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Use of *Lachancea thermotolerans* for the Bioacidification of White Grape Musts: Assays from the Bench to the Cellar Scale

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Abstract: To date, there are no specific guidelines for the use of bioacidifying yeasts in winemaking. In this work, we aimed to characterize an oenological strain of *Lachancea thermotolerans* (Lt), a *non-Saccharomyces* lactic acid-producing yeast, and to test different sequential inoculation conditions with *Saccharomyces cerevisiae* (Sc). The results of bench scale vinifications showed that both the strategy of inoculating Sc 12 h after Lt and the mixing of Lt and Sc during fermentation delivered an acceptable increase in lactic acid (2 g/L) and a decrease in pH (about 0.15 units). Therefore, both strategies were implemented in winery experiments. Our results at the cellar scale showed no increase in acidity, which was likely due to the presence of indigenous yeasts. Overall, our experience shows the difficulty of translating laboratory protocols into cellar experiments and calls for further research into new strategies for implementing acidifying yeasts.

Keywords: *Lachancea thermotolerans*; fermentation; lactic acid

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1. Introduction

Global warming has been affecting all agricultural sectors. In viticulture, high temperatures and long periods of drought can cause water stress in the berry and a reduced ability to absorb or synthesize nitrogen compounds. In addition, hot and dry periods can induce early grape ripening, resulting in a decrease in acids, especially malic acid, and an increase in sugar content [1]. Without any intervention in the vineyard or the cellar, the wines obtained from these grapes have a high alcohol content and are poorly balanced due to their lack of acidity. These wines may also be compromised in terms of microbial stability, with insufficient levels of molecular SO_2 [2]. Possible interventions in the vineyard are aimed at delaying ripening and, thus, maintaining a high malic/tartaric acid ratio [3]. In the winery, the winemaker has several tools at his/her disposal to increase acidity. In addition to electrochemical techniques, such as electrodialysis, which increases the level of H+ ions after K+ extraction, it is possible to add a certain amount of organic acids, such as tartaric, malic, or lactic acid (LA), to musts and finished wines. Each of these acids has specific solubility characteristics, but in general, they can be used effectively within regulatory limits [4]. However, this process is typically not well received by consumers who prefer wines with minimal enological additives [5]. In fact, the external addition of acids could be perceived as a "non-natural" method of acidification. Therefore, along with today's trend of reducing the use of ingredients in favor of more sustainable production, producers are looking for other ways to address such problems in the wineries.

In the last decade, so-called bioacidification alternatives have been presented, such as the use of *Saccharomyces* and non-*Saccharomyces* yeasts that produce organic acids [6].

The most widely used non-*Saccharomyces* yeast for this purpose is *Lachancea thermotolerans* (Lt), which converts fermentable sugars partly to ethanol and partly to lactate through the enzyme lactic dehydrogenase [7]. The main advantage of Lt is that it produces large amounts of LA, which significantly affects the acidity of the must. Unfortunately, Lt is very sensitive to the level of alcohol produced (it remains viable down to about 8% vol/vol of ethanol) and, thus, does not complete the process of alcoholic fermentation (AF) [8]. For this reason, it is common to add *Saccharomyces cerevisiae* (Sc) to complete AF. Despite the large amount of data in the literature that have been obtained through different experimental models of microvinification [9], there are no clear guidelines available to help winemakers implement the rational use of Lt yeast that guarantees a satisfactory acidification result. In fact, besides data showing little or no acidification, there have also been cases in which the must at the end of fermentation had an unacceptable level of acidity [10]. In this work, we have characterized a commercial strain of Lt and tested different acidification protocols at the bench and cellar scale on the 2023 vintage.

2. Materials and Methods

2.1. Bench Scale Fermentations

In the bench scale experiments, fermentations were carried out in 500 mL glass bottles sterilized at 121 °C for 15 min. Chasselas must (from the domain of Pully, Vaud, Switzerland), processed and frozen in 2022 vintage at −20 °C in 3 L bag-in-boxes, was used for the fermentation. The must was brought to 4 °C the day before the experiment and then equilibrated in a thermostatically controlled bath at 25 °C. Bottling, yeast inoculation, and sampling were performed under a horizontal laminar flow hood. The bottles containing the must were pasteurized at 60 °C for 20 min in a thermostatic bath and returned to room temperature by cooling with running water. Lt (Levulia Alcomeno, AEB, Brescia, Italy) was inoculated at 30 g/hL in duplicate or triplicate conditions, depending on the experiments. Briefly, the yeast was rehydrated in non-chlorinated water (in ten parts of water) at 25° C with slow mixing and then inoculated into the must. Sc (Fermivin 7013, Oenobrands, Montpellier, France) was rehydrated and inoculated at 20 g/hL in different conditions from day 0 to day +3 after Lt inoculation. Once a day, 5 mL of fermenting must was collected for densitometric evaluation using a DMA 35 portable densitometer (Antoon Paar, Graz, Austria) and for pH measurement with a 691 pH meter (Metrohm, Zofingen, Switzerland). In some experiments (see Section 3.5), a simplified sensory analysis of the experimental wines was performed by a trained panel of four Agroscope tasters. The tasters rated the intensity of acidity in the different conditions and expressed an overall qualitative preference.

2.2. Cellar Scale Experiments

2.2.1. Chasselas Vinification

Chasselas grapes (about 500 kg) from the Grand Brûlé domain of Leytron (Wallis, Switzerland) were harvested and processed on 20 September 2023. Grapes were crushed, pressed, and then subjected to static settling for 16 h at about 10 °C. The must was then divided into two 200 L stainless steel tanks. As an enrichment strategy, 20 g/hL of Fermaid O (Lallemand, France) was added to the must at the beginning and after 1/3 of the fermentation process had been completed for a total of 40 g/hL (about 16 mg/L of assimilable nitrogen in the form of amino acids). The control condition was inoculated with rehydrated Fermivin 7013 at 20 g/hL. The sequential fermentation condition was inoculated with Lt (Levulia Alcomeno) and Sc after 12 h. AF was carried out at a controlled temperature (19–21 °C) and monitored daily by densitometry. The AF was considered complete when, after 5–7 consecutive days of negative densitometric readings, the detection of residual sugar was found to be <1 g/L. The wine was racked and chemically stabilized with SO_2 (40 mg/L) and then physically stabilized by cooling at 4 °C for one month. Bottling was preceded by an additional cartridge filtration step at 0.65 and 0.45 µm. Bottles were stored under controlled conditions (10–12 °C in the dark) prior to analysis.

2.2.2. Divona Vinification

The Divona grapes vintage 2023 from the domain of Pully (Vaud, Switzerland) were crushed and pressed, and the must was treated with 1 g/hL of Trenolin Opti (Erbslöh, Geisenheim, Germany) and subjected to static sedimentation at 13 °C for 24 h without the addition of SO_2. Before inoculation, the must was divided into three 60 L tanks and brought to a temperature of 20 °C. The control must (with 50 mg/L SO_2 added) was inoculated with Sc Lalvin Cy3079 (Lallemand, Montreal, Canada) at the recommended dose of 20 g/hL and then rehydrated according to the manufacturer's directions. On day 6, oxygenation was performed, and 30 g/hL of FermoBent (Erbslöh) was added to the tank. Another identical condition (without SO_2) was prepared to blend with the Lt must in fermentation.

Lt was rehydrated according to the manufacturer's directions with 10 g/hL of Fermoplus Energy Glu 3.0 (AEB) and inoculated in must (without SO_2) at the recommended dose of 30 g/hL. All the tanks were maintained at a controlled temperature of 20 °C for the entire AF. The AF was considered complete when, after 5–7 consecutive days of negative densitometric readings, the detection of residual sugar was found to be <1 g/L. The wine was racked, chemically stabilized with SO_2 (50 mg/L), and then filtered (through tangential filtration). Then, the wine was physically stabilized by cooling at 1 °C for one month. Bottling was preceded by an additional cartridge filtration step at 0.65 and 0.45 µm. Bottles were stored under controlled conditions (10–12 °C in the dark) prior to analysis.

2.3. Flow Cytometry (FCM)

For the FCM analysis, 50 µL of fermenting must (for both the bench scale fermentations and cellar scale experiments) was diluted at a ratio of 1:20 in Phosphate Buffered Saline (PBS) and stained with 5-carboxy-fluorescein diacetate-acetoxymethyl ester (CFDA-AM; Fisher Scientific, Waltham, MA, USA) at a final concentration of 2 µM, Hoechst 33258 (Fisher Scientific, Hampton, VA, USA) at a concentration of 1 ug/mL, and propidium iodide (PI, Sigma-Aldrich, Taufkirchen, Germany) at a concentration of 0.5 µg/mL. After incubation for 15 min at room temperature, the sample was collected using a MACSQuant 10 analyzer (Miltenyi, Bergisch Gladbach, Germany). Offline analysis of the FCM files was performed using Flow Logic software v. 8.7 (Inivai Technologies, Mentone, Australia) or FCS Express v.7 (De Novo Software, Pasadena, CA, USA). The parameters analyzed included the cell count (expressed as the log of live cells/mL) and the relative fluorescence intensity of CFDA (expressed as the median fluorescence intensity [MFI]), a general indicator of the metabolic activity of the cell.

2.4. Wine Analysis by Enzymatic Methods and Fourier Transform Infrared (FT-IR)

2.4.1. Bench Scale Fermentations

Samples of must in fermentation were collected and analyzed for LA content by enzymatic methods (L-lactic kit, Biosystems, Barcelona, Spain) using a Y25 Biosystems instrument (Biosystems). Total and free SO_2 were determined by colorimetric methods using an automated analyzer (Y15, Biosystems). Other biochemical parameters (i.e., acids, sugars, and ethanol) were assessed at the end of fermentation using an FT-IR Wine Scan (Foss, Hillerød, Denmark). Key parameters, such as sugar and acid concentrations, were calibrated against standard methods.

2.4.2. Cellar Scale Assays

Enzymatic methods were used to measure LA and nitrogen content during the vinification of both Divona and Chasselas. FT-IR and enzymatic methods were used for the analysis of bottled wine.

2.5. Statistical Analysis

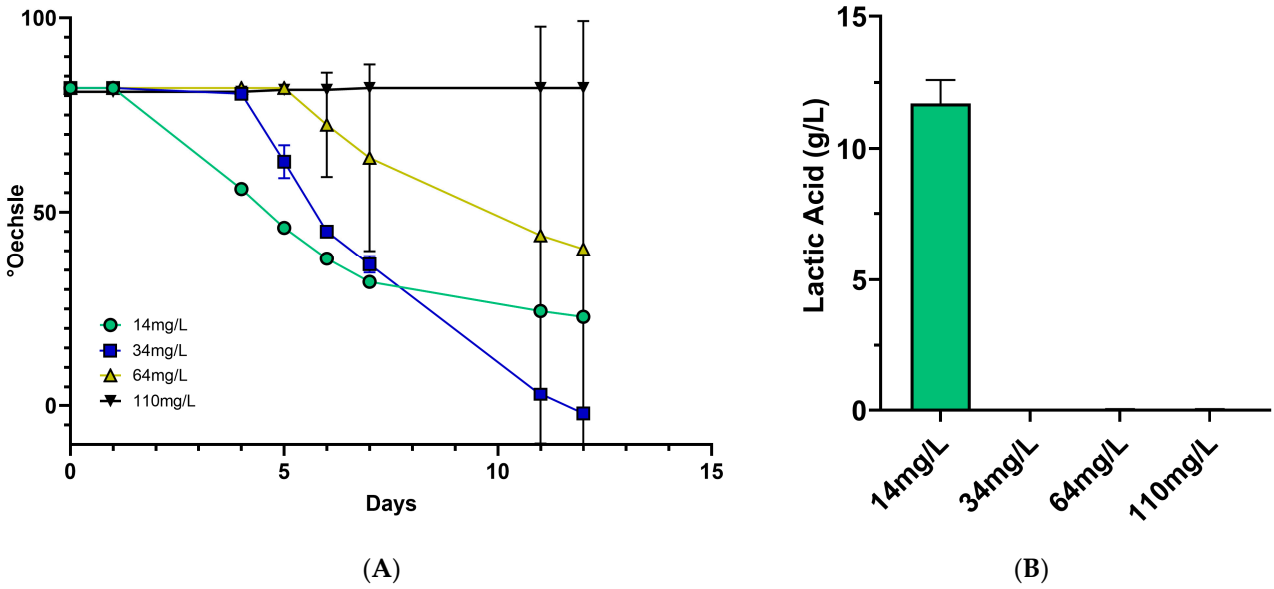
Statistical analysis was performed using Prism 10.3 (Dotmatics, Boston, MA, USA). Results were expressed as the mean of two or three biological replicates with standard

deviation (SD). One-way Analysis of Variance (ANOVA) or *t*-test were used to compare different experimental conditions.

3. Results

3.1. Increasing Sulfite Concentration Prevents Lt Development and LA Production

We started the bench scale experiments by testing the resistance of Lt to increases in sulfite concentration. It should be noted that the frozen must (Chasselas 2022) already contained a certain amount of dissolved SO_2 (14 mg/L total, 9 mg/L free). Therefore, we added increasing concentrations of SO_2 and evaluated the response of Lt. Our results show that, already at concentrations of 34 mg/L (20 mg/L free), there was no more LA production aside from sugar consumption (Figure 1A,B).



(**A**) (**B**)

Figure 1. LA production (**A**) and sugar consumption (**B**) at different sulfite concentrations after inoculation with *Lt*. A: the mean of LA production (two biological replicates) in fermentations at different sulfite concentrations; the bars indicate the standard deviation (SD). B: Sugar consumption in fermentations at different sulfite concentrations. The symbols represent the mean of the densitometry values of two biological replicates, and the bars indicate the SD. The SO_2 values indicate the total SO_2 present in the sample.

Interestingly, we observed fermentative activity from day + 5 of inoculation under the 34 and 64 mg/L (40 mg/L free) sulfite conditions with biomass formation, as shown by flow cytometry (Figure 2), but without LA production. Neither fermentative activity nor cell growth was observed at 110 mg/L (70 mg/L free). These results showed that the investigated Lt strain studied could resist and produce LA at concentrations between 14 to 34 mg/L total SO_2 (9 to 20 mg/L free).

3.2. Nitrogen Level Does Not Affect LA Production in Bench Scale Experiments

The Chasselas must used in these experiments contained an amount of yeast-assimilable nitrogen (YAN) generally considered to be at the limit of deficiency (about 140 mg/L). We, therefore, carried out supplementation (about 60 mg/L YAN) with diammonium phosphate (DAP) and evaluated its effect on the biomass and LA production. Our results showed no significant differences in either the number of live cells or the amount of LA. However, to rule out the possibility of stuck fermentation in successive experiments, we supplemented all subsequent bench fermentations to a level of 200 mg/L (Figure 3A,B).

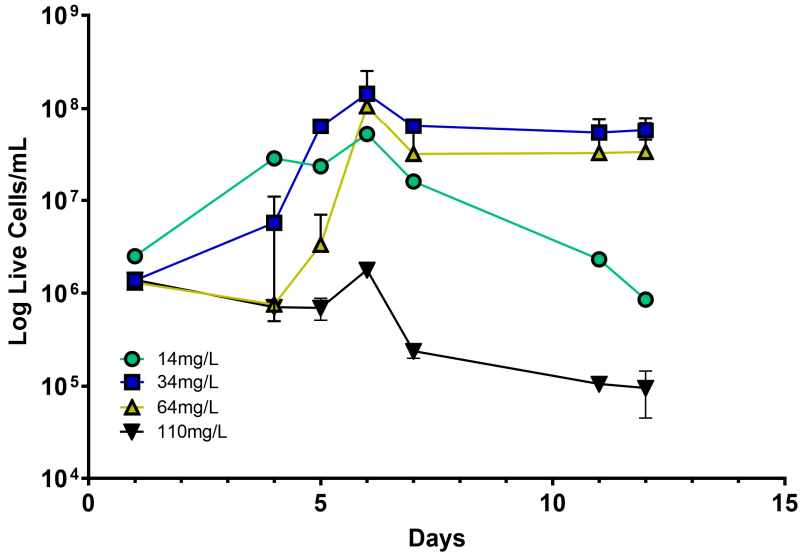


Figure 2. Cell growth after inoculation with Lt at increasing sulfite concentrations. The symbols represent the mean cell concentration in two biological replicates, and the bars indicate the SD. The SO_2 values indicate the total SO_2 present in the sample.

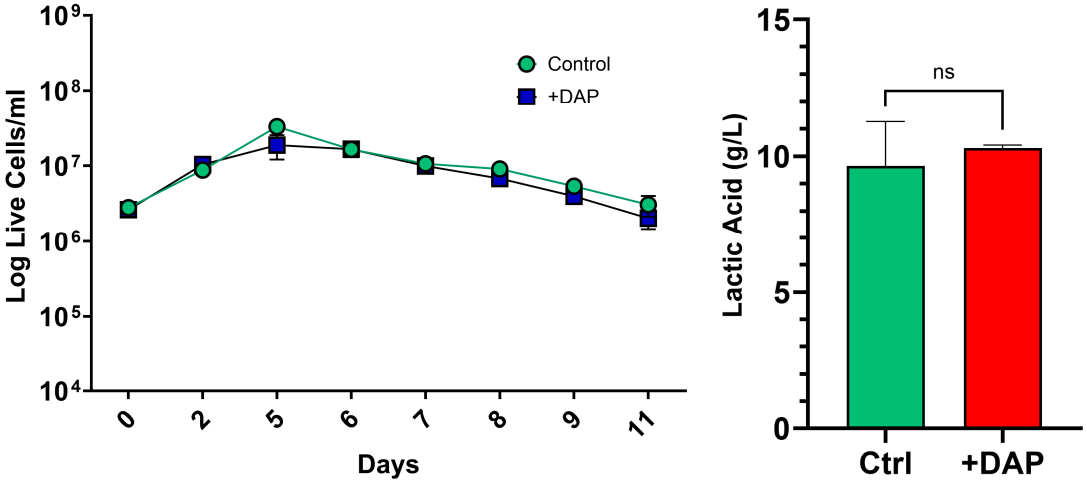


Figure 3. Cell growth (**A**) and LA production (**B**) in the control and DAP-supplemented conditions. A: the symbols represent the mean of cell concentration in three biological replicates; the bars indicate SD. B: mean of LA production (three biological replicates); the bars indicate SD. ns: not statistically significant differences between the mean of the two conditions after unpaired *t*-test.

3.3. Plateau of LA Production Reached after Three Days

In the following series of bench-scale experiments, we evaluated the kinetics of LA production by Lt using a must supplemented with nitrogen and fermentation at a temperature of 20–22 °C. Our results showed that after the resting period, Lt proliferated and started to produce LA after about 24 h until it reached a plateau on day 3 (Figure 4A). Interestingly, both the increased proliferation and metabolic activity temporally preceded the detection of LA (about 10 h before), and the peaks of cell proliferation and activity were consistently reached before day 3 of fermentation (Figure 4B,C).

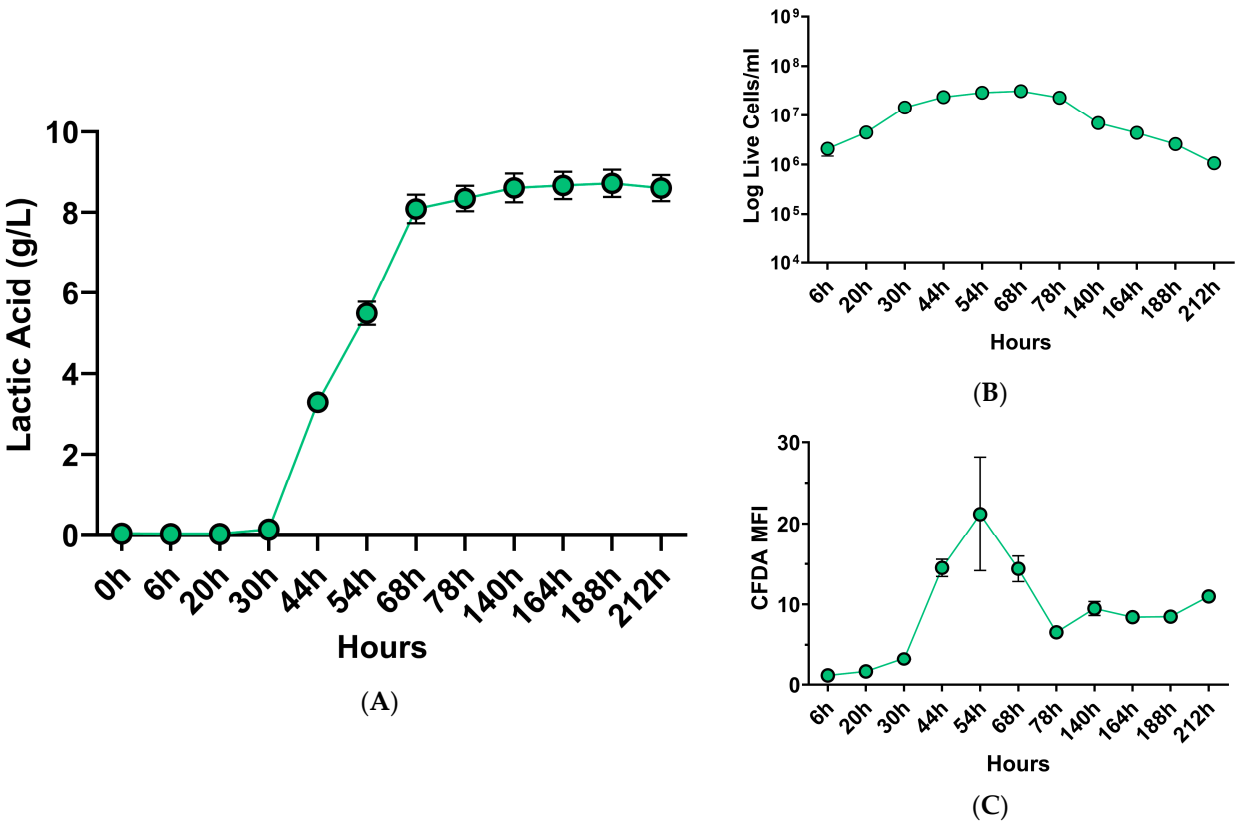


Figure 4. Kinetics of LA production (**A**). Cell proliferation and metabolic activity (defined as CFDA MFI value) during the kinetics determination experiment (**B**,**C**). The symbols represent the mean LA production (**A**), cell concentration (**B**) or metabolic activity (**C**) in three biological replicates. The bars indicate the SD.

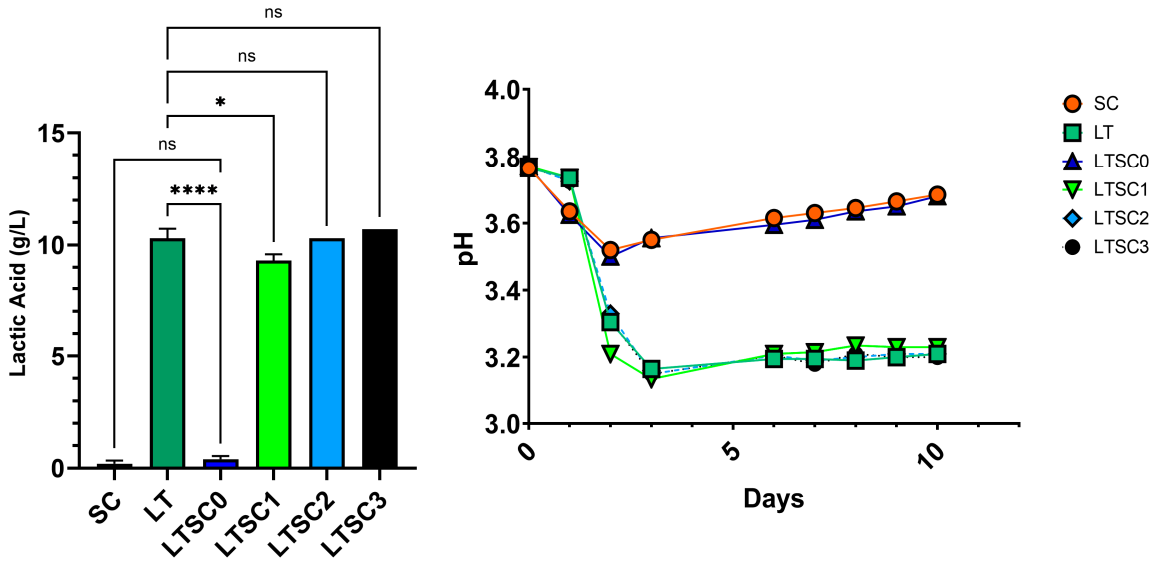
3.4. Time-Dependent Sequential Sc Inoculation Protocols Minimally Affect LA Production by Lt

The experimental data suggested that the inoculation of Sc beyond day 3 would result in a wine with excessive LA content. The protocols recommended by the yeast producer and also used by other investigators suggest the sequential inoculation of Sc on day +1 or +2 from the inoculation of Lt to obtain a modulation of the total acidity. Therefore, in this experiment, we prepared different bench-scale fermentation conditions (single Sc, single Lt, and Lt + Sc on days 0, +1, +2, and +3) to evaluate the best time points for Sc inoculation in terms of LA production and, thus, for a decrease in pH. Our results showed that the co-inoculation (day 0) did not result in significant LA production, and the pH at the end of fermentation was not different from that of the control with Sc (Figure 5A,B). In contrast, the final amount of LA produced in all conditions of sequential inoculation was very high (>9 g/L, Figure 5A) and not significantly different from the single LT fermentation, except for the condition LTSC1. Consistently, the decrease in pH was very large, with a value of about 0.4 units, which was practically the same as the fermentation condition with Lt alone (Figure 5B).

3.5. Alternative Sc Inoculation Protocols Provide Acceptable Acidification in Bench-Scale Fermentation

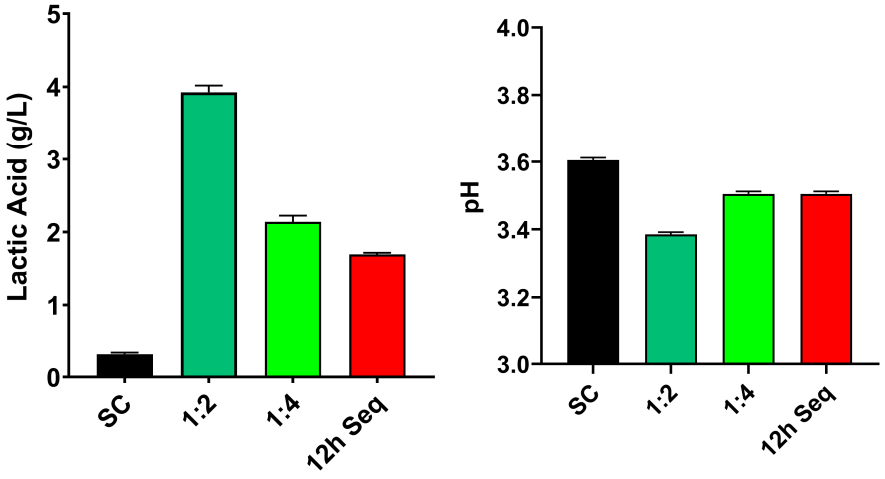
In our model, the inoculation time of Sc did not consistently impact LA production. Therefore, we tested two alternative methods to try to control LA production using Lt. In the first method (mixing), we allowed the maximum amount of LA to be produced by Lt (about 8–9 g/L) and then mixed an appropriate volume to a single fermentation condition with Sc. In the second method (12 h Seq), we tested whether a very short culture time (about 12 h) and subsequent inoculation of Sc could result in a lower amount of LA.

Our results show that both methods yielded an acceptable amount of LA (about 2–4 g/L, Figure 6A). Moreover, the Lt-to-Sc 1:4 mixing and the Sc inoculation conditions at 12 h after Lt were also acceptable from a sensory point of view, according to bench scale tasting tests focused on acidity. Therefore, we decided to apply the two methods in the Chasselas and Divona acidification assays at the cellar scale.



(**A**) (**B**)

Figure 5. LA production (**A**) and pH (**B**) in the sequential fermentation experiments. SC: *Saccharomyces cerevisiae*; LT: *Lachancea thermotolerans*; LTSC0: co-inoculation of Lt and SC; LTSC1: sequential inoculation of Sc on day +1 after LT; LTSC2: sequential inoculation of Sc on day +2 after LT; LTSC3: sequential inoculation of SC on day + 3 after LT. A: the columns represent the mean LA values of two biological replicates. The bars represent the SD. The asterisks represent *p*-values after pairwise comparison (Sidak test): **** = $p < 0.0001$; * = $p < 0.05$; ns: not significant. B: the symbols represent the mean pH values of two biological replicates. The bars represent SD.



(**A**) (**B**)

Figure 6. LA production (**A**) and pH (**B**) after the application of alternative protocols. 1:2 and 1:4: mixing ratios of Lt fermenting must to Sc fermenting must. 12 h Seq: inoculation of Sc 12 h after Lt inoculation. The columns represent the mean LA or pH values of two biological replicates. The bars represent the SD.

3.6. Cellar Scale Assays Did Not Show Consistent Acidification When Using 12 h Seq and Mixing Protocols

The Divona and Chasselas musts had a low malic acid content (less than 0.8 g/L for Divona and less than 1.9 g/L for Chasselas), which was reflected in their rather high pH values (3.4 for Divona and 3.7 for Chasselas), thereby making them suitable for this type of experiment. It should be noted, however, that before inoculation with Lt, the Chasselas must exhibited a certain amount of active indigenous yeasts, as shown by FCM analysis (Figure 7). At the cellar level, the results did not show a significant increase in LA produced in the Lt condition, either for Chasselas or Divona (Tables 1 and 2). Interestingly, the condition of Divona with Lt showed signs of fermentation before being mixed with Sc (from 96 to 43 degrees Oechsle in four days of fermentation). As for Chasselas, a slight difference was observed in the two conditions, which could be attributed to spontaneous malolactic fermentation as measured by a decrease in malic acid.

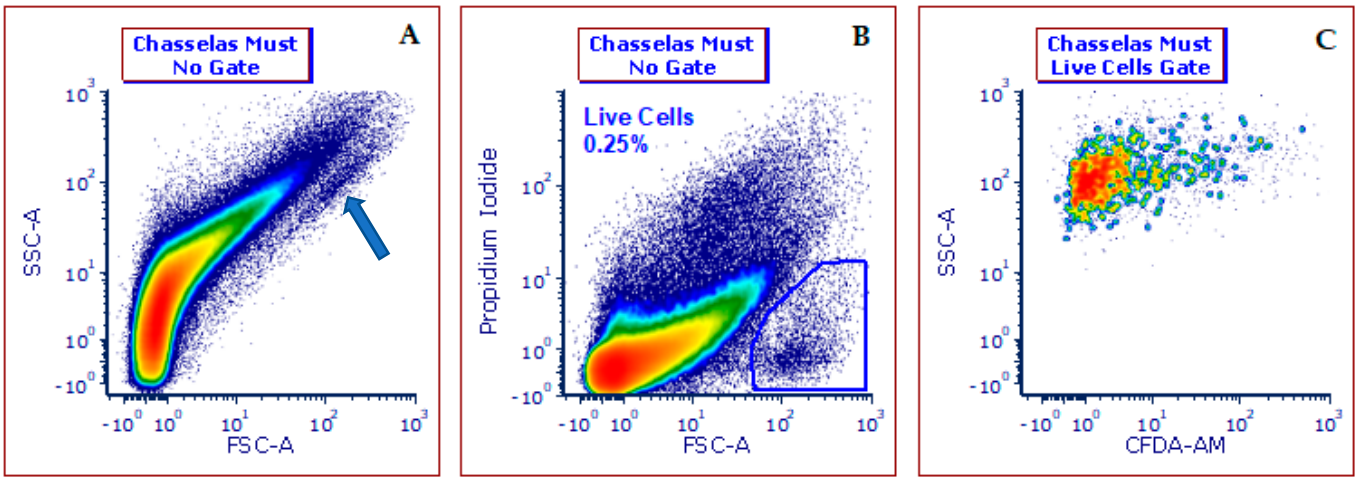


Figure 7. Flow cytometry determination of indigenous yeasts in must after settling before Lt inoculation. (**A**): Arrows indicate yeasts in a scatter parameter plot. (**B**): Live cells are gated. (**C**): General metabolic activity as indicated by CFDA fluorescence.

Table 1. Main chemical parameters of the Chasselas must (left side of each cell) or wine (right side). Titratable acidity is expressed in g/L of tartaric acid. ND: not determined. Bioac.: bioacidification. YAN: Yeast Assimilable Nitrogen.

	Densitometry (°Oe)	pH	Sugars (g/L)	EtOH (% *v*/*v*)	Titratable Acidity (g/L)	Lactic Acid (g/L)	Malic Acid (g/L)	Tartaric Acid (g/L)	YAN (mg/L)
Control (*Sc*)	74/<0	3.70/3.65	ND/<1	ND/10.4	4.50/5.12	0.3/1.07	1.70/0.62	ND/1.89	283/ND
Bioac. (*Lt* + *Sc*)	78/<0	3.73/3.66	ND/<1	ND/11.1	4.35/5.33	0.23/1.14	1.90/0.81	ND/1.89	310/ND

Table 2. Main chemical parameters of Divona must (left side of each cell) or wine (right side) Titratable acidity is expressed in g/L of tartaric acid. ND: not determined. Bioac.: bioacidification. YAN: Yeast Assimilable Nitrogen.

	Densitometry (°Oe)	pH	Sugars (g/L)	EtOH (% *v*/*v*)	Titratable Acidity (g/L)	Lactic Acid (g/L)	Malic Acid (g/L)	Tartaric Acid (g/L)	YAN (mg/L)
Control (*Sc*)	93/<0	3.40/3.44	233.3 < 1	ND/13.5	4.7/5.5	0/0	0.8/0.8	6.1/4.5	303.2/ND
Bioac. (*Lt* + *Sc*)	93/<0	3.40/3.42	234.2 < 1	ND/13.4	4.7/5.7	0/0.03	0.80.88	6.0/4.4	301.5/ND

4. Discussion

The use of acidifying yeasts, such as non-*Saccharomyces* Lt, is a strategy that can be effective in counteracting the lack of acidity in musts in warm vintages. However, these yeasts are difficult to manage because they are very sensitive to the conditions of the fermentative environment, and to date, there are no proper implementation guidelines to ensure successful winemaking in such cases. In this work, we characterized a commercial Lt strain at the bench scale and tested different bioacidification protocols to be transferred to the cellar scale (100–200 L). First, we tested a critical factor in the implementation of Lt, namely, sulfite resistance. Our results showed that the level of sulfite resistance was rather limited for the strain used, reaching about 30 mg/L of total SO_2. At values equal to 34 mg/L and 64 mg/L of total SO_2, no LA production was observed despite the presence of active biomass-fermenting sugars, which was not detectable in the high SO_2 level (110 mg/L) condition. The fermentation observed may have been caused by indigenous yeasts that were resistant to both the pasteurization process and SO_2, at least at concentrations of up to 64 mg/L. This hypothesis is consistent with the delay in the onset of these fermentations (more than four days from the time of Lt inoculation). Indeed, the very low numbers of resistant yeasts needed a certain amount of time to produce enough active biomass to initiate fermentation in the absence of active Lt. Overall, our data are consistent with previous reports describing the limited resistance of Lt to sulfites, ranging between 10 and 20 mg/L free SO_2 [11]. In general, the available data suggest that, above a certain threshold, the proliferation of Lt is prevented, and LA is not produced.

Next, we tested whether the amount of YAN could have a direct relationship with LA production. This was because we wanted to supplement the must to avoid stuck fermentations, especially under sequential inoculation conditions with Sc. Our results showed no difference in LA production between the two conditions (140 vs. 200 mg/L YAN). Interestingly, Sainz et al. [12] observed that LA production by Lt was dependent on the amount of YAN. Our results did not point in this direction, which can be explained by the use of a different experimental model (i.e., a different strain of Lt and artificial must vs. frozen natural must) and, most importantly, by the experimental conditions tested. Specifically, Sainz et al. prepared three concentrations ranging from a nitrogen-deficient must (60 mg/L) to a nitrogen-rich must (300 mg/L), whereas we tested two conditions relatively similar in terms of YAN. Therefore, it is possible that the differential production of LA may be observed in our system when using musts containing very different amounts of nitrogen. These observations highlight the importance of YAN assessment not only for Sc but also for non-*Saccharomyces* yeasts [13].

We then proceeded to determine the kinetics of LA production to establish an appropriate time interval for the sequential inoculation of Sc. The results showed that LA production was proportional to Lt growth and metabolic activity. The FCM data, both in terms of growth and metabolic activity, as indicated by the MFI of the CFDA dye, showed an anticipation of LA production, which may, therefore, have an important predictive value for yeast functionality and the success of the acidification process. The production plateau was reached on the third day, with a value of about 8 g/L of LA. Furthermore, the pH drop was about 0.5 units. This value is considerably high and may not be acceptable to consumers. In fact, in a winery trial with Lt carried out in the 2021 vintage on Chasselas grapes with Sc inoculated on day 3 after Lt, we obtained an LA value of approximately 10 g/L (unpublished data). Sensory analysis showed low preference indices for the wine obtained. We, therefore, tried to obtain lower LA values (2 to 4 g/L), which we thought could be achieved by adding Sc after 24 or 48 h. We set up sequential fermentations with Sc inoculated with Lt from day 0 to day +3, working with the hypothesis that inoculation of Sc after 24 or 48 h could arrest Lt growth and limit LA production. Our results showed that the sequential strategy did not lead to acceptable results since LA production did not significantly decrease under different conditions, whereas it was completely abolished in the co-inoculation condition. Therefore, although we could not distinguish the population of Sc from that of Lt in fermentation (it was not possible to discriminate the two yeasts

in FCM with the available dyes), it is reasonable to hypothesize that Sc did not prevent the growth and production of LA in all sequential inoculation conditions, resulting in a decrease in pH that was hardly acceptable at the sensory level (more than 0.4 units).

The strategy of co-inoculation or sequential inoculation has been used by many experimenters in the last 10 years. As in our case, despite the different experimental methodologies used (different Lt. strains, synthetic vs. natural musts, and yeast preparation for inoculation), the sequential strategy was also found to be more effective in the acidification process, leading to pH decreases of up to 0.4 units, while the co-inoculation strategy did not lead to appreciable changes in pH and acidity [14–16]. However, these findings also revealed that the increase in acidity could not be accurately predicted and managed, and this can be particularly problematic in vintages where only mild acidity corrections are required in the must. Therefore, we wondered if the best way to obtain a certain level of lactic acid would be to let Lt produce as much LA as possible (8–12 g/L in our case) and add an adequate amount of this must to the Sc fermenting must. In this way, the Sc would complete the fermentation, and the wine would have the desired level of LA. In addition, we tested a condition (not yet proposed by yeast producers) of co-inoculation of Sc 12 h after Lt. This short interval could have led, at least theoretically, to better control of LA production, in the sense that Lt could have developed enough to produce an acceptable amount of LA before Sc would take over. The results of these experiments were satisfactory since the mixing method allowed us to control the amount of LA in the wine, resulting in sensory-acceptable acidification, especially in the 1:4 mixing condition. The short sequential inoculation also gave satisfactory results, as we found a sweet spot between Sc dominance, Lt activation, and subsequent LA production.

We then applied these protocols at the cellar level, and we chose the mixing protocol to acidify Divona, while the short inoculation protocol was used for Chasselas. Our data showed that the application of such protocols at the cellar level did not result in a significant degree of LA production and acidification. We hypothesized that this result might be due to the presence of indigenous yeasts that prevented Lt implantation. Both Divona and Chasselas musts were not treated with SO_2, which led to the undesirable development of indigenous yeasts, and this probably prevented the development of Lt. This hypothesis was directly supported by the FCM data in the Chasselas after settling before Lt inoculation. For Divona, we did not have direct microbiological evidence, but the fact that fermentation occurred in the first few days under the Lt condition (without further evidence of LA production) suggests that a yeast population may have prevented the implantation of Lt. This result highlights the difficulty of translating protocols from the bench scale to the cellar scale, especially in terms of managing the must microbiome, a characteristic that can vary from vintage to vintage. Specifically, the pasteurization protocol used at the lab scale ensured that the indigenous yeast load did not limit the implantation of the selected yeast. In this way, our bench-scale model provided the necessary robustness and guaranteed the reproducibility of the results. In winery assays, where the must cannot be pasteurized, and sulfites are not used to avoid Lt blockage, alternative solutions must be found. For example, lowering fermentation temperatures can be effective in controlling indigenous yeasts [17]. It should be noted that Lt can be functional at low temperatures and, therefore, the first stages of fermentation could be carried out at 15–17 °C to secure an advantage over indigenous yeasts.

Today, there is great interest in the application of biocontrol methods for regulating indigenous microbiomes. The main yeast used for biocontrol purposes is *Metschnikowia pulcherrima* (Mp), of which several oenological strains are already available. Mp, through different mechanisms (e.g., killer toxins, iron chelation, and nitrogen depletion, as reviewed in [18]), can prevent the development of indigenous yeasts and has already been successfully used in the settling phase [19,20]. In recent work, our group also observed a putative bioprotective effect of Mp at the prefermentative stage [21]. Thus, the use of Mp could facilitate the development of Lt, and this should be the subject of further experiments.

Another promising method for controlling the indigenous microbiome was applied by Morata et al. [22]. In this case, the authors used high-pressure homogenization technology (ultra-high-pressure homogenization, or UHPH) to reduce the indigenous microbiome load and facilitate Lt implantation in Verdejo musts. The authors obtained successful implantation of Lt and significant acidification of the must when using UHPH technology in comparison to conditions with or without SO_2.

Finally, it would be desirable to isolate and characterize Lt strains that are resistant to the amount of sulfites commonly used in musts (around 50 mg/L), for example, by applying adaptive evolutionary strategies, as already tested in Sc strains [23].

5. Conclusions

In this work, we characterized an enological strain of Lt in the laboratory and developed protocols to achieve an acceptable sensory level of acidification. Both the strategy of mixing in fermentation and the short sequential inoculation allowed for controlled acidifications, which can be useful for modulating wine acidity in very hot vintages. However, the transfer of the protocols to the cellar scale remains difficult, as the presence of active indigenous yeasts can prevent proper Lt implantation and subsequent LA production. Future experiments must be aimed at limiting the proliferation of indigenous yeasts in order to fully exploit the acidifying power of Lt.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/fermentation10090458/s1, Figure S1: Chasselas vinification assay at cellar scale; Figure S2: Divona vinification assay at cellar scale.

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