *L. plantarum* strains studied, UNQLp11 and UNQLp155, on sAP in comparison with classical growth in the commercial MRS broth. The results showed that the growth of both strains in the commercial medium MRS was

similar to that in the alternative medium (sAP), reaching a stationary state after 48 h of incubation. UNQLp11 showed a higher number of cells and faster growth than UNQLp155 (1×10^{10} CFU/g vs. 5×10^{9} CFU/g). Also, UNQLp11 showed no significant differences in either the MRS or sAP media.

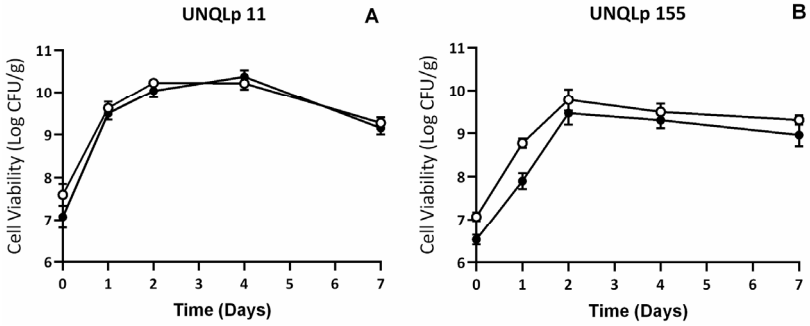

Figure 1. Growth kinetics of UNQLp11 (**A**) and UNQLp155 (**B**) in a commercial MRS medium (○) or supplemented apple-based medium (●). Data are presented as mean ± standard deviation.

The LAB used as commercial starters depend on their preservation technology. To obtain information on bacterial recovery after freezing or freeze drying, cell survival was evaluated before and after preserving the cultures using 20% m/v trehalose (as a protective agent), 1 M potassium phosphate buffer (to neutralize the pH), a combination of both, and a 0.9% sodium chloride solution as a control.

UNQLp11 and UNQLp155 showed different survival behaviors depending on the preservation conditions. A dramatic decrease was observed in the samples with the sodium chloride solution, where non-viable cells were observed in a dilution order corresponding to 10^4 CFU/g (survival less than 10^4 CFU/g after freezing). In the presence of trehalose, survival decreased from approximately 10^{10} to 10^8 CFU/g by the freezing process and to 10^4 CFU/g or non-detected after freeze drying.

No significant differences were observed before and after freezing and/or freeze drying with the phosphate buffer or with the combination of trehalose and phosphate buffer. For both strains, UNQLp11 and UNQLp155, grown in MRS (positive control), no significant difference was observed in regards to the fresh cultures.

The cultures that showed high survival in Figure 2 were then inoculated in synthetic wine (pH 3.8 and 12% v/v of ethanol) at 1% of initial inoculum (1 g of rehydrated sample in 100 mL of wine). Viability and malic acid consumption were evaluated under these stress conditions for fresh cultures and after being preserved by freezing or freeze drying. Samples from the inoculated wine were taken on days 0, 6, and 12 of incubation at 21 °C.

The results of this assay showed that for UNQLp11 (Figure 3), the highest MAC in the synthetic wine was in the frozen and freeze-dried samples in the presence of the phosphate buffer and its combination with trehalose. After 12 days of fermentation, UNQLp11 presented a survival in the order of approximately 8.0 log units in the frozen samples preserved in trehalose and trehalose + phosphate buffer and in the order of approximately 7.0 log units after freeze drying. For frozen samples preserved in the phosphate buffer and trehalose + phosphate buffer, the MAC after 12 days of fermentation was approximately 100% (Figure 3). For freeze-dried samples, the best preservation condition was the addition of the trehalose and phosphate buffer, where the MAC after 12 days of fermentation was approximately 100%. The preservation with a phosphate buffer alone showed slow kinetics of MAC, but with good results. Preservation with trehalose (without the buffer addition) was analyzed only in frozen samples, but the low cell viability after the process showed low percentages of MAC.

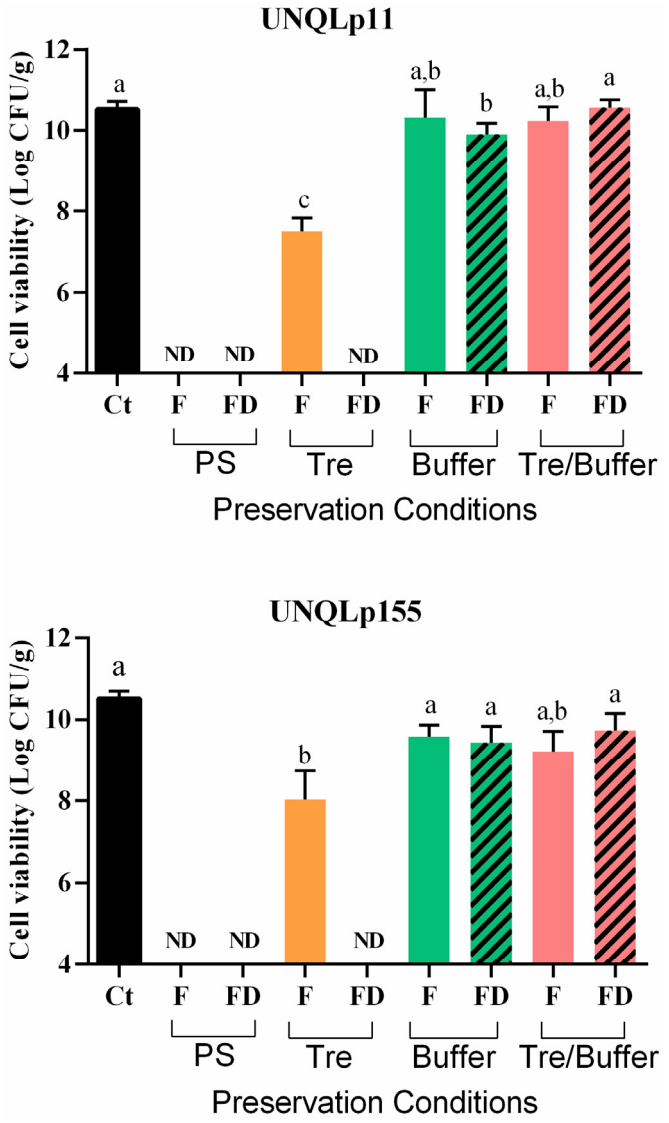

Figure 2. Cell survival of UNQLp11 and UNQLp155 grown in supplemented apple-based medium (sAP), frozen (F), or freeze dried (FD) compared to fresh culture (Ct—Black bars). Cultures were supplemented with trehalose (Tre), phosphate buffer (Buffer), trehalose and phosphate buffer (Tre/Buffer), or 0.9% sodium chloride (physiological solution: PS) as a negative control. ND: not detected (counts $< 10^4$ CFU/g). Data are presented as mean of $Log_{10} \pm$ standard deviation. The different letters (a, b, c) represent groups with significant differences ($p > 0.05$) according to the ANOVA statistical analysis and Tukey's test.

The performance of UNQLp155 in wine was different from that of UNQLp11 (Figure 4). The fresh culture was the most susceptible to wine stress, and the MAC kinetics were slower than for UNQLp11. After the freezing process, the best preservation condition was the combination of trehalose and phosphate buffer, where the MAC in the synthetic wine was 100% despite the decrease in cell viability, which was approximately 3 log. Frozen samples preserved in trehalose or the buffer showed a MAC less than 50% after 12 days. For freeze-drying conditions, the preservation with trehalose was not included due to the dramatic loss of viability (Figure 2). The viability of samples protected with the buffer or trehalose plus buffer showed the same decrease (approximately 3–4 log), but only the sample preserved with trehalose and the phosphate buffer showed a considerable MAC of nearly 80% after 12 days of fermentation.

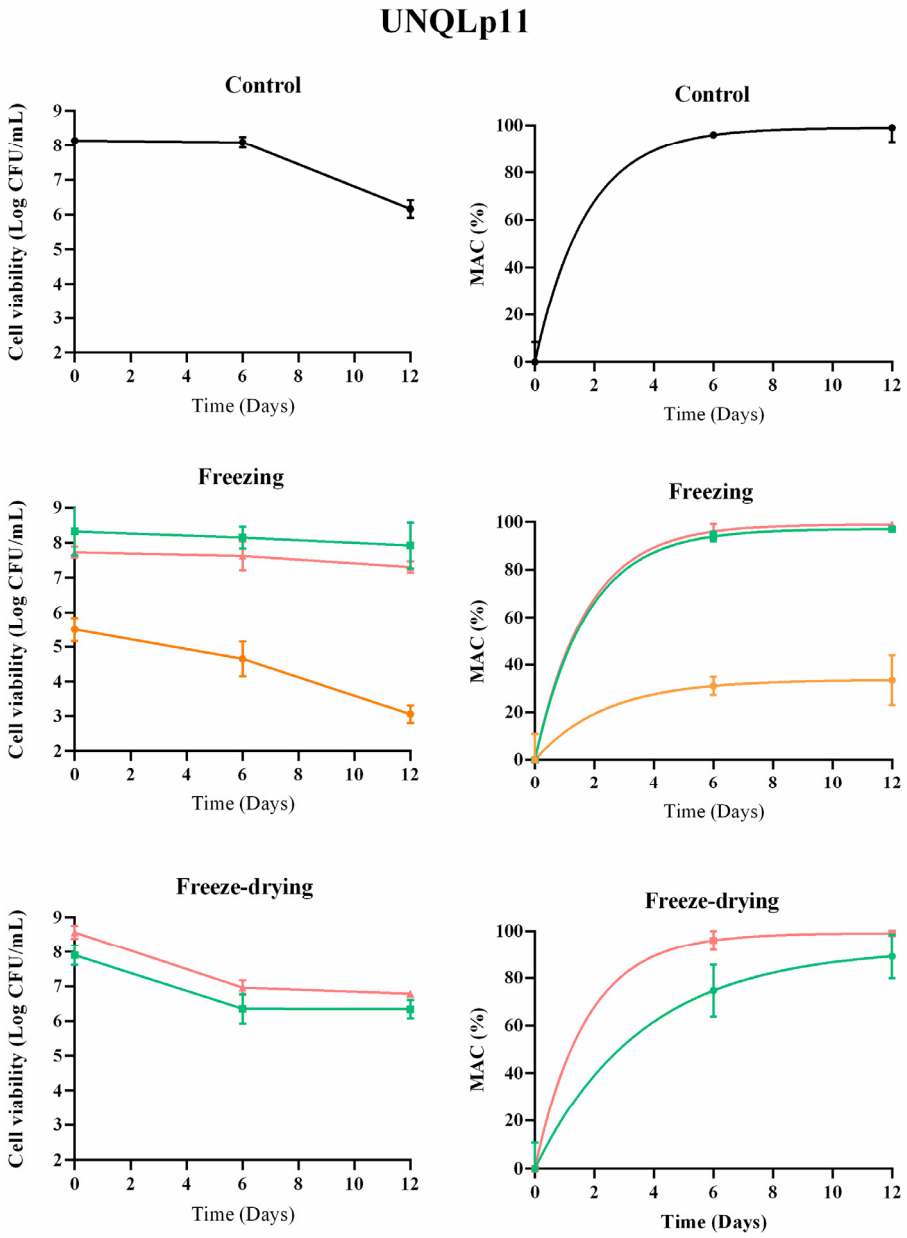

Figure 3. Cell viability (left column) and percentage of malic acid consumption (% MAC, right column) in synthetic wine fermentations (pH 3.8 and 12% ethanol) for 12 days inoculated with UNQLp11 cultures grown in sAP: fresh culture (control, black) and frozen and freeze dried with different preservation conditions: trehalose (orange), potassium phosphate buffer (green), and trehalose/potassium phosphate buffer (pink). Data are presented as mean of Log_{10} ± standard deviation. The results of %MAC were fitted according to a non-linear regression of exponential decay (Equation (2)).

Figure S1 (Supplementary Material) shows the results of control growth in MRS. The decay of cell viability in UNQLp 11 was similar to the results observed in cultures grown in sAP (Figure 3). For UNQLp155, the growth and previous acclimation showed higher tolerance to wine conditions than cultures growth in sAP (Figure 4). The MAC% was greater than 95% for both strains (Figure S1), being similar to cultures grown in sAP and freeze dried with the combination of trehalose + buffer.

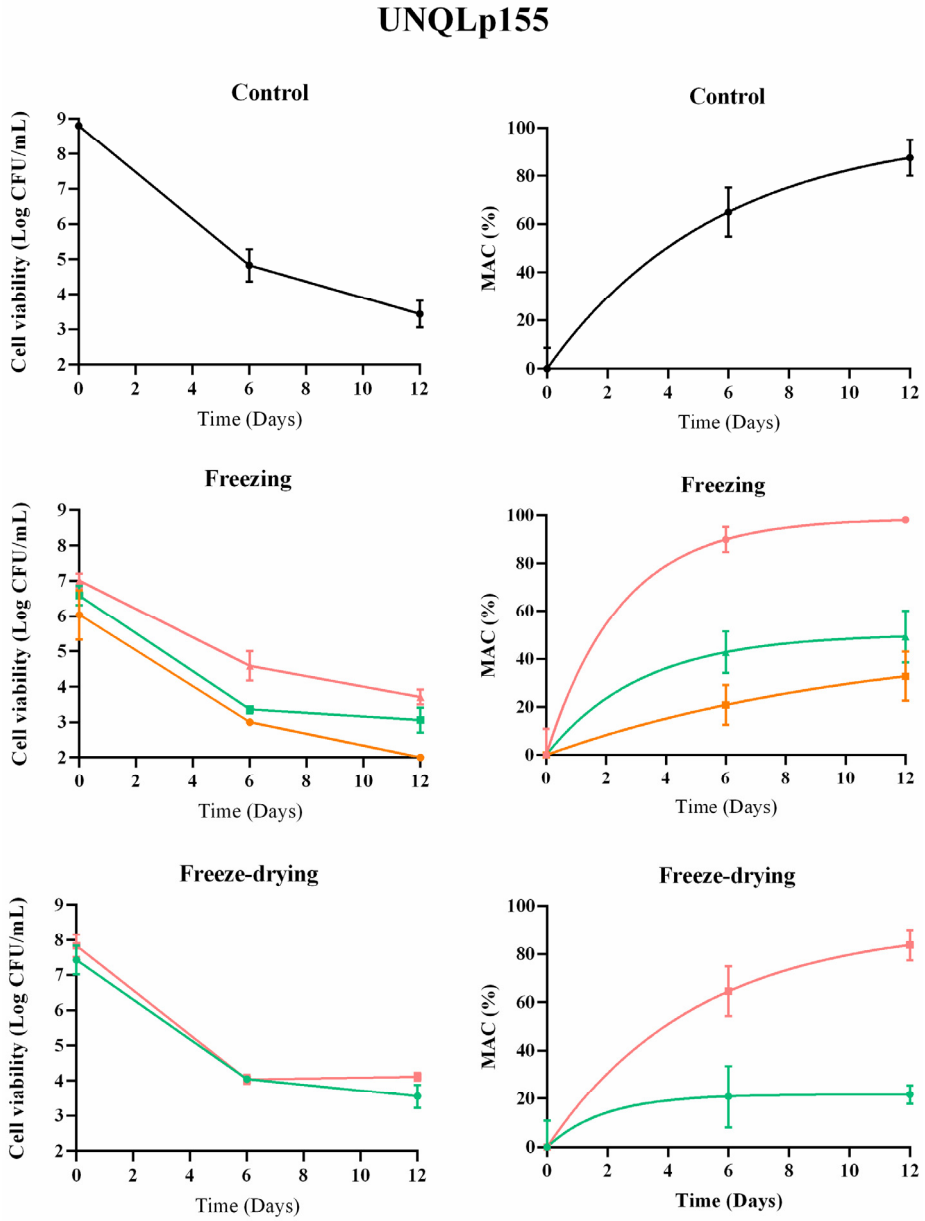

Figure 4. Cell viability (left column) and percentage of malic acid consumption (% MAC, right column) in synthetic wine fermentations (pH 3.8 and 12% ethanol) for 12 days inoculated with UNQLp155 cultures grown in sAP: fresh culture (control, black) and frozen and freeze-dried with different preservation conditions: trehalose (orange), potassium phosphate buffer (green), and trehalose/potassium phosphate buffer (pink). Data are presented as mean of Log_{10} ± standard deviation. The results for %MAC were fitted according to a non-linear regression of exponential decay (Equation (2)).

4. Discussion

LAB strains are generally grown in a rich and complex commercial medium such as MRS, which has a high cost and represents a problem for large-scale biomass production, since their use becomes unfeasible at an industrial level for the generation of starters for beverage production. Therefore, the use of by-products from the food industry appears to be a solution that reduces cellular production costs.

Recently, interest has grown in the use of industrial waste for the production of quality malolactic starter cultures (capable of remaining viable during storage time; growing in the medium in which it is inoculated; and, most importantly, maintaining its malolactic activity) at low cost and in a sustainable manner. In this work, supplemented AP was

used as a semi-solid medium for the growth of *L. plantarum* and compared with traditional commercial media.

Many LAB strains require minerals, especially Mg^{2+} and Mn^{2+}, as essential enzymatic cofactors. The ammonium citrate and phosphates contained in the added salt mixture generate a buffer effect that favors the development of LAB, and Tween 80 provides oleic acid that is essential for the synthesis of lactobacillic acid, one of the fatty acids of membrane lipids that are associated with tolerance to various LAB stress factors [10].

As a result, growth of approximately 10^9–10^{10} CFU/g was obtained for both *L. plantarum* strains, which indicates that this alternative and sustainable medium could have the potential to be used at an industrial level (previously carrying out scale-up tests and pilot scale tests). These results are better than previous ones obtained in an AP supernatant with the same supplementation [12]. This could be due to the fact that bacterial cells are able to metabolize part of the semi-solid by-product, improving the obtention of biomass. In addition, the use of semi-solid AP reduces the cost and time of centrifugation to obtain the supernatant from the AP.

In LAB preservation processes, such as freezing and freeze drying, cells are exposed to low water activity, which can damage cellular structures. For this reason, different compounds (polymers, sugars, amino acids, etc.) are usually added before the freeze-drying process to limit stress and improve the tolerance of LAB to dehydration [19–21]. In this work, trehalose was used as a cryoprotectant, potassium phosphate buffer pH 7 to neutralize the pH of the sample, and a combination of both compounds for freezing and freeze drying.

Trehalose is a sugar synthesized by plants, bacteria, and fungi. It is a protector composed of two glucose molecules (disaccharides). Its high viscosity, high solids content, adequate solubility in water, and high glass transition temperature makes it a good matrix to stabilize biological samples during freezing and drying [14]. During the conservation process, the membrane is the main site of damage, and its repair is crucial before inoculation into wine. Trehalose has the function of protecting the integrity of the cell membrane, which is decisive for survival in wine conditions and carrying out MLF [22]. In previous works, we demonstrated that trehalose is a good protectant for freeze drying of UNQLp11 and UNQLp155 [18,23,24]. However, in the present work, the addition of trehalose alone to the sAP culture was not sufficient to protect cell viability. This could be due to the pH of the sample previous to the freezing and freeze-drying process and to the fact that when MRS broth was used, the cell biomass was separated and resuspended in a cryoprotectant. However, the growth in semi-solid sAP requires neutralization of the pH with other methods. In this context, the use of a potassium phosphate buffer was vital. Frozen and freeze-dried samples preserved in a buffer showed no significant differences with the control (Figure 2) and also showed good MAC under wine conditions (Figure 3). pH neutralization previous to freeze drying has been previously reported by other authors [16,25]. The pH of the culture medium based on sAP goes from a value of 4.7 to 3.2 with the growth of the bacterial biomass. Under these conditions, the organic acids are protonated and uncharged, which makes them bacteriostatic and allows them to cross the bacterial membrane, generating variations in the intracellular pH. The addition of the potassium phosphate buffer showed a protective effect, particularly for freeze-dried samples. On the other hand, some compounds present in AP, such as polysaccharides, fiber, and polyphenols, can act as protective agents [26]. The combination of phosphate buffer and trehalose was the most efficient alternative to protect the cultures after the freeze-drying process and inoculation in wine, particularly for the MAC of more susceptible strains such as UNQLp155 (Figures 3 and 4).

Finally, the results obtained in the present work could represent a sustainable way to obtain dried LAB for the wine industry, as well as for other food and beverage industries, in a short time with low cost and simple steps, while also adding value to apple juice by-products.

5. Conclusions

Apple pomace supplemented with yeast extract and salts proved to be a good medium for the growth and preservation of *L. plantarum* strains. This medium yielded bacterial biomass values comparable to those obtained with commercial media and has the potential to mitigate the environmental impact because it is an industrial by-product. Furthermore, our findings indicate that the optimal cryoprotection treatment, both for freezing and freeze drying, involved the use of a potassium phosphate buffer combined with trehalose. Both *L. plantarum* strains evaluated after preservation exhibited robust tolerance to inoculation in synthetic wine, demonstrating significant cell viability and L-malic acid consumption capacity.

The development of this work also represents an innovative solution for starter culture production considering the high costs involved in biomass generation at the industrial scale and the environmental impact generated by the waste used. More studies are necessary to evaluate the storage stability of dried cultures as well as the impact of the inoculation of the malolactic starter, produced in sAP, on the flavor of wine.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/beverages10030052/s1, Figure S1. Cell viability (left) and percentage of malic acid consumption (% MAC, right) in syn-thetic wine fermentations (pH 3.8 and 12% ethanol) for 12 days, inoculated with freeze-dried UNQLp11 (●) or UNQLp155 (▲).

Author Contributions: Formal analysis, M.E.N., B.M.B.-F. and E.E.T.; Investigation, M.E.N., N.S.B., M.M., N.E.F., D.V.L.H. and A.C.C.; Methodology, M.E.N., N.S.B. and B.M.B.-F.; Project administration, L.C.S. and E.E.T.; Writing—original draft, M.E.N., B.M.B.-F. and E.E.T.; Writing—review & editing, M.E.N., N.S.B., B.M.B.-F., D.V.L.H., A.C.C., L.C.S. and E.E.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by grants from Agencia Nacional de Promoción Científica y Técnica (ANPCyT; PICT 2020 N° 3372, PICT 2019 N° 0008, PICT 2021 ApliCatI 0013) and the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) (PIP-2021—11220200100394CO), Argentina.

Data Availability Statement: The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Acknowledgments: N.S.B., B.B.-F., E.E.T., and M.M. are members of the Research Career of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). N.F. is a fellow of CIC –PBA. L.S. and D.V.L.H. are members of the Research Career of the CIC-PBA.

Conflicts of Interest: The authors do not have any conflicts of interest.

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