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

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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.

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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

(Agilent, USA) was employed. Five microliters of sample were automatically injected by using the Agilent G1367B device. The separation of the components was carried out using an Aminex HPX-87H pre-column (Bio-Rad, USA) coupled with two ion exclusion columns of 300 mm by 7.8 mm Aminex HPX-87H (Bio-Rad, USA) thermostatically controlled at 65 °C. Compounds were detected by a G1314B variable-wavelength detector (Agilent, USA) set to 210 nm and a refractive index detector (Agilent G1362A) in series. The elution time was 45 min. External calibration was performed with different concentrations of commercial standards. All samples were centrifuged at rotational speed of 8000 rpm for 10 min and filtered through a membrane filter with a mean pore size of 0.22 μm before used. Quantification was performed by measuring peak height compared to external standards.

For a rapid detection of malic acid degradation, paper chromatography was used. 20 μl of the MRS inoculated media were spotted on Whatman 3 MM paper. The solvent-developer mixture consisted of 2 solutions: bromophenol blue 0.1% (w/v) in n-butanol and 40% (v/v) glacial acetic acid, 10% (v/v) formic acid and 50% (v/v) distilled water. Five volumes of the first solution were added to two volumes of the other to obtain the final solution (Pardo and Zúñiga, 1992).

2.7. Reverse transcription and real-time RT-PCR quantitative analysis (qRT-PCR)

Total RNA was extracted from mid-exponentially *L. plantarum* cultures (OD_{600nm} of 2.5 for UNIF44 and 1 for UNIFG 30) grown in MRS either at pH 3.5 and pH 6.2, by using the UltraClean Microbial Isolation Kit (Mo Bio Laboratories, USA) according to the manufacturer's instructions. cDNAs were synthesized from 0.5 μg of total RNA by using the Quantitect Reverse Trascription Kit (Qiagen, USA). Absence of chromosomal DNA contamination was confirmed by real-time PCR. Real time PCR was performed on 7300 Real-Time PCR System (Applied Biosystems, USA). After twenty-fold dilution of cDNA, 5 μl were added to a 15 μl of a real-time PCR mix containing Power SYBR Green PCR Master Mix (Applied Biosystems, USA) and 100 nM of each primer and the amplification was carried out according to the manufacturer's instructions. The constitutive *ldhD* gene was chosen as an internal control for these experiments (Fiocco et al., 2008, 2009; Bove et al., 2012). The *hsp1, hsp2, hsp3, ftsH* and *ctsR* cDNA were respectively amplified by using their specific primer pairs previously reported (Fiocco et al., 2008, 2009, 2010). Thermal cycling conditions were set as follows: initial denaturation at 95 °C for 10 min, followed by 35 cycles of 20 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C (Russo et al. 2012). A melting curve analysis was performed in order to verify the specificity of real-time PCR. Real-time PCRs were performed in triplicate for each sample of cDNA. For each measurement, a threshold cycle (CT) value was determined. This was defined as the number of cycles necessary to reach a point in which the fluorescent signal is first recorded as statistically significant above background. In this study, the threshold value was determined automatically. The results were calculated by the comparative critical threshold (ΔΔCT) method by normalizing to the expression with the non-stress condition (pH 6.2). The significance of the differences was determined by a two-tailed Student t-test. The confidence interval for a difference in the means was set at 95% ($P \leq 0.05$) for all comparisons.

2.8. Gene bank accession number

The accession numbers of *16S rDNA* genes *L. plantarum* strains UNIFG 30 and UNIFG 44 are KP899075.1 and KP899090.1, respectively.

3. Results

3.1. Evaluation of pH and ethanol tolerance

In order to investigate the behaviour at low pH, growth of *L. plantarum* strains was monitored in MRS at pH 3.5. In general, growth was strain dependent although different groups of strains were clearly distinguishable. A first group with strains UNIFG 44, 49, 88, and 98, showed high tolerance to pH with an efficiently grew at the tested condition with an OD_{600nm} between 5 and 6 after 100 h of incubation. A second group of strains including strains UNIFG 30, 45, 70, and 81 was affected by pH and their growth was significantly lower after 5 days of incubation with an OD_{600nm} between 3 and 3.5 (Fig. 1). A third group of strains was constituted by the remainder fifty four *L. plantarum* strains and showing an intermediate behaviour between the first and the second group of *L. plantarum* strains. Indeed, they were able to growth more than the strains belonging to the second group but less than strains belonging to the first group (data not shown).

In order to analyse the relationship between growth, tolerance to pH and tolerance to ethanol, *L. plantarum* strains UNIFG 44, 49, 88, 98 were selected as potential useful microbial starter for MLF and their behaviour compared to strains UNIFG 30, 45, 70, 81. The ethanol tolerance of the *L. plantarum* strains was studied by monitoring their growth level in MRS media containing different concentrations of ethanol (see Section 2). All the strains were unable to grow in MRS medium containing 10% and 12% (v/v) of ethanol. As reported in Fig. 2, a slight growth was achieved when all the strains were inoculated in MRS supplemented with 8% (v/v) of ethanol. Notably, the differences observed within strains at pH 3.5, partially disappeared in presence of ethanol. For example, the behaviour of strains UNIFG 45 and 70 was similar to that of strains UNIFG 44 and 49, previously selected for their ability to growth at pH 3.5.

3.2. Growth at high sugar concentration

Growth rates were analysed at MRS broth supplemented with glucose (100 g/l) and fructose (100 g/l) (pH 3.6) by turbidimetric assay. The pH tolerant *L. plantarum* strains UNIFG 44, 49, 88 and 98 started to grow before and the final biomass was higher than for the

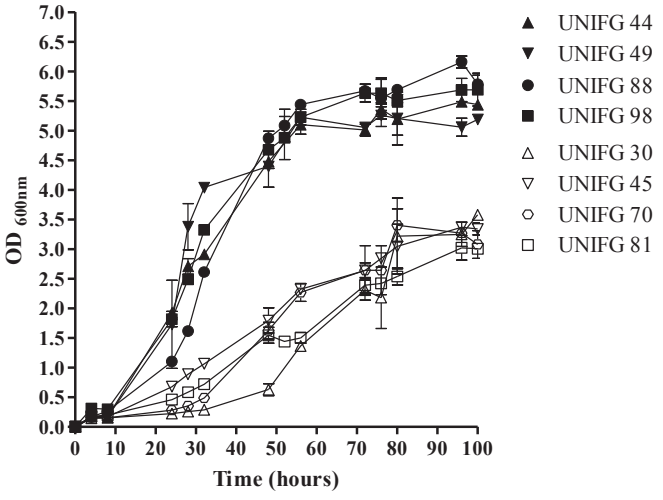

Fig. 1. Growth of *L. plantarum* strains in MRS broth (pH 3.5) at 30 °C. The increase in OD_{600nm} is shown as a function time (hours) and monitored over 100 h for all strains. Data shown are means ± standard errors of the results from two independent experiments.

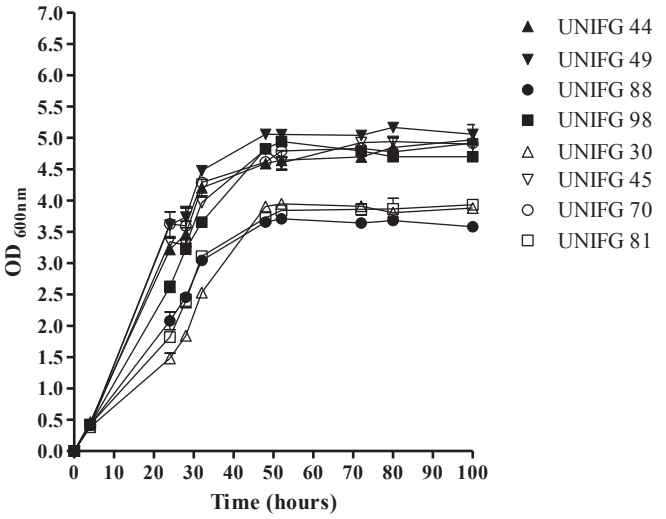

Fig. 2. Growth of *L. plantarum* strains in MRS broth (pH 6.2) containing 8% (v/v) of ethanol at 30 °C. The increase in OD_{600nm} is shown as a function time (hours) and monitored over 100 h for all strains. Data shown are means ± standard errors of the results from two independent experiments.

other strains (Fig. 3a). However, *L. plantarum* strains UNIFG 45, 70, 81 and, in particular, the strain 30, grew poorly and showed a longer lag phase (Fig. 3a). During bacterial growth the pH decreased, revealing an acidification of the medium. Those strains that reached the highest biomass (UNIFG 44, 49, 88 and 98), decreased the pH level in 0.7–0.9 units (Fig. 3b).

The eight *L. plantarum* strains were further investigated for their growth rates and for their ability to lower the pH by culturing them in MRS supplemented with malic acid (10 g/l; pH 3.6) (Fig. 4). In this case, the differences in the growth rates between the pH tolerant *L. plantarum* strains UNIFG 44, 49, 88 and 98 were not significant (Fig. 4a). Concerning the strains UNIFG 45, 70, 81 the addition of malic acid enhanced their growth, whereas the strain UNIFG 30 was still the strain with the lowest growth and with the longest lag phase even in this experimental condition. During the first 35 h, *L. plantarum* strains increased the pH level around 0.3 units, except for the strain UNIFG 30, which presented a pH rise after 80 h of incubation. The increase of pH during the first 35 h was due to the occurrence of MLF as paper chromatography revealed. Once the malic acid was completely consumed, the pH level decreased in the medium because of the production of lactic acid from sugars utilization. The faster growth of the *L. plantarum* strains implies the faster MLF and the subsequent medium acidification observed. In this case, strains UNIFG 44 and 49 were the best in terms of MLF and medium acidification (Fig. 4b).

3.3. Malolactic fermentation and acidification in grape must

In order to evaluate the ability to perform MLF in conditions close to wine fermentation, *L. plantarum* strains UNIFG 44 and UNIFG 30 were chosen on the base of their very different oenological properties. In particular, *L. plantarum* strains UNIFG 44 was selected for its high tolerance to pH and ethanol 8% and high L-malic acid consumption rate, while UNIFG 30 was selected for its reduced growth rate in MRS at pH 3.5 or less tolerance to ethanol 8% (v/v) and with a low L-malic consumption rate.

Red grape must was subjected to fermentation by the two selected *L. plantarum* strains and the commercial *S. cerevisiae* strain UVAFERM VN following a co-inoculation approach. In Figs. 5 and 6 the results obtained from growth and glucose, fructose, L-malic acid, and lactic acid kinetics are reported. The UNIFG 44 grew faster during the first 24 h, from 5×10^6 CFU/ml up to 1.6×10^8 CFU/ml, whereas UNIFG 30 reached a concentration of 3.2×10^7 CFU/ml in the same period. When the yeast starter was inoculated with the UNIFG 44 strain, it reached its maximum biomass (5.8×10^7 CFU/ml) after 3 days of inoculation, whereas when co-inoculated with UNIFG 30, the yeast population increased up to 1.8×10^8 CFU/ml (Fig. 5a and b). The growth of UNIFG 44 produced 8.58 g/l of lactic acid during the first 7 days of fermentation, while the UNIFG 30 strain produced 2.76 g/l of this organic acid. When *S. cerevisiae* was co-inoculated with UNIFG 44 produced 11.58% (v/v) of ethanol, whereas with UNIFG 30 produced 12.22% (v/v) (Fig. 6a and b).

3.4. Bacterial growth and consumption of L-malic acid after lyophilisation

The ability of strains UNIFG 44 and UNIFG 30 to tolerate lyophilisation process was determined by comparing the cell count of rehydrated lyophilized cells with the cell counts of the initial culture. Strain UNIFG 30 was included in this experimental trial as control, since it showed a low tolerance to lyophilisation stress. After lyophilisation treatment, *L. plantarum* strain UNIFG 44 displayed a viability of 42%, whereas strain UNIFG 30 presented a

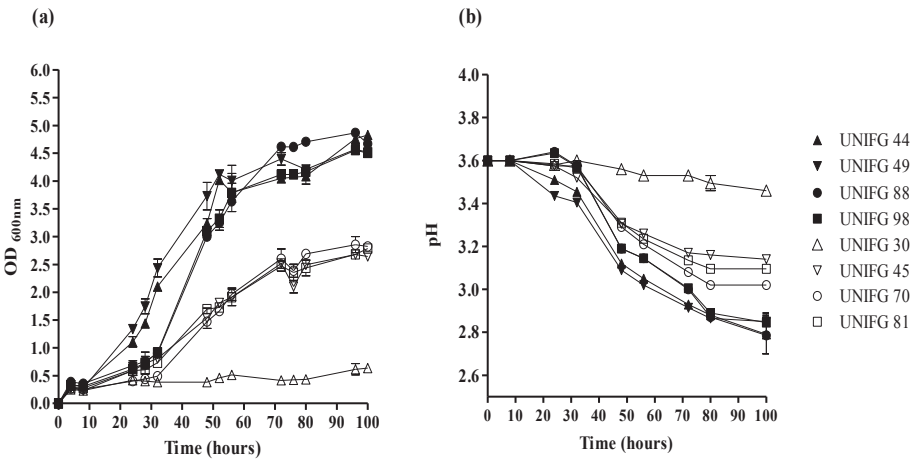

Fig. 3. (a) Growth of *L. plantarum* strains and (b) pH variations in MRS broth (pH 3.6) supplemented with glucose (100 g/l) and fructose (100 g/l) at 30 °C. The increase in OD_{600nm} and pH are shown as a function time (hours) and monitored over 100 h for all strains. Data shown are means ± standard errors of the results from two independent experiments.

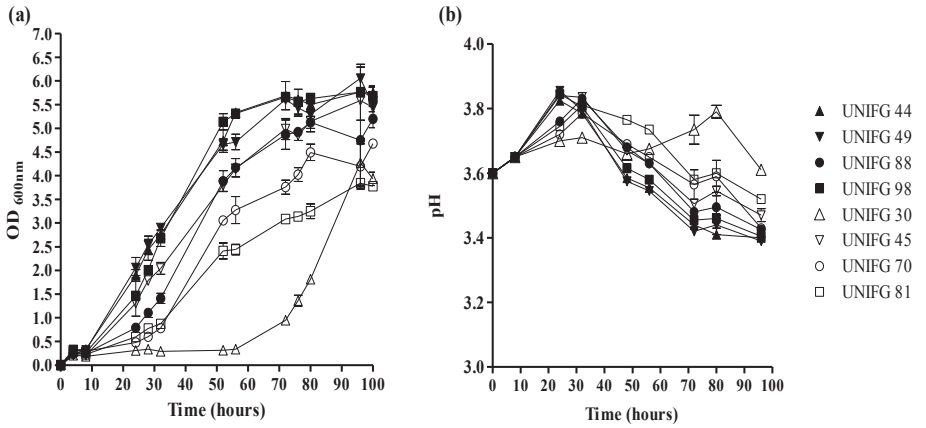

Fig. 4. (a) Growth of *L. plantarum* strains and (b) pH variations in MRS broth (pH 3.6) supplemented with malic acid (10 g/l) at 30 °C. The increase in OD_{600nm} and pH are shown as a function time (hours) and monitored over 100 h for all strains. Data shown are means ± standard errors of the results from two independent experiments.

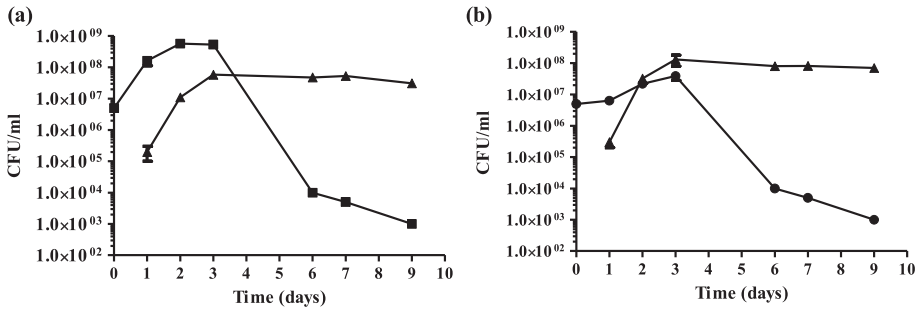

Fig. 5. Dynamics of yeast and bacterial population during AF and MLF, in grape must inoculated with (a) *L. plantarum* UNIFG 44 (■) and *S. cerevisiae* VN (▲) and, (b) *L. plantarum* UNIFG 30 (●) and *S. cerevisiae* VN (▲). The yeast has been inoculated 24 h (1 day) after the bacteria. Data shown are means ± standard errors of the results from two independent experiments.

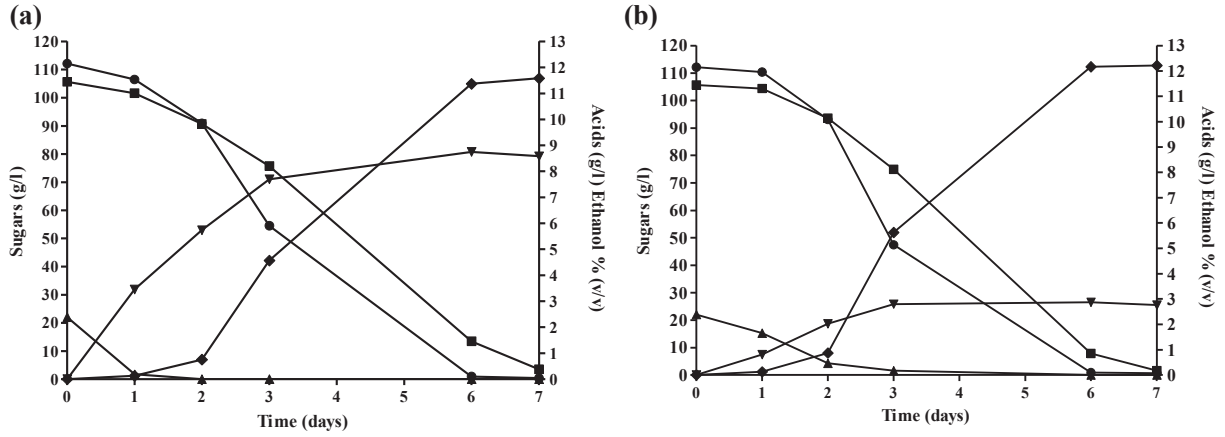

Fig. 6. Evolution of glucose (●), fructose (■), L-malic acid (▲), lactic acid (▼) and ethanol (◆) during AF and MLF in grape must carried out by (a) *L. plantarum* UNIFG 44 and *S. cerevisiae* VN and, (b) *L. plantarum* UNIFG 30 and *S. cerevisiae* VN. Data shown are means ± standard errors of the results from two independent experiments.

viability of 14%. Both lyophilised strains were able to grow and carry out the MLF in MRS supplemented with L-malic acid (10 g/l) (pH 3.5). However, strain UNIFG 30 started to grow later than strain UNIFG 44 (Fig. 7a). In this case, the glucose consumption was faster and the L-malic acid was completely consumed in 2 days (Fig. 7b and c), thus enhancing a faster formation in lactic acid, 2 days after strains inoculation, with a 4.7-fold difference compared to UNIFG 30 (Fig. 7d).

Overall, the ability to recover the viability and perform the malolactic activity observed after the lyophilisation process, suggest that strain UNIFG 44 may be commercially suitable as starter culture.

3.5. Real-time RT-PCR quantitative analysis (qRT-PCR)

Relationship between the tolerance to pH and expression levels of stress response genes of strains UNIFG 44 and UNIFG 30 was investigated. Strains were grown in MRS at pH 6.2 and in MRS at pH

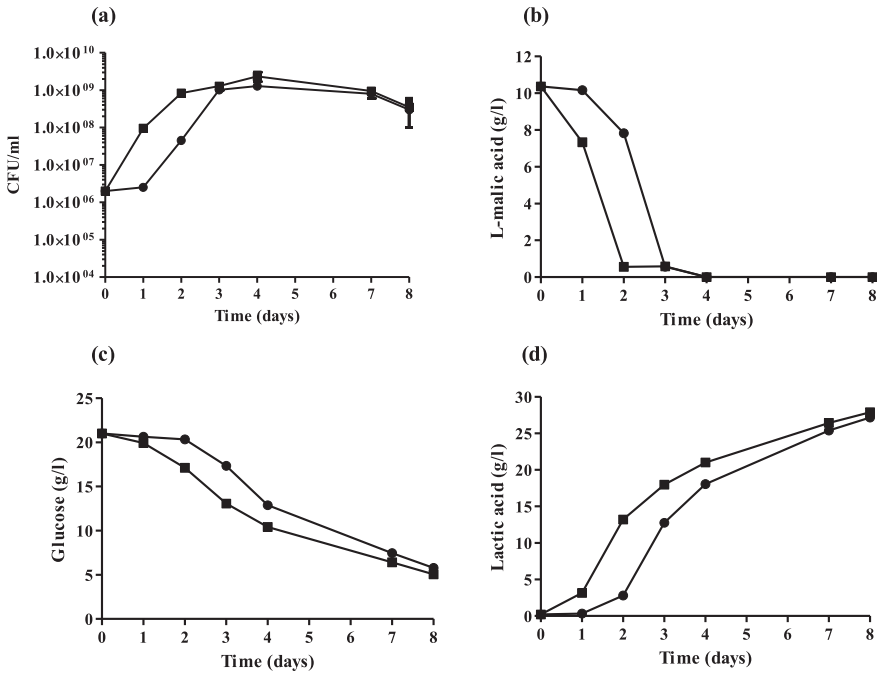

Fig. 7. (a) Viable cells, (b) L-malic acid consumption, (c) glucose consumption, and (d) lactic acid formation by lyophilised *Lactobacillus plantarum* strains UNIFG 44 (■) and UNIFG 30 (●) in MRS supplemented with malic acid (10 g/l) (pH 3.5). Data shown are means ± standard errors of the results from two independent experiments.

3.5 and the expression of *hsp1, hsp2, hsp3, ftsH* and *ctsR* genes monitored by qRT-PCR after normalization related to *lghD* gene. No clear differences were observed between strains when the expression of *hsp 2* and *ftsH* genes was analysed at pH 3.5 (data not shown). However, the expression value of the *hsp1*, *hsp3* and *ctsR* genes, and particularly that of *hsp1* (22-fold increase) gene, was higher in strain UNIFG 44 than in UNIFG 30 (Fig. 8). This result suggests a difference in stress response between the analysed strains.

4. Discussion

The production of efficient malolactic starter cultures has become one of the main challenges for oenological research in recent years. The success of these starter cultures depends on the strain used and it is influenced by a variety of factors, including adaptation to the characteristics of each wine (Gónzalez-Arenzana et al., 2012; Lerm et al., 2011). Thus, the use of autochthonous starter cultures, well adapted to the conditions of a specific wine-producing area, has been recommended (Bauer and Dicks, 2004; Carreté et al., 2006; Solieri et al., 2009; Capozzi et al., 2010; Ruiz et al., 2010; Bravo-Ferrada et al., 2013; Nisiotou et al., 2015). Although *O. oeni* is by far the most studied LAB species of oenological origin and the most used MLF starter in commercial cultures, several investigations have stated the potential of *L. plantarum* as starter culture in winemaking (G-Alegría et al., 2004; López et al., 2008; Miller et al., 2011; Onetto and Bordeu, 2015). Moreover, alternative MLF starters such as *L. plantarum*, may be considered as a potential solution for some recurrent problems of winemaking (Onetto and Bordeu, 2015).

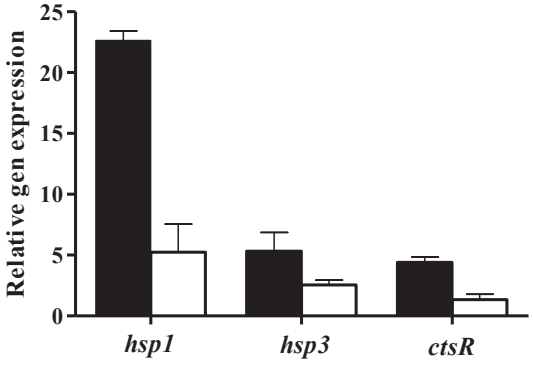

Fig. 8. Relative gene expression in *L. plantarum* UNIFG 44 (black bar charts) and *L. plantarum* UNIFG 30 (white bar chars) of *hsp1*, *hsp3* and *ctsR* genes determined by quantitative real-time RT-PCR. The relative levels of expression were calculated normalizing the levels of *ldhD* gene, and the respective gene expression level under unstressed condition (pH 6.2).

The aim of this study was to perform a technological characterization of *L. plantarum* strains, in order to select suitable starters able to promote MLF in wines produced in Apulia region and denoted by high sugar content (about 250 g/l) and elevate total acidity (about 4.5 g/l).

To evaluate the ability of *L. plantarum* strains to grow and perform MLF, the level of expression of stress gene markers was estimated in addition to pH, sugar and ethanol tolerance, viability recovery after lyophilisation stress and behaviour in grape must.

During this investigation, all the strains were grown in MRS with a pH of 3.5, with ethanol 8%, 10% and 12% (v/v), supplemented with glucose and fructose or with malic acid. Eight *L. plantarum* strains were selected, considering differences in their pH tolerance. Among them, only strains UNIFG 44, 49, 88 and 98 were able to growth and reach higher biomass in a low pH medium suggesting their potential application as MLF starters.

All the eight selected strains were unable to grow in medium containing 10% or 12% (v/v) of ethanol, but a slight increase of LAB biomass concentration was observed when an 8% (v/v) of ethanol was supplied. These results are in accordance with Guerzoni et al. (1995), who showed that when the ethanol concentration was lower than 6% (v/v), *L. plantarum* was more resistant than *O. oeni* to

the combined action of various stresses, such as pH and malate concentration (Guerzoni et al., 1995).

Co-inoculation approach has several clear benefits compared to the sequential inoculation, since the introduction of LAB inoculum before AF helps the bacteria to improve its adaptation to the medium. Moreover, it is now well known that co-inoculation reduces the total fermentation time (Jussier et al., 2006; Rosi et al., 2006; Massera et al., 2009; Pan et al., 2011; Abrahamse and Bartowsky, 2012; Knoll et al., 2012) and may limits the risk of spoilage by other microorganisms, such as the *Brettanomyces* species (Jussier et al., 2006; Curtin et al., 2007; Gerbaux et al., 2009).

In our case both strains were able to consume all L-malic acid from grape must, but differences between strains in terms of growth and L-malic acid degradation were observed. Furthermore, the co-inoculation of *S. cerevisiae* and *L. plantarum* strains permitted to complete both AF and MLF in 17 days, thus confirming the advantages of the co-inoculation approach.

The ability to survive to lyophilisation process was also evaluated in the *L. plantarum* strains analysed. The adopted lyophilisation process can be considered as a gentle drying procedure. Both strains preserved the malolactic activity after the lyophilisation process and, as in the case of the fresh liquid cultures, the strain UNIFG 44 showed better performances in terms of growth rate and L-malic acid degradation. These results are in agreement with those reported by G-Alegría et al. (2004), who observed similar growth rate and maximal population in *L. plantarum* strains after and before lyophilisation.

Stress genes are considered molecular markers for the fitness of starter cultures and could be used as positive indicators for a culture that is fully adapted to resist a stress condition (Spano and Massa, 2006; Beltramo et al., 2006; Fiocco et al., 2010; Russo et al., 2012). In this work, the expression levels of the *hsp1*, *hsp 2*, *ftsH*, *hsp3* and *ctsR* genes in *L. plantarum* strains UNIFG 30 and 44 were evaluated using a quantitative PCR approach. The expression level of the *hsp1*, *hsp3* and *ctsR* genes was much higher in strain UNIFG 44, the strain presenting high tolerance to pH and a good MLF performance.

Overall, our results suggest that analyses of the expression of *hsp1*, *hsp3* and *ctsR* genes can be successfully used ascertain the response to stress in selected *L. plantarum* strains.

5. Conclusions

In this study, a technological characterization of *L. plantarum* strains isolated from Apulian wines has been carried out in order to select suitable starters for MLF. Several parameters such as pH, sugar and ethanol tolerance, ability to overcome lyophilisation stress and ability to grow and perform MLF in grape must were evaluated. Moreover, the expression of stress gene markers was investigated. To our knowledge, this is the first report that focuses on the technological characterization of *L. plantarum* isolates from Apulia region and their heterogeneous behaviour in grape must. This diversity may have a significant technological importance which calls on deeper studies of viability during MLF as well as the behaviour of *L. plantarum* isolates at industrial scale vinification.

Finally, the proposed program, mainly based on technological and stress tolerance features, may be generalized as a standard method for the selection of bacterial resources for the design of *L. plantarum* strains suitable for MLF and tailored for high pH must.

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