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Selection and technological potential of *Lactobacillus plantarum* bacteria suitable for wine malolactic fermentation and grape aroma release

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ABSTRACT

Lactobacillus plantarum strains have resistance mechanisms that enable them to survive and proliferate in wine, which makes them potential malolactic fermentation (MLF) starter cultures. This work focused on the technological characterization of 11 *L. plantarum* strains isolated from Southern Italian wines that undergo spontaneous MLF, and proposes a selection of new *L. plantarum* malolactic starters. These strains were characterized according to their oenological characteristics, their ability to produce biogenic amines and bacteriocins, their response to the presence of phenolic compounds, their enzymatic activities and their ability to produce wine odorant aglycones from odourless grape glycosidic aroma precursors. Finally, the malolactic activity of one selected strain was assessed in Cabernet Sauvignon wine, using two inoculation methods. *L. plantarum* strains tested were not producers of biogenic amines. In particular, the M10 strain showed a good resistance to high levels of ethanol and low pH, it has a good malolactic performance and β-glucosidase activity, this last one demonstrated both directly through the measurement of this enzymatic activity and indirectly by following the release of volatile aglycones from commercial and natural grape glycosidic odourless precursors. These results demonstrated the potential applicability of M10 as a new MLF starter culture, especially for high-ethanol wines.

1. Introduction

Malolactic fermentation (MLF) plays an important role in the production of wine, especially red wines, resulting in microbial stability, biological deacidification, as well as contributing to the aroma profile (Bartowsky, Costello, & McCarthy, 2008; Moreno-Arribas & Polo, 2005). Nowadays, the use of lactic acid bacteria (LAB) strains as malolactic starter cultures to improve wine quality is a common winemaking practice.

Spontaneous MLF is often unpredictable. It may occur during, or many months after the completion of alcoholic fermentation (Henschke, 1993; Wibowo, Eschenbruch, Davis, Fleet, & Lee, 1985), and it may also fail because of very harsh environmental conditions in the wine, impeding bacterial survival and growth, such as low pH, high alcohol content, high SO_2 concentrations and low temperatures (Lafon-Lafourcade, Carre, & Ribéreau-Gayon, 1983; Wibowo, Fleet, Lee, & Eschenbruch, 1988). Moreover, some LAB have also undesirable effects on wine quality, because they produce off-flavours, a reduction in colour (Liu & Pilone, 2000) and the formation of biogenic amines (Moreno-Arribas, Polo, Jorganes, & Munoz, 2003). The overall effects of MLF are largely dependent on the strains that carry out the process and on the type of wine being manufactured. *Oenococcus oeni* is the major bacterial species found in wines during spontaneous MLF, as it is well adapted to the low pH and high ethanol concentration of wine. However, *O. oeni* can also be detected with other LAB, mainly *Lactobacillus* spp., and in particular *Lactobacillus plantarum* species (Bravo-Ferrada et al., 2013; Lerm, Engelbrecht, & Du Toit, 2011; Lonvaud-Funel, 2001). In 1988 the potential of *L. plantarum* as a malolactic starter culture was realised by Prahl (1988)

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with the first freeze-dried culture being released. Today there are a few *L. plantarum* strains commercially available as MLF starter cultures (Fumi, Krieger-Weber, Déléris-Bou, Silva, & Du Toit, 2010; Lerm et al., 2011). Some relevant characteristics of *L. plantarum*, such as the ability to function well at low pH conditions, the tolerance of ethanol up to 14%, has a similar SO_2 tolerance to *O. oeni*, and it has a more diverse array of enzymes that could lead to more aroma compounds being produced, all contribute to making *L. plantarum* as the up-to-date generation wine MLF starter cultures (Du Toit, Engelbrecht, Lerm, & Krieger-Weber, 2011; Lerm et al., 2011; Spano, Beneduce, Tarantino, Zapparoli, & Massa, 2002). The selection criteria for enological malolactic starters should include: (i) technological challenges (resistance to the main wine parameters and withstanding the production processes); (ii) malolactic performance and flavour production (malic acid degradation; impact on wine aroma); (iii) production of ensured enhancement of the wholesomeness of wine (no production of biogenic amines) (Du Toit, 2012). A minor but also important aspect to be considered is the susceptibility of LAB to polyphenols, which are one of the most abundant groups of chemical compounds in wine (and in red wines in particular) and can have an extremely important impact on wine sensorial characteristics. Several studies have shown different effects of wine polyphenols on the growth and metabolism of enological LAB (Campos, Couto, & Hogg, 2016; García-Ruiz et al., 2008, 2013a). Particularly *O. oeni* and *L. plantarum* may be inhibited by tannins and phenolic acids, and so they have a negative impact on the development of malolactic fermentation, while anthocyanins and gallic acid seem to have a stimulatory effect (Alberto, Farias, & De Nadra, 2001; Campos, Figueiredo, Hogg, & Couto, 2009; Reguant, Bordons, Arola, & Rozes, 2000).

Recently, some authors have evidenced that the *L. plantarum* species shows a different enzymatic profile to other LAB species, which could play an important role in the wine aroma profile (Lerm et al., 2011; Swiegers, Bartowsky, Henschke, & Pretorius, 2005). The use of malolactic starter cultures has become widespread to control the MLF process and to prevent the production of off-flavours. However, the induction of malolactic fermentation by use of commercially available strains is not always successful. Several reports have shown that the success of MLF starters depends of the strain and is influenced by several factors, including geographical origin and adaptation to the winemaking conditions of each wine (Ruiz, Izquierdo, Seseña, & Palop, 2010; Testa et al., 2014; Valdés La Hens, Bravo-Ferrada, Delfederico, Caballero, Semorile, 2015). Because the resistance to wine conditions is strictly strain-dependent, the development of new malolactic starters is a multiphasic approach, whose identification and oenological characterization of *L. plantarum* strains naturally occurring in wines that have undergone spontaneous MLF are relevant steps.

With the final aim of proposing a selection of potential *L. plantarum* malolactic fermentation starter cultures, this study was focused on the oenological characterization of 11 *L. plantarum* strains previously isolated from Southern Italian red wines. The first objective was to characterize the isolates by assessing their capacity to survive at low pH and high alcohol content, and their malic acid degradation performance in synthetic wine. Also, the production of bacteriocins and biogenic amines was examined, as well as the production of enzymatic activities that play a role in wine production; furthermore, the transformation of odourless glycosidic aroma precursors into odorant aglycones was investigated. The second objective was to evaluate the malolactic activity of one selected strain in a Cabernet Sauvignon wine using two inoculation methods: co-inoculation with yeast and sequential inoculum at the end of alcoholic fermentation.

2. Materials and methods

2.1. Microorganisms and starters preparation

L. plantarum V22 (Lallemand Inc., Montreal, Canada) and 11 strains of *L. plantarum*, selected from southern Italian wines (Testa et al., 2014), were used in the characterization tests of MLF, after a first screening including 58 *L. plantarum* strains isolated from these wines. A commercial strain of *Saccharomyces cerevisiae* AM37 (Enobiotech, Novara Italy) was used to carry out the alcoholic fermentation. The AM37 and V22 strains were rehydrated according to the manufacturer's specifications before use.

At time of use, the strains of *L. plantarum*, were propagated overnight in Man, Rogosa and Sharpe (MRS) medium (Oxoid Ltd., UK) at 30 °C, reinoculated into a new MRS medium and incubated until the exponential phase growth was reached. The cells were pelleted by centrifugation at 10,000 rpm for 15 min at 4 °C, washed twice with sterile water and resuspended in must at a concentration of 10^8 CFU/mL (colony-forming units per millilitre).

2.2. Characterization of the L. plantarum strains in synthetic wine medium

In the first test, 58 *L. plantarum* strains were screened in synthetic wine (SW) media [4 g/L yeast extract, 2 g/L glycerol, 6 g/L D,L-malic acid] (Carreté, Vidal, Bordons, & Constant, 2002). The pH was adjusted to 3.5 with 4N NaOH and the ethanol concentration to 14% (v/v). Cells grown at exponential phase on MRS (Oxoid Ltd., UK) for 48 h at 28 °C were washed with physiological solution and resuspended in SW at a final concentration of 10^8 CFU/mL. The viable cell number was measured by plating diluted SW aliquots on MRS agar at different times on days 5, 10, 15 of incubation at 30 °C under anaerobic conditions.

The second screening panel was performed on the selection of strains (11 *L. plantarum*). Their capacity to grow in SW with the following combination of pH and ethanol concentrations was evaluated: a) pH 3.5 and 11% (v/v) ethanol; b) pH 3.5 and 13% ethanol; c) pH 3.2 and 11% ethanol; d) pH 3.2 and 13% ethanol; e) pH 3.0 and 10% ethanol; each medium was incubated at 24 °C for 15 days. The cell counts were monitored at four different stages during MLF (0, 10, 15 days) by conducting plate counts on MRS agar plates incubated at 30 °C in anaerobic conditions. The L-malic acid concentration was determined with a malic acid enzymatic assay (Steroglass, San Martino in Campo, Italy) at different times (0, 5, 15 days).

2.3. Multi-enzymatic activities

The strains used in this study were assayed for their enzymatic activities using the Api-Zym galleries (BioMérieux, Montalieu-Vercieu, France) as described by the manufacturer. Rapid semi-quantitative evaluation of 19 hydrolytic enzymes was carried out. The colour that developed in each enzymatic reaction was graded from (+) positive to (−) negative and (W) weakly positive by the API-ZYM colour reaction chart.

2.4. Odourless glycosidic aroma precursor transformation by L. plantarum strains

As an indirect measurement of β-D-glucosidase activity in *L. plantarum*, each of the strains tested in this study was first incubated with a commercial glucoside (Octyl-β-D-glucopyranoside) (Sigma-Aldrich, St. Louis, MO, USA) and then with a natural odourless glycosidic aroma precursor extract, which can better represent the ability of these microorganisms to release positive