ADAPTATION OF LACTOBACILLI TOWARDS LOW pH AND SO_2 TO DEVELOP MLF IN BASE MUSTS FOR SPARKLING WINES

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Introduction

The MLF involves the bioconversion of malic acid into lactic acid and CO_2, but many other biochemical reactions occur simultaneously producing many other molecules that can improve quality, or be detrimental to the sensory quality or health (Eveline J. Bartowsky, Francis, Bellon, & Henschke, 2002; Cappello, Zapparoli, Logrieco, & Bartowsky, 2017; Cavin, Andioc, Etievant, & Diviès, 1993; Gil-Sánchez, Bartolomé Suáldea, & Victoria Moreno-Arribas, 2019; Hernández-Orte et al., 2009; Lerm, Engelbrecht , & du Toit, 2010; Lonvaud-Funel, 2010; Swiegers, Bartowsky, Henschke, & Pretorius, 2005). The MLF reduces the acidity of the wine, stabilizes wine by removing potential nutrients for other microorganisms, and produces aroma and flavour changes (E. J. Bartowsky, 2014; Virdis, Sumby, Bartowsky, & Jiranek, 2021).

In the past, MLF was mainly used to produce red wine, and sometimes very acidic white wine in order to reduce the excessive acidity of cool climates wines, or to improve the sensory complexity of white wines with lighter flavors, such as Chardonnay (Morenzoni, 2015). In recent years, winemakers have paid more and more attention to induce MLF in white wines, among them sparkling wines such as “Cava” (Pardo & Ferrer, in press). MLF is especially difficult for some wines of low pH values and high SO_2 content, as usually whites are. Besides organoleptic changes, FML can have a beneficial effect by bringing a bioprotection to the wines, preventing the growth of spoilage microorganisms, the synthesis of biogenic amines, or the decreases of wine quality, as hazing in sparkling wine bottles when a late and undesired MLF is produced (Carmen Berbegal et al., 2017; C Berbegal, Spano, Tristezza, Grieco, & Capozzi, 2017; Pardo & Ferrer, in press; Virdis et al., 2021).

Oenococcus oeni is the preferred bacterium to conduct MLF (Pardo & Ferrer, 2019). However, there is an increasing interest in other bacteria such as *Lactobacillus*, because of their particular organoleptic contribution to the wine (Cinquanta, De Stefano, Formato, Niro, & Panfili, 2018; Esteban-Torres et al., 2015; Gambetta, Bastian, Cozzolino, & Jeffery, 2014; Lucio, Pardo, Krieger-Weber, Heras, & Ferrer, 2016; Sereni, Phan, Osborne, & Tomasino, 2020; Volschenk, 2006). One of the advantages of using *Lactobacillus* is that some species can ferment sugars homofermentatively to lactic acid without producing acetic acid when growing. This fermentation acidifies the final wine and can counterbalance the relative increase in pH produced by MLF (Lucio et al., 2016).

One disadvantage of *Lactobacillus*, however, is their greater sensitivity to low pH and SO_2 than *Oenococcus*. This work aimed to adapt already selected strains of the genus *Lactobacillus* to grow, perform an early MLF, and to decrease the final pH in Macabeo white grape musts. The adaptation to low pH and presence of SO_2 is related to the expression of a series of stress genes associated with proton pumps to keep the internal pH and homeostasis (Beltramo, Desroche, Tourdot-Maréchal, Grandvalet, & Guzzo, 2006; Bon et al., 2009; Darsonval, Julliat, Msadek, Alexandre, & Grandvalet, 2018; Jean Guzzo & Desroche, 2009; J. Guzzo et al., 2000; Liu et al., 2017; Margalef-Català, Araque, Bordons, & Reguant, 2017; Margalef-Català, Felis, et al., 2017; Olguín, 2010; Olguín, Bordons, & Reguant, 2010). Several stress genes have been related to the resistance towards ethanol in *O. oeni* (Cafaro, Bonomo, & Salzano, 2014; G. da Silveira, 2003; M.G. da Silveira & Abee, 2009; M. Graça da Silveira, Golovina, Hoekstra, Rombouts, & Abee, 2003; M.G. da Silveira, San Romão, Loureiro-Dias, Rombouts, & Abee, 2002; Maitre et al., 2014; Margalef-Català, Araque, Weidmann, et al., 2017; Silveira, Baumgartner, Rombouts, & Abee, 2004; Steensels, Gallone, Voordeckers, & Verstrepen, 2019; Tourdot-Maréchal, Gaboriau, Beney, & Diviès, 2000), pH (Bourdineaud, 2006; Thuy, Huong, & Son, 2011), osmotic pressure (Le Marrec, Bon, & Lonvaud-Funel, 2007), sulphites (J. Guzzo, Jobin, M. P. and Diviés, C. , 1998), etc. This resistance has been also correlated to the synthesis of exopolysaccharides by Dimopoulou et al. (2018). Extensive transcriptomic and proteomic studies have been performed during the adaptation period of *O. oeni* cells before MLF start (Margalef-Català, Araque, Bordons, Reguant, & Bautista-Gallego, 2016). For all that, *O. oeni* cells have been successfully adapted to acidity and phenol presence (Bordas, Araque, Bordons, & Reguant, 2015; Breniaux et al., 2018; García-Ruiz et al., 2013), and ethanol (Costantini et al., 2015). The stress response has also been evaluated in lactobacilli (Papadimitriou et al., 2016; Ricciardi et al., 2012; van de Guchte et al., 2002), and also proteomic studies have been performed to know the effects of the stress response (Hussain, Hosseini Nezhad, Sheng, & Amoafo, 2013). Regarding resistance and adaptation, different species of *Lactobacillus* have been trained to grow under unfavorable conditions (Golod et al., 2009; Vrancken, Rimaux, Weckx, De Vuyst, & Leroy, 2009), adapting them towards temperatures (high or low), acidity, ethanol, solvents, and other stresses (Bravo-Ferrada, Gómez-Zavaglia, Semorile, & Tymczyszyn, 2015; Fiocco, Capozzi, Goffin, Hols, & Spano, 2007; Montanari, Sado Kamdem, Serrazanetti, Etoa, & Guerzoni, 2010; Rimaux et al., 2012; Su, Schlicht, & Gänzle, 2011; Succi et al., 2017; Suutari & Laakso, 1992; van Bokhorst-van de Veen et al., 2011; Vrancken, Rimaux, Wouters, Leroy, & De Vuyst, 2009).

The main objective of this study is the isolation, selection and adaptation of strains from the *Lactobacillus* genus to decreasing values of pH and increasing concentrations of SO_2, so they can carry out a successful MLF and acidification of the final wine.

Materials and methods

Lactic acid bacteria were isolated from Macabeo grape musts. After identification at the species level by 16S-ARDRA analysis (Ana MarÍa Rodas, Ferrer, & Pardo, 2003), they were typed by RAPD analysis with M13 primer (A. M. Rodas, Ferrer, & Pardo, 2005). Digitized images of all band patterns were processed by Bio-Numerics software version 6.6 (Applied Maths), which carried out the conversion, normalization and similarity analysis of these patterns. 16S-ARDRA patterns were grouped by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering method using the Dice similarity coefficient. RAPD patterns were analysed using Pearson's product moment correlation coefficient and the UPGMA clustering method.

Once the different strains were established, they were subjected to stress and adaptation rounds consecutively towards decreasing pH values in Macabeo grape musts: Therefore, from the initial pH value of 6.5, 5 consecutive stress and adaptation rounds were made to 4.5, 3.8, 3.2, 3 and 2.9 pH units. In every round, the cell concentration of cultures, once inoculated, was between 2 x 10^6 CFU/mL and 5 x 10^6 CFU/mL. The incubation temperatures were always kept at 18 ºC. Cell populations were quantified by OD_{600nm} and plate-counting, and metabolites as sugars, organic acids and ethanol were quantified by HPLC. The assays were performed in triplicate.

Once the cells have been adapted to low pH values, a similar strategy was designed to adapt them to sulphur dioxide. They were independently inoculated in a 3.2 pH Macabeo white grape must at a final concentration of 5x10^6 CFU/mL. Once they had grown, they were consecutively inoculated at 5x10^6 CFU/mL in grape musts with increasing concentrations of SO_2 (1, 1.5, 2 and 3 g/hL). Growth dynamics were determined by the plate count method, and malolactic fermentation was monitored by HPLC. All assays were performed at 18 ºC to mimic winemaking conditions.

Results and discussion

Sixty lactic acid bacteria were isolated from Macabeo grape must, and were identified at the species level by 16S-ARDRA analysis (Table 1) and strain level by M13 RAPD analysis (Figure 1). From the 60 isolates, 4 belonged to two strains of *O. oeni*, and the rest divided into 16 strains belonging to three species of lactobacilli: *Lactiplantibacillus plantarum* (syn. *Lactobacillus plantarum*), *Lacticaseibacillus casei* (syn. *Lactobacillus casei*), and *Levilactobacillus brevis* (syn. *Lactobacillus brevis*).

STRAIN NAME	SPECIES	PROFILE	NUMBER OF ISOLATES
I	*Lactobacillus plantarum*	P1	6
J		P2	1
O		P3	11
D, E, N		P4	13
G		P5	2
M		P6	1
B		P7	4
A		P8	2
C		P9	2
O	*Lactobacillus casei*	P10	3
H		P11	1
L	*Lactobacillus brevis*	P12	3
F		P13	1
K		P14	1
Q		P15	3
R		P16	2
S	*Oenococcus oeni*	P17	2
T		P18	2

***Table 1**. Distribution in species and strains of the Macabeo grape must isolates.*

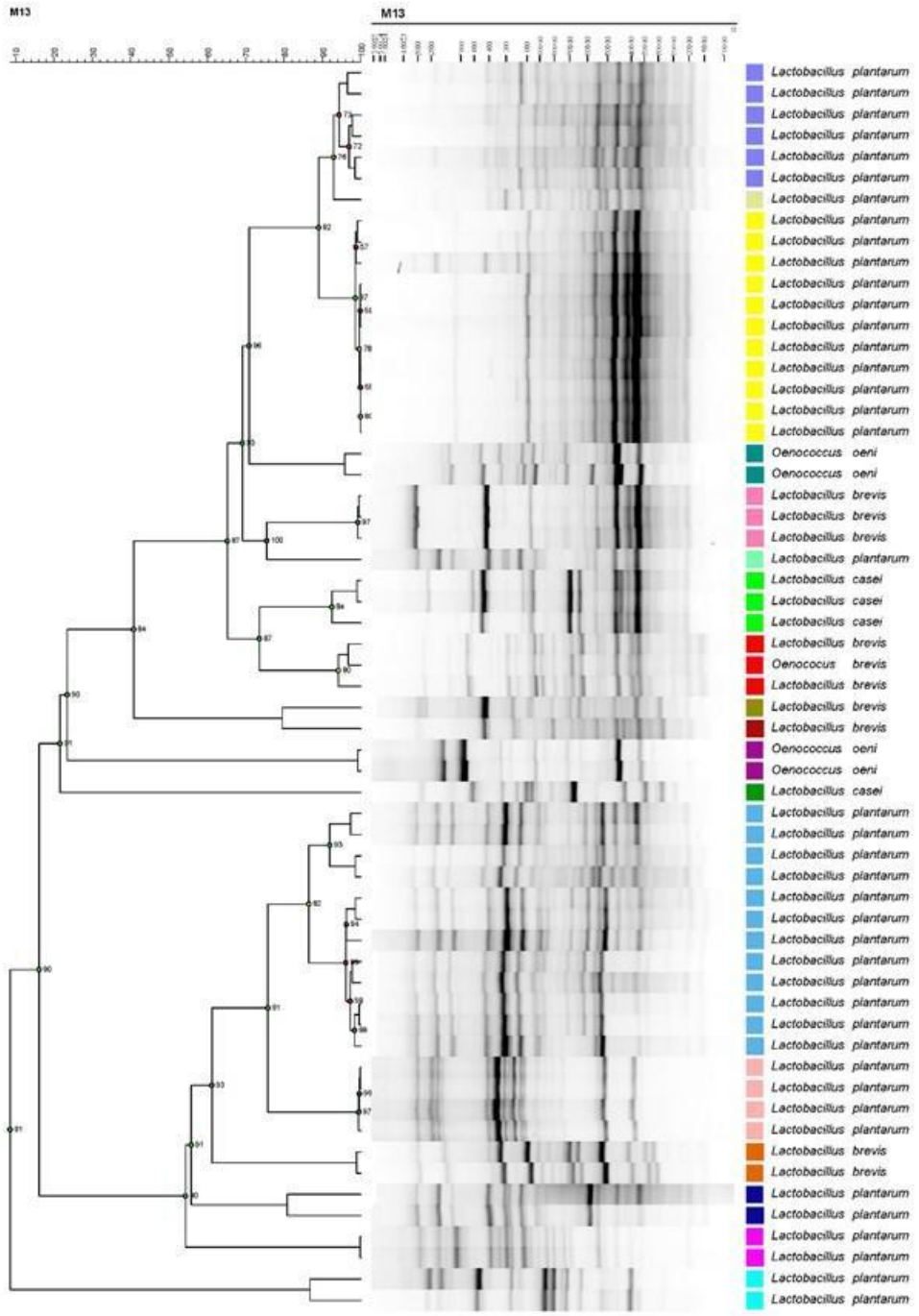

Figure 1. *Dendrogram derived from a comparison of the M13 band profile, obtained from the Macabeo grape must isolates. The clustering analysis was carried out in software BioNumerics 6.6. using the Unweighted Pair Group Method with Arithmetic Average (UPGMA) and the Pearson correlation coefficient.*

After stress, all the *Lactobacillus* strains could be able to grow at pH values among 4.5 and 3.2. At pH 3.2 they reached a cell density between 10^7 and 10^8 CFU/mL (Figure 2). However, we could not detect any growth at the pH value of 2.9, although cells remained viable and metabolically active (data not shown). The growth parameters (inflection point and maximum population) of the different *Lactobacillus* strains in Macabeo grape must with progressively decreasing pH values are shown in Figure 3. These growth parameters help in the choice of the best strains for the selection and adaptation programme.

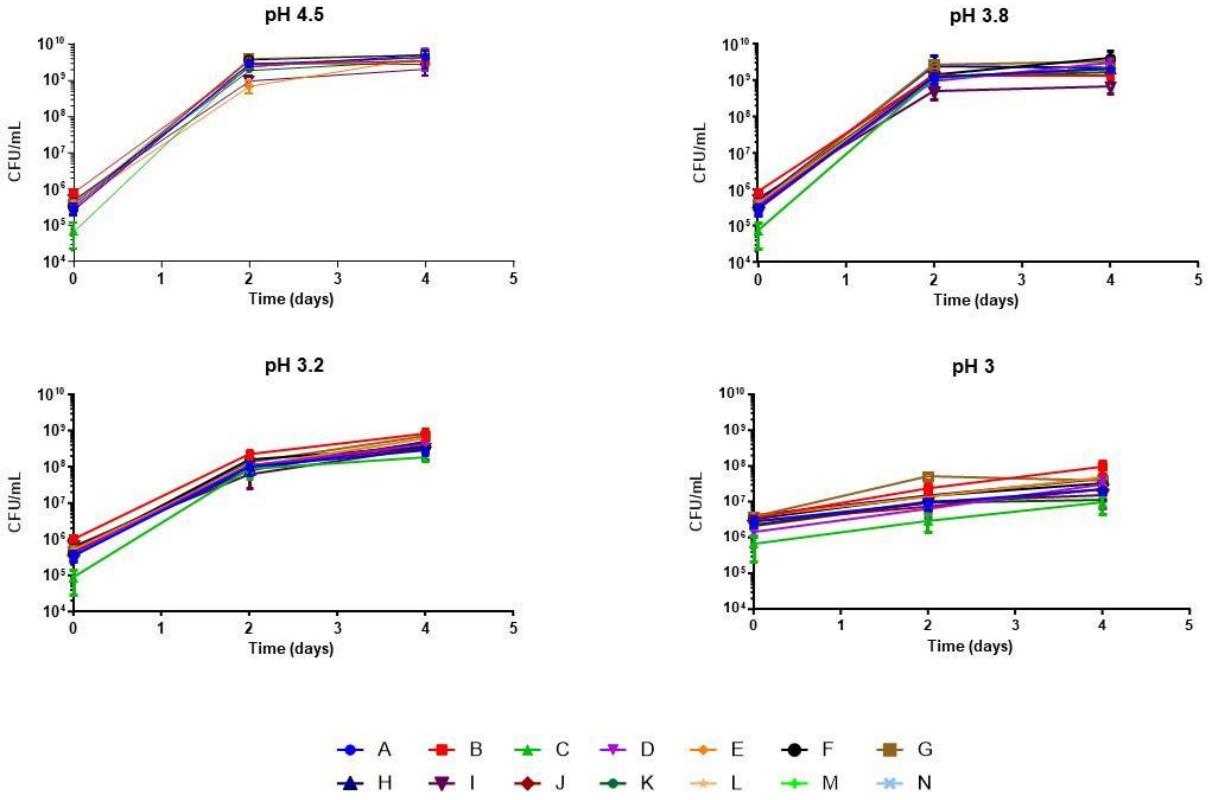

***Figure 2**. Ability of the different* Lactobacillus *strains to grow in Macabeo grape must with progressively decreasing pH values.*

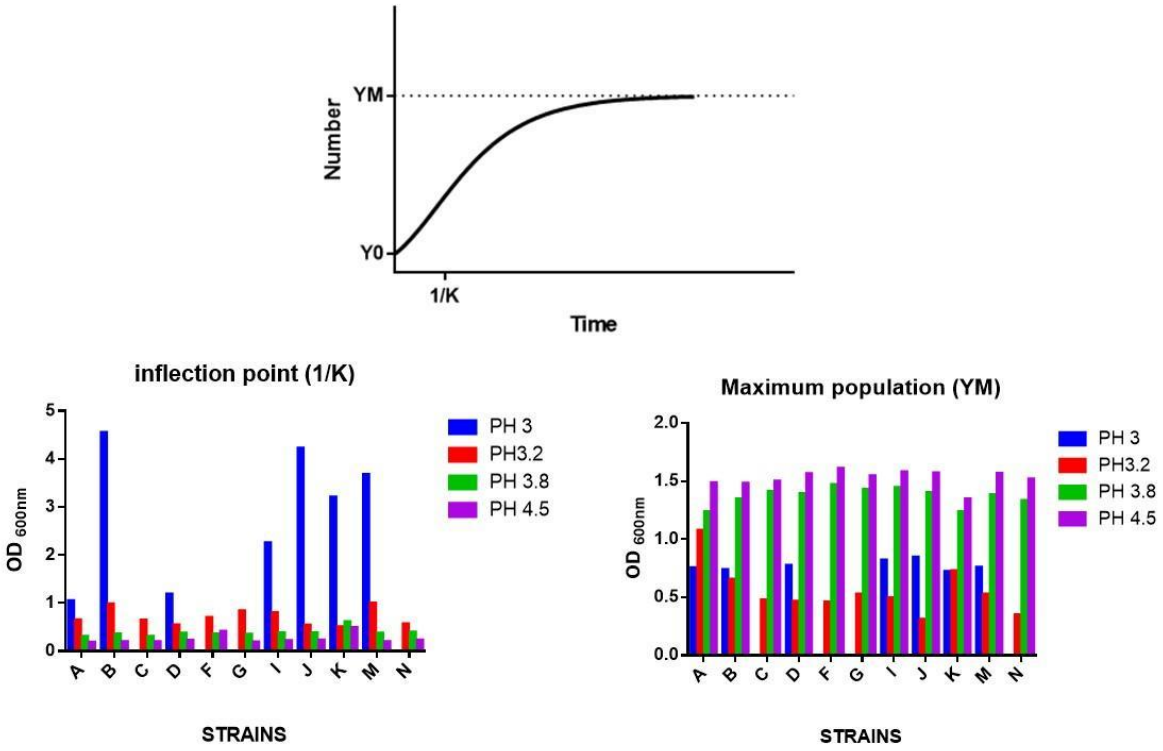

***Figure 3**. Growth parameters (inflection point and maximum population) of the different* Lactobacillus *strains in Macabeo grape must with progressively decreasing pH values.*

Once the cells had been adapted to low pH and were able to grow in 3.2 pH Macabeo grape must but not before, they were subjected to a new stress programme before adapting them to increasing SO_2 concentrations (Figure 4). All *Lactobacillus* strains grew successfully, maintaining or even increasing their biomass in all cases.

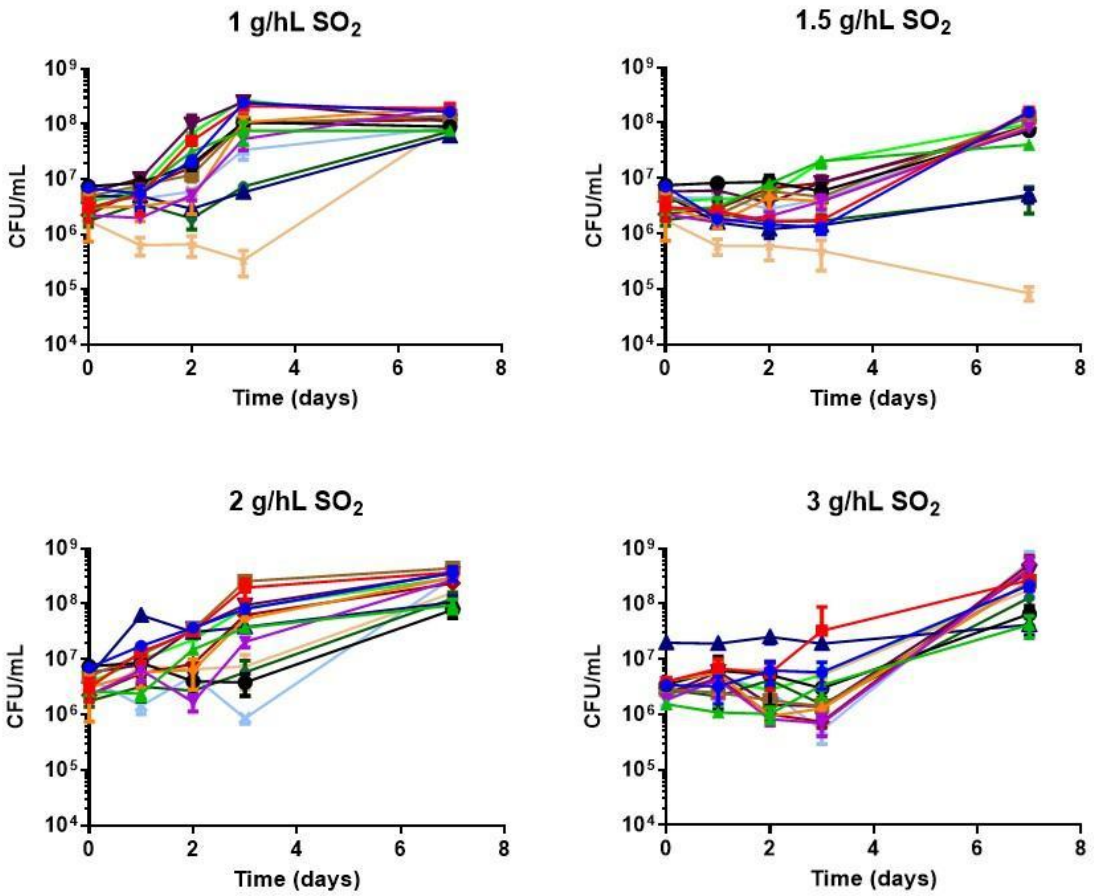

***Figure 4**. Ability of the different* Lactobacillus *strains to grow in Macabeo grape must with progressively increasing SO_2 values.*

Regarding malolactic fermentation, our findings show that it was carried out by all strains at most after 7 days at any condition, and many times in less than 2-3 days, depending on the strain (Figure 5). Malic acid consumption and lactic acid production rates were, however, dependent on the strain and sulphur dioxide concentration. For SO_2 lower concentrations, most strains transform malic acid into lactic acid in three days or less, but as SO_2 concentrations increase, so does the degradation time. Similarly, lactic acid production seems to decrease for most strains at higher concentrations of sulphur dioxide.

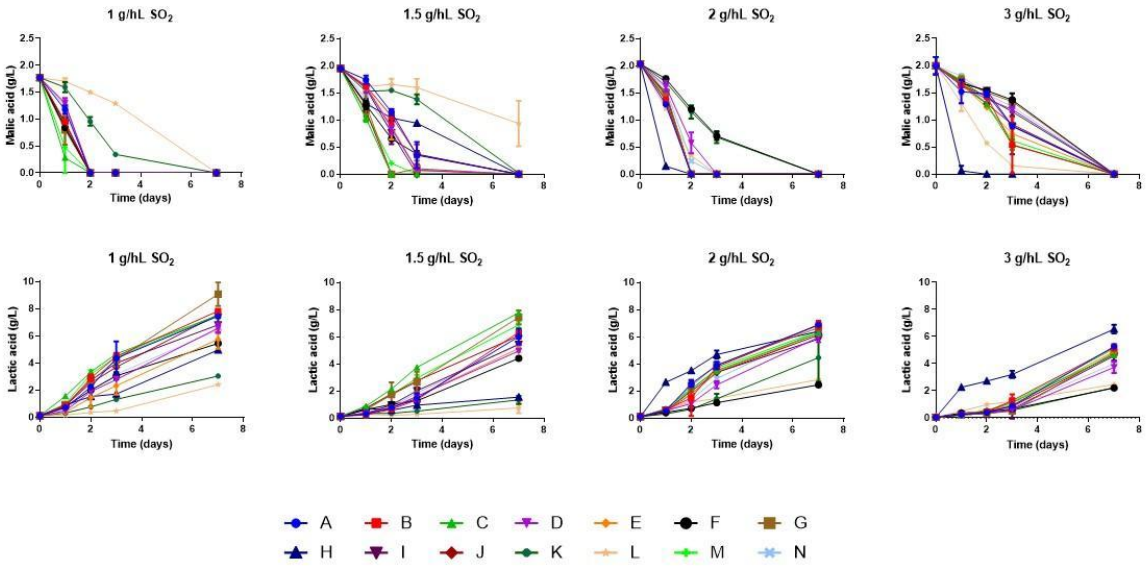

***Figure 5**. Malic acid degradation and lactic acid production of the different* Lactobacillus *strains in Macabeo grape must with progressively increasing SO_2 values.*

It can be observed that the stoichiometry of the MLF reaction is not one-to-one in terms of malic and lactic acid molar relationship in these dynamics (Figure 5). For examples, for 1 g/hL SO_2, up to some 9 g/L of lactic acid were synthesized while only 1.75 g/L malic acid were degraded. For 3 g/hL SO_2, up to some 6 g/L of lactic acid were synthesized while 1.75 g/L malic acid were degraded. This is one of the advantages of inoculating these bacteria in grape must: the ability to transform sugars exclusively into lactic acid (Carmen Berbegal et al., 2016; Lucio et al., 2016; Onetto & Bordeu, 2015; Pardo & Ferrer, 2019, in press). The lactic fermentation (LF) yield of these strains was calculated as the amount of lactic acid synthesized from the sugars consumed, independently of the lactic acid produced from the MLF (Figure 6). Lactic acid production by LF is strain- and species-dependent. Obviously, lower amounts of lactic acid were synthesized in increasing SO_2 concentrations, accordingly to the ability to grow every strain under these circumstances (Figure 4).

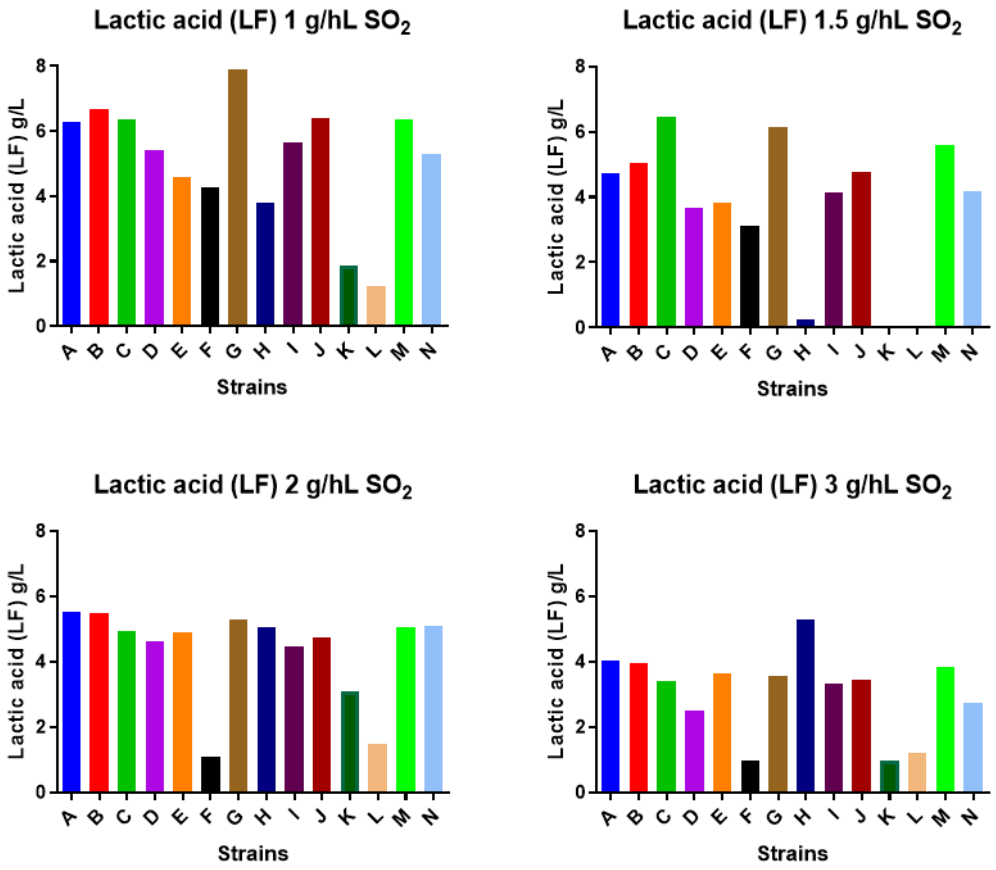

***Figure 6**. Lactic fermentation (LF) calculated as the amount of lactic acid synthesized from the sugars consumed by different strains in 3.2 pH Macabeo grape must at different SO_2 concentrations, independently of the lactic acid produced from the MLF.*

Lactic acid bacteria can convert glucose and fructose present in grape musts into lactic acid in a process called lactic fermentation. This process might be useful in white wines, as the production of this acid can counterbalance the pH increase due to MLF, or even ending in a more acidic wine although MLF is done. Besides, a lower pH provides extra freshness that is desired in this type of wines. Our data show that, in most cases, strains can produce lactic acid from sugar fermentation at all sulphur dioxide concentrations. The behaviour of the lactic bacteria regarding SO_2 concentration depends on strain and species; however, at higher sulphur dioxide concentrations, there is a tendency to decrease the production of lactic acid from sugars.

The final pHs of the wines after both alcoholic and malolactic fermentations were measured as well. A grape must not inoculated with bacteria but subjected to alcoholic fermentation was used as a control to compare the behaviour of the strains (Figure 7). As the graphs show, there was a pH decrease in grape musts fermented with most of the strains compared to a non-inoculated control. *Lactobacillus plantarum* and *L. casei* strains decreased more the pH, between 0.2 and 0.4 units compared to *Lactobacillus brevis,* according to the results from lactic fermentation (Figure 4).

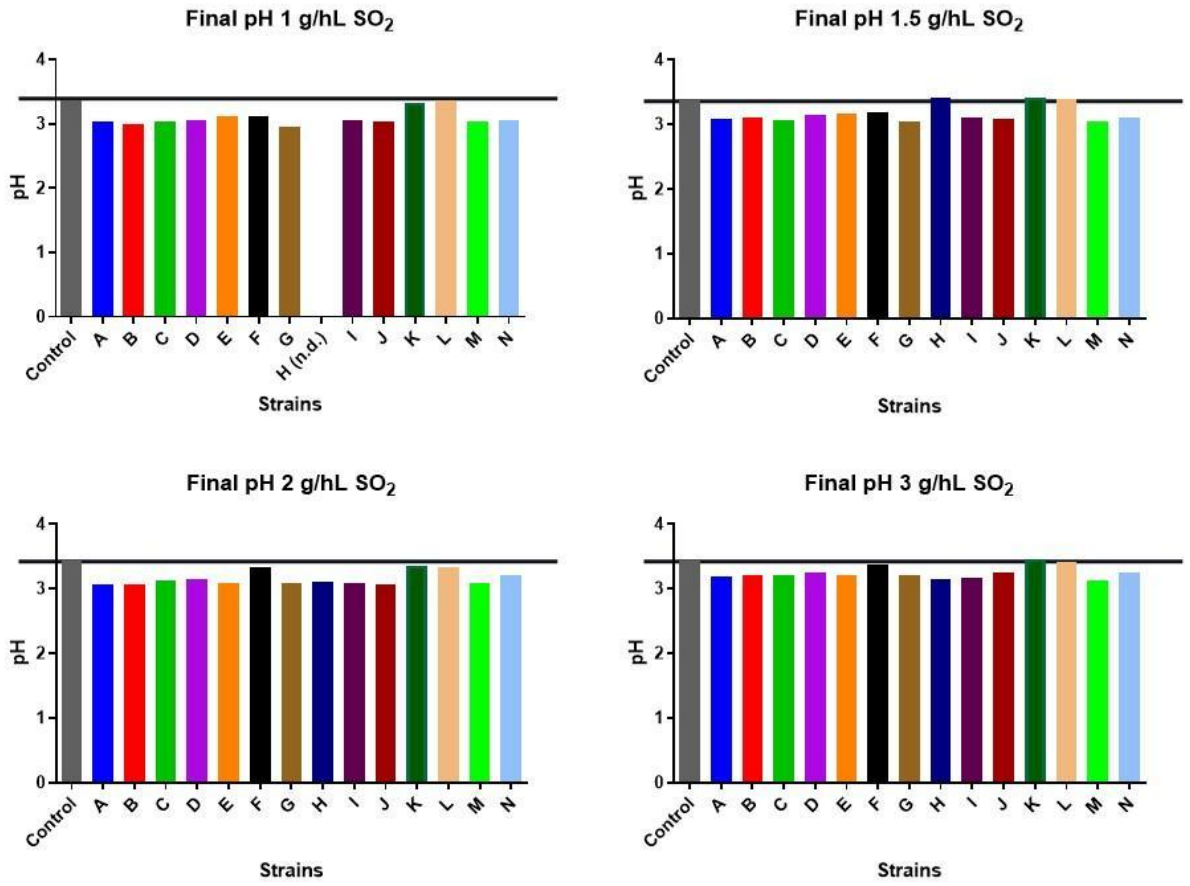

***Figure 7**. pH in final wines, once both MLF and alcoholic fermentations were completely finished.*

As a demonstration of the usefulness of the stress and adaptation strategies, a comparison in growth ability between the same non-adapted and adapted strain is shown in Figure 8. In can be observed that at pH 3.2 and 0 g/hL SO_2 (Figure 8C), only the adapted strain can grow. Even more dramatically, at pH 3.2 and 3 g/hL SO_2 (Figure 8B), the non-adapted strain drops immediately, whereas the adapted one is not only able to survive but grow actively in a few days to more than 10^8 CFU/mL.

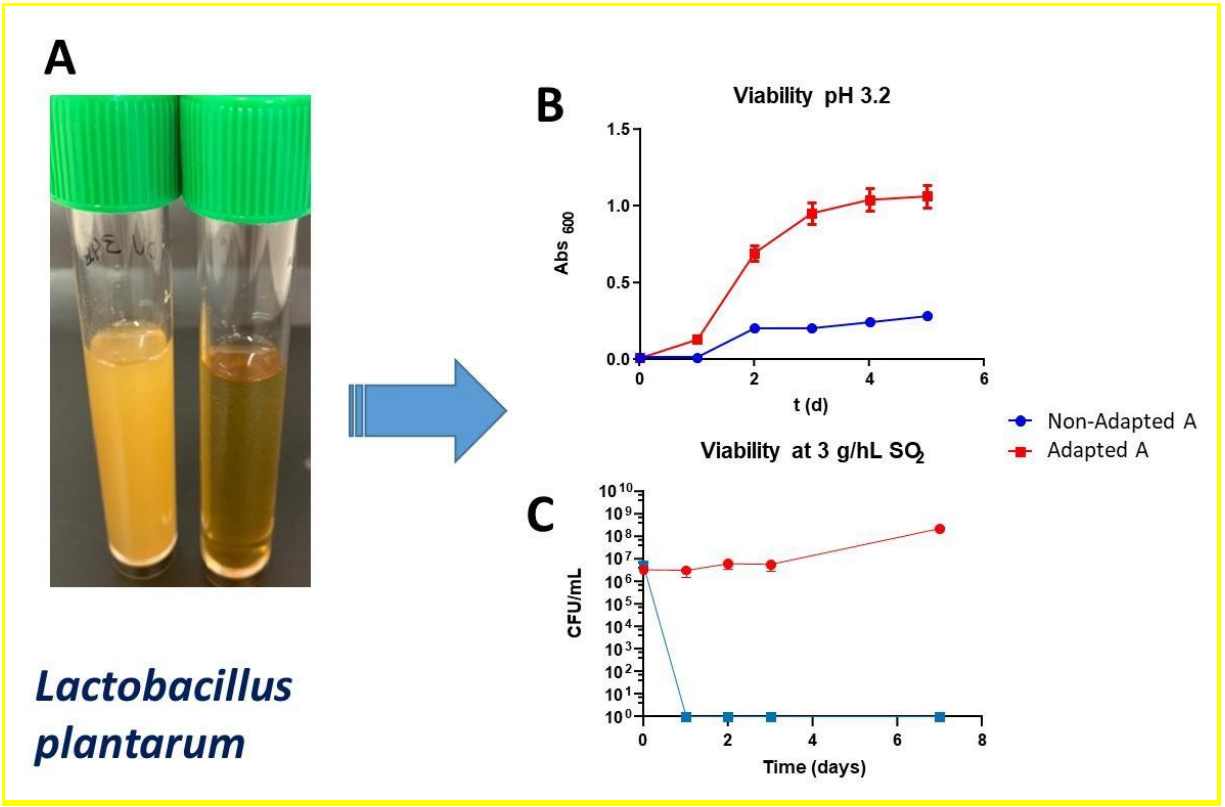

***Figure 8**. Demonstration on the adaptation ability of a* Lactobacillus plantarum *strain to grow in pH 3.2 and 3 g/hL SO_2 (B), and in pH 3.2 and 0 g/hL SO_2 (C) compared to the original non-adapted strain.*

Conclusions

We have isolated, identified, typified, and successfully adapted several *Lactobacillus* strains to SO_2 at low pH. They were able to maintain or even increase their biomass in all cases. Most of the *Lactobacillus* strains reach their maximum cell concentration within 48 h. *Lactobacillus* strains adapt progressively to low pH, being *L. plantarum* strains the best performers, secondly *L. casei*, and thirdly *L. brevis* strains. MLF was carried out by all strains in less than 7 days. Most of the strains were able to produce additional lactic acid from sugars and decreased the final pH after alcoholic and malolactic fermentations. At pH 2.9, no growth is detected, although the *Lactobacillus* strains remain viable during the first days of fermentation. MLF is completely and rapidly performed, and *Lactobacillus* strains were able to degrade 2 g/L of malic acid in 3 days or less. As a strategy, to induce the MLF in a grape must it is much better to inoculate with cells grown at a lower pH value than the pH in the grape must where they must grow, whenever possible. When compared to the same not adapted strain to low pH, these bacteria grow much better, produce more lactic acid from sugars, and develop a faster MLF.

Benefits for wine production

A fast, reliable, and safe MLF is possible after adapting lactobacilli to low pH and the presence of SO_2 in grape musts. This inoculation of these adapted bacteria is compatible with co-fermentation with *Saccharomyces* and non-*Saccharomyces* yeasts. Just after alcoholic fermentation, wines can be completely stabilized, which saves time, money, and spoilage risks. Organoleptic improvements are achievable because of the metabolism of LAB while actively growing in grape musts; this brings new wine characteristics to obtain new products on the market. This strategy is applicable to a wide variety of wines, both reds and whites, and not only t white sparkling wines. The growth and early FML produces bioprotection against spoilage microorganisms and avoids undesired late MLF (haze, ropiness, etc.). The growth of these bacteria in grape musts produces simultaneous biological acidification (lactic acid) from sugars, counterbalancing the pH increase due to MLF, or even ending in a more acidic wine although MLF is done. Finally, the consumption of sugars by actively growing bacteria reduces the final ethanol content in wines as fewer sugars are available to yeasts.

Abstract

In some white wines, malolactic fermentation (MLF) is very interesting, and for low pH wines this process is particularly difficult. Although MLF is generally not recommended for sparkling white wine, some winemakers prefer to promote it to contribute to organoleptic complexity and to avoid undesirable MLF in the bottle. *Oenococcus oeni* is generally the bacterium of choice for performing MLF. However, people's interest in other species as alternative (such as *Lactobacillus*) is increasing. However, one disadvantage of lactobacilli is that they are more sensitive to low pH and SO_2 than *O. oeni*, and to promote MLF with these bacteria some starters' producers inoculate high doses of non-growing cells in grape musts. This work aims to accomplish the growth of some selected strains of *Lactobacillus* in grape juice and to perform an early MLF. With this strategy, beyond performing the MLF homofermentative and facultative heterofermentative bacteria can contribute clearly to maintain or even decrease the final pH in wines by producing lactic acid from sugars; they also produce more complex wines and prevent the spoilage of an undesired late MLF in bottles.

To perform this adaptation, fourteen *Lactobacillus* selected strains were successively inoculated after stress and adaptation steps to the lowering of pH and the increasing concentration of SO_2. The cell concentration of the inoculum was in the order of x 10^6 CFU/mL to allow the growth and synthesis of lactic acid. All *Lactobacillus* strains gradually adapted to low pH and SO_2 and could grow at pH 3.2 and 3 g/hL SO_2 concentration, thereby maintaining or even increasing their final biomass. After 7 days, all strains underwent MLF. Malic acid consumption rate and lactic acid production depended on the strain. The final pH of wines was maintained or even decreased, even when complete MLF was achieved. This biological acidification of wines helps to avoid the loss of acidity and pH increase of wines in a scenario of climate change.

Acknowledgements

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