Food Microbiology 57 (2016) 187–194

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Technological properties of *Lactobacillus plantarum* strains isolated from grape must fermentation

Carmen Berbegal [a, c], Nuria Peña [a], Pasquale Russo [a, d], Francesco Grieco [b], Isabel Pardo [c], Sergi Ferrer [c], Giuseppe Spano [a, *], Vittorio Capozzi [a, d]

[a] Dipartimento di Scienze Agrarie, degli Alimenti e dell'Ambiente, Università di Foggia, via Napoli 25, 71122, Foggia, Italy
[b] Istituto di Scienze delle Produzioni Alimentari, Consiglio Nazionale delle Ricerche, Unità Operativa di Supporto di Lecce, Lecce, Italy
[c] ENOLAB — Laboratori de Microbiologia Enològica, ERI/ISIC BioTecMed, Universitat de València, Spain
[d] Promis Biotech srl, via Napoli 25, 71122, Foggia, Italy

ARTICLE INFO

Article history:
Received 23 October 2015
Received in revised form
2 March 2016
Accepted 2 March 2016
Available online 4 March 2016

Keywords:
Lactobacillus plantarum
Wine
Saccharomyces cerevisiae
Starter cultures
Co-inoculation

ABSTRACT

Malolactic fermentation (MLF) is a secondary fermentation in wine that usually takes place during or at the end of alcoholic fermentation. *Lactobacillus plantarum* is able to conduct MLF (particularly under high pH conditions and in co-inoculation with yeasts), and some strains are commercially used as MLF starter cultures. Recent evidences suggest a further use of selected *L. plantarum* strains for the pre-alcoholic acidification of grape must. In this study, we have carried out an integrated (molecular, technological, and biotechnological) characterization of *L. plantarum* strains isolated from Apulian wines in order to combine the two protechnological features (MLF performances and must acidification aptitudes). Several parameters such as sugar, pH and ethanol tolerance, resistance to lyophilisation and behaviour in grape must were evaluated. Moreover, the expression of stress gene markers was investigated and was linked to the ability of *L. plantarum* strains to grow and perform MLF. Co-inoculation of *Saccharomyces cerevisiae* and *L. plantarum* in grape must improves the bacterial adaptation to harsh conditions of wine and reduced total fermentation time. For the first time, we applied a polyphasic approach for the characterization of *L. plantarum* in reason of the MLF performances. The proposed procedure can be generalized as a standard method for the selection of bacterial resources for the design of MLF starter cultures tailored for high pH must.

1. Introduction

Malolactic fermentation (MLF) is a secondary fermentation in wine that usually takes place during or at the end of alcoholic fermentation (AF) and it is carried out by one or more species of lactic acid bacteria (LAB) (Carr et al., 2002). The MLF contributes to the stabilisation of wine by de-acidification and removal of residual substrates able to be metabolized by spoiling microorganisms (Wibowo et al., 1985; Davis et al., 1988; Drici-Cachon et al., 1996). Nevertheless, depending on the wine, MLF can be beneficial or detrimental (Lonvaud-Funel, 1999). Five genera of LAB were identified as the principal organisms involved in winemaking: *Lactobacillus, Leuconostoc, Oenococcus, Pediococcus* and *Weissella*. Among all LAB species, *Oenococcus oeni* is probably the best adapted to resist the harsh wine conditions, thus making it the most utilized species for commercial MLF starter preparation (Betteridge et al., 2015).

However, *Lactobacillus* spp. have been shown that they can survive in winemaking conditions and that they possess many favourable biological properties that would make them suitable candidate for MLF starter cultures (Du Toit et al., 2011; Bravo-Ferrada et al., 2013). In fact, during the fermentation process, they are also able to carry out a number of secondary metabolic reactions of great importance for aroma and flavour in wines which include citrate metabolism, amino acid metabolism, metabolism of polysaccharides, metabolism of polyols, catabolism of aldehydes, hydrolysis of glycosides, synthesis and hydrolysis of esters, degradation of phenolic acids, lipolysis, proteolysis and peptidolysis (Liu, 2002; Matthews et al., 2004). Several authors have demonstrated that many wine-associated lactobacilli contain genes encoding

Abbreviations: AF, alcoholic fermentation; LAB, lactic acid bacteria; MLF, malolactic fermentation.

* Corresponding author.
E-mail address: giuseppe.spano@unifg.it (G. Spano).

http://dx.doi.org/10.1016/j.fm.2016.03.002
0740-0020/

important enzymes active under winemaking conditions (Cavin et al., 1997; Vaquero et al., 2004; Grimaldi et al., 2005; Spano et al., 2005; De las Rivas et al., 2009; Mtshali et al., 2010; Du Toit et al., 2011; Lerm et al., 2011). Moreover, they exhibit a wider and higher spectrum of enzymatic activities than *O. oeni*, and can contribute to a greater modification of wine aromas (Fumi et al., 2010; Du Toit et al., 2011). The pH of wine is one of the most selective parameters for bacterial survival in wine; in fact, at pH below 3.5 only strains of *O. oeni* can generally survive and express malolactic activity. In contrast, when the pH value is above 3.5, some *Lactobacillus* species have also shown a good ability to conduct MLF (Beneduce et al., 2004). Together with the ability to induce MLF under high pH conditions, *Lactobacillus plantarum* strains are usually unable to produce acetic acid from glucose and fructose because they are homofermenters. Moreover, corrections with organic acids are commonly used to secure the alcoholic fermentation and improve the organoleptic characteristics of red grape musts characterized by high pH and sugar concentration. Recently, an alternative biological acidification method using the ability of *L. plantarum* to produce high concentrations of lactic acid has been suggested (Onetto and Bordeu, 2015). Taken together, the above considerations indicate that *L. plantarum* retains excellent potential and characteristics that would make it suitable to be used as MLF starter.

In this work, we report, for the first time, the technological characterization of *L. plantarum* strains isolated from grape must from Apulia region. Moreover, stress markers were used to establish a link between technological features and tolerance to stress factors commonly found in wine. Finally, the suitability of the characterized *L. plantarum* strains as starters for MLF is discussed.

2. Material and methods

2.1. Microorganisms

Sixty two *L. plantarum* strains previously isolated from Nero di Troia wine undergoing malolactic fermentation during vintages 2010, 2011 and 2012 and available in the culture collection of the Industrial Microbiology Laboratory (Dipartimento di Scienze Agrarie, degli Alimenti e dell'Ambiente, University of Foggia) were used. A preliminary identification of all the strains was performed by amplification and sequencing of the *recA* gene (Torriani et al., 2001). Furthermore, most of the gene encoding the 16S ribosomal RNA was amplified by PCR using primers pA (5′ AGAGTTTGATCCTGGCTCAG 3′) and pH (5′ AAGGAGGTGATCCAGCCGCA 3′) (Capozzi et al., 2012; Edwards et al., 1989). The resulting sequences were compared with sequences available at NCBI database using the standard nucleotide–nucleotide homology search Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov/BLAST).

L. plantarum strains were grown in MRS broth (de Man et al., 1960) (pH 6.2) at 30 °C, 48 h until the end of exponential phase, and then bacterial cultures and sterile glycerol at 30% (v:v) in water were mixed 50% each. The mixture was frozen at −80 °C in tubes of 2 ml The *Saccharomyces cerevisiae* strain UVAFERM VN (Lallemand, USA) was used to promote alcoholic fermentation during experimental vinifications.

2.2. Evaluation of pH and ethanol tolerance in MRS medium

L. plantarum strains were pre-grown in MRS broth (pH 6.2) to early stationary phase (OD_{600nm} between 3 and 4). An inoculum consisting of 1×10^6 CFU/ml of each *L. plantarum* strain was used to inoculate MRS either at pH 3.5 and pH 6.0, both supplemented with ethanol 8%, 10% and 12% (v/v). The cultures were incubated at 30 °C for 5 days. Microbial growth monitored by turbidimetry measuring the optical density (OD) at 600 nm.

2.3. Growth at high sugar concentrations

L. plantarum strains were pre-grown in MRS broth (pH 6.2) to early stationary phase. An inoculum consisting of 1×10^6 CFU/ml of each *L. plantarum* strain was used to inoculate MRS (pH 3.6) supplemented either with glucose (100 g/l) and fructose (100 g/l) or with L-malic acid (10 g/l). The cultures were incubated at 30 °C for 5 days. Microbial growth was monitored by turbidimetry measuring the optical density (OD) at 600 nm. The pH values were monitored throughout the experimental period.

2.4. Evaluation of lyophilisation tolerance

Lyophilisation was carried out after growing the bacteria until the end of the exponential phase in 50 ml of MRS medium. Cells were recovered through centrifugation and washed twice with 0.067 M sodium L-glutamate 1-hydrate (Panreac, Spain), recovered with the same above centrifugation conditions, and suspended in 2 ml of 0.067 M sodium L-glutamate 1-hydrate. 200 μl of bacterial solution were distributed in each tube. Tubes were frozen at −80 °C, 1 h. The freeze-dying was performed at −60 °C for 18 h under vacuum by using a Virtis system (Sentry, USA). The tubes were vacuum sealed and stored at 4 °C under darkness. Lyophilized cultures were rehydrated with 200 μl of NaCl (9 g/l). Cell viability was studied by plate counting from samples before and after lyophilisation. The volume of 0.1 ml of decimal serial dilutions in 0.9% NaCl of rehydrated cell suspension were spread by duplicate on MRS agar plates and were incubated at 30 °C for 5 days and then the colonies were counted. An inoculum consisting of 1×10^6 CFU/ml of each *L. plantarum* lyophilized strain was used to inoculate MRS broth (pH 3.5) supplemented with L-malic acid (10 g/l). The cultures were incubated at 30 °C for 8 days. Cell viability was studied by plate counting on MRS agar plates.

2.5. Alcoholic and malolactic fermentations in grape must by yeast and bacteria co-inoculation

Red must from Nero di Troia grapes containing glucose (112.10 g/l), fructose (105.60 g/l), L-malic acid (2.39 g/l) was used. pH value was adjusted to 3.5 with NaOH. Grapes were skimmed and the grape must was then sterilized by autoclave at 115 °C for 30 min. Malolactic fermentation was induced by direct inoculation with *L. plantarum* strains, pre-grown in MRS pH 3.5 for 16 h, to a final concentration of 1×10^6 CFU/ml in 50 ml of must. The grape must was incubated at 20 °C for 24 h and then inoculated at the concentration of 1×10^6 CFU/ml, with the UVAFERM VN commercial starter (Lallemand, USA). Thereafter, the containers were incubated at 20 °C. Yeast and bacterial numeration were performed by counting cells (CFU/ml) spread on GPYA (Martorell et al., 2005) and MRS supplemented with natamycin (0.15 mg/l) agar media respectively, after incubation at 30 °C for 5 days. Vinifications were carried out in triplicate and a control without yeast and bacteria inoculation was performed to verify any spontaneous fermentation.

2.6. Analytical methods

Organic acids and sugars were quantified by high pressure liquid chromatography (HPLC) (Series 1200; Agilent, USA) provided with an isocratic pump (Agilent G1310A) following the procedure described by Frayne (1986) with minor modifications. The mobile phase consisted of a solution of 0.75 ml of 85% H_3PO_4 per litre of deionised water with a flow rate of 0.7 ml/min. A G1322A degasser