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Selection of *Lactobacillus* strains to induce biological acidification in low acidity wines

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ABSTRACT

Because of global warming, wines are obtained nowadays with high pH values and low acidity. This results in wines with disturbed flavor and increased susceptibility of microbial spoilage. The aim of this work was the selection of *Lactobacillus* strains with ability to induce biological acidification in low acidity grape musts to obtain more acidic wines. A screening of *Lactobacillus* strains was carried out using several selection criteria. *Lactobacillus* strains that grew in must, carried out the malolactic fermentation, acidified grape must, synthesized lactic acid from sugars, and showed high resistance to lysozyme and sulfur dioxide were selected. Selected strains were characterized based on their metabolism in grape must and their ability to synthesize biogenic amines.

1. Introduction

Some of the consequences of global warming have been the sharp decrease in acidity of wines in many regions, and an imbalance between phenological stage and ripeness. This warming leads to vintages with low total acidity and high sugar concentration, producing wines organoleptically disrupted and very susceptible to microbial spoilage. An example of this happens in La Rioja (Spain) where in recent years an increase of 0.5 units in the pH of some wines has been observed, reaching sometimes pH values of 3.8–4.0 (Hidalgo, 2003; Martinez de Toda & Balda, 2014; Mira de Orduña, 2010; Palacios García, Suárez Martinez, & Heras Manso, 2006).

Currently, the chemical acidification is commonly used to solve this important problem. It is based on the addition of authorized and limited organic acids such as malic, tartaric or lactic acid in grape must and/or in wine (Carvalho, Costa, Franco, & Curvelo-Garcia, 2001; Hidalgo, 2003). Other techniques to decrease the pH of wines based on physical methods are treatment with ion exchange resins (Hidalgo, 2003; Minguez, 2003) or electrodialysis (Hidalgo, 2003; Ochoa, Valle, Hilera, Belaustegui, & Elejalde, 1999). These treatments are regulated by the "Organisation Internationale de la Vigne et du Vin" (OIV) for grape musts, wines, and special products (OIV, 2015). For musts, accepted practices are acidification, (6/79, Oeno 4/03, Oeno 360/2010), chemical acidification (Oeno 3/99, Oeno 13/01), microbiological acidification (Oeno 5/03), acidification by *Saccharomyces* (Oeno 4/02), acidification by electromembrane treatment (Bipolar membrane electrodialysis) (Oeno 360/2010), and acidification by cation exchanger treatment (Oeno 442/2012). For wines, accepted practices include acidification (6/79), chemical acidification (Oeno 4/99, Oeno 14/01), treatment with calcium sulphate (plastering) (3/85), treatment with ion exchangers (6/76) (Oeno 443-2012), acidification by electromembrane treatment (Bipolar membrane electrodialysis) (Oeno 361/2010), and acidification by cation exchanger (Oeno 443-2012). Special products can be acidified by chemical acidification (Oeno 439-2012). Nevertheless, chemical and physical methods have several disadvantages such as being very expensive for cellars and strictly regulated by legislation. Conversely, biological methods can be a good alternative to perform acidification in wines by use of lactic acid bacteria because they are allowed in oenology, even in organic wines (OIV, 2016). Moreover, lactic acid synthesized by microorganisms is more stable as it is not lost by precipitation, it is more integrated and provides more equilibrated acidity, better taste and flavor (Dequin, Baptista, & Barre, 1999; Uthurry, Navascués López-Cordón, González, & Suárez Lepe, 2005; Val, Ferrer, & Pardo, 2002; Yeramian, 2003).

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The optimal way to induce biological acidification is the use of starter cultures. The development of starter cultures is a multidisciplinary approach requiring not only an ecological study of man-made food ecosystems (Vogel, Ehrmann, & Gänzle, 2002), but also the characterization of useful technological and physiological features in order to select those strains most suitable for industrial applications. Selection of bacterial strains for induction of malolactic fermentation (MLF) have been essentially based on *Oenococcus oeni* because is the wine-related species most adapted to harsh conditions of wine. For this, selection criteria such as the good survival in wine, the rapid consumption of malic acid and positive organoleptic properties were usually used (Henick-Kling, Sandine, & Heatherbell, 1989; Ruiz, Izquierdo, Seseña, & Palop, 2010). However, to develop acidifying starters, many other useful oenological properties need to be assayed, such as the high synthesis of lactic acid from grape must sugars, the pH decrease in grape must, the performing of MLF, the incapacity to synthetize biogenic amines (BA) and acetic acid (volatile acidity), or the high resistance to wine antiseptics such as lysozyme or sulfur dioxide.

Among different lactic acid bacteria found in the vinification process, homofermentative or facultative heterofermentative *Lactobacillus* are good candidates to be used as acidifying starters. These can synthesize only lactic acid from grape must sugars by lactic fermentation, and have no danger of acetic acid synthesis (Ribéreau-Gayon, Dubordieu, Donèche, & Lonvaud, 2006). The synthesis of lactic acid during the vinification of high pH grape musts produces a beneficial decrease in the final pH (Estela, Rychtera, Melzoch, Quillama, & Egoavil, 2007; Garde, Jonsson, Schmidt, & Ahring, 2002; Kious, 2000; Mercier, Yerushalmi, Rouleau, & Dochain, 1992; Pardo, 2003; Roy, Blanch, & Wilke, 1982). In addition, *Lactobacillus* strains, as *O. oeni*, also contain the malolactic enzyme. Therefore, these bacteria could carry out the MLF and the acidification simultaneously during vinification (Bravo-Ferrada et al., 2011; Caspritz & Radler, 1983; Chagnaud, Naouri, Arnaud, Galzy, & Mathieu, 1989; Lucio, Ferrer, Krieger, Heras, & Pardo, 2011; Claus; Prahl, 1989; Strasser de Saad, Pesce de Ruiz Holgado, & Oliver, 1984; Velázquez et al., 1991). Therefore, the unpleasant and strong taste of acid malic is removed from wine, resulting in a relative increase of pH, which is rapidly surpassed by the synthesis of lactic acid by fermenting sugars.

To select *Lactobacillus* strains to become acidifying starters, they should show a rapid and good growth in grape must, with high malolactic and lactic fermentation ability, with high acidification capacity, and high resistance to antiseptics used in vinification such as lysozyme and sulfur dioxide (Bartowsky, Costello, Villa, & Henschke, 2004; Cejudo-Bastante et al., 2010; Lafon-Lafourcade & Peynaud, 1974; Lasanta, Roldán, Caro, Pérez, & Palacios, 2010; Ribéreau-Gayon et al., 2006; Romano & Zambonelli, 1993). Another important property is the inability to synthesize BA since these compounds detract the quality and acceptability of wines, and also pose a risk to consumer health (Caruso et al., 2002; Aline; Lonvaud-Funel, 2001; Moreno-Arribas, Polo, Jorganes, & Muñoz, 2003).

The aim of this work was the selection of *Lactobacillus* strains for their use as acidifying starter cultures to carry out a biological acidification in low acidity grape musts.

2. Material and methods

2.1. Strains, media and growth conditions

Thirty-one *Lactobacillus* strains of Enolab collection (University of Valencia) were used in this work (Table 1). Previously, these strains had been isolated in spontaneous vinifications at different cellars. *Lactobacillus* strains were grown in 10 mL of MRS broth (Scharlab) supplemented with 0.5 g/L of cysteine (Merk), pH 6.5 at 28 °C for 48 h.

MC broth was used to perform different assays in this work, simulating white grape must. MC broth containing concentrated grape must of "Airén" variety, diluted in distilled water (1:4), supplemented with 0.3 g/L of Nutrient VitEnd® (Lallemand), adjusted to pH 3.5 and sterilized at 115 °C for 30 min. Composition of MC broth was 93 g/L of glucose, 103 g/L of fructose, 1.6 g/L of malic acid, 0.46 g/L of citric acid and 1.42 g/L of tartaric acid.

2.2. Screening of Lactobacillus strains

The selection of *Lactobacillus* strains for their use as acidifying starter cultures based on first and second order criteria was performed. Rapid growth and malolactic activity in grape must, high synthesis of lactic acid from must sugars and high decrease of grape must pH were used as first order criteria, and high resistance to lysozyme and sulfur dioxide were used as second order criteria.

For this assay, cultures of the 31 *Lactobacillus* strains were grown in MC broth in 96-well microplates (Falcon™Microplates BD). Approximately 2×10^6 cells/mL of each strain were inoculated in 200 μL of MC broth per well, in triplicate. A non-inoculated MC broth was established as a control. Cultures were incubated in a Fluostar Optima Microplate Reader (BGM LABTECH, GmbH) at 28 °C for 7 days and O.D. readings were performed at 600 nm every 12 h.

2.2.1. Evaluation of growth

The growth curves of the strains were obtained as described, and the Area Under Curve (AUC) parameter was determined (Herbers, Elder, & G.Woo, 2011).

2.2.2. Evaluation of malolactic ability

The malolactic ability of *Lactobacillus* strains was determined by the consumption of malic acid in cultures at the end of the assay, calculated by High Performance Liquid Chromatography (HPLC) following the procedure described by Berbegal, Benavent-Gil, Pardo, and Ferrer (2015).

2.2.3. Evaluation of lactic acid synthesis

The lactic acid synthesis ability was determined by the quantification of this acid in cultures at the end of the assay. Concentration of lactic acid was determined by HPLC following the procedure described by Berbegal, Benavent-Gil, et al. (2015). Lactic acid from MLF was determined by the corresponding stoichiometric calculation. Lactic acid from lactic fermentation from sugars was calculated by the difference between total lactic acid and lactic acid from MLF.

2.2.4. Evaluation of grape must acidification ability

Grape must acidification ability of strains was determined by measure of differences in pH between control medium and inoculated medium at the end of assay. A pHmeter pH 330i (WTW) coupled to an electrode SLIMTRODE (Hamilton) were used to measure the pH of cultures.

2.2.5. Evaluation of resistance to lysozyme and sulfur dioxide

Strains which provided the best results in the first order criteria were tested in a microplate assay (Falcon™ Microplates, BD) to evaluate its resistance to lysozyme and sulfur dioxide. In each assay, MC broth was supplemented with 0.2 mg/mL of lysozyme and with 0.05 mg/mL of sulfur dioxide (0.1 mg/mL of potassium metabisulfite (Agrovin)). Cultures of strains were carried out as described above, in duplicate. A control was set without lysozyme and sulfur dioxide addition. The growth of strains in these conditions was determined as described in 2.2.1.