Effect of exogenous proline on the ethanolic tolerance and malolactic performance of *Oenococcus oeni*

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Revised: 27 March 2020 / Accepted: 3 April 2020 / Published online: 10 April 2020
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Abstract The use of malolactic starter cultures, often offer no guarantee of microbiological success due to the chemical and physical factors (pH, ethanol, SO₂, nutrient availability) that occur during the winemaking process. This study was born with the aim of improving the performance of the lactic acid bacteria used as a starter culture in the de-acidification of wines. Two commercial strains of *Oenococcus oeni*, were used. Was evaluated the effect of exogenous L-proline added during the bacterial growth, on the improvement of their survival in the presence of different ethanol concentrations and their ability to degrade L-malic acid in synthetic wine with the presence of 12% (v/v) and 13% (v/v) of ethanol. The results showed that L-proline improve ethanol tolerance and so the malolactic performances of *O. oeni*. This work represents an important strategy to ensure good vitality and improve the performance of the malolactic starter.

Keywords L-proline · Lactic acid bacteria · Ethanol tolerance · Malolactic fermentation

Introduction

The use of bacteria as starter cultures in the food industry is fundamental for their technological importance, for their metabolic activities and for their possible antimicrobial

activities, and therefore product stability (De Leonardis et al. 2015; Testa et al. 2019). During food processing bacteria are subject to several stress conditions such as acid environment, high and low temperatures, oxidative stress, osmotic stress, ethanol content, presence of toxic compounds etc. (Van de Guchte et al. 2002), to which they have evolved complex stress responses that can promote their survival under restrictive growth conditions and then improve the performance of the microorganism itself (Baliarda et al. 2003). Bacteria have developed significant strategies to grow and survive under stressful conditions. Stress can be defined as a change in the genome and in the proteome of the bacterium and results in a decrease in the growth rate and its survival. In fact, stress factors can be chemical, physical or biological, some are of environmental origin (temperature, osmotic pressure, pH, ethanol concentration, low availability of nutrients). Therefore, both the physiological state of the cells and the environmental factors will affect the mechanism of stress response. Lactic acid bacteria (LAB) have developed stress detection systems that enable defensive mechanisms to be activated to withstand harsh conditions or change environmental problems (Van de Guchte et al. 2002). One of the most powerful adaptive strategies that bacterial cells have evolved to counteract low activities of their growth media is the accumulation to high intracellular levels of a set of organic solutes (osmo-protectants) that are synthesized de novo or actively taken up from the growth medium. In bacteria and plants, the principal osmo-protectants are glycine betaine, carnitine and L-proline. L-proline improves the stability of proteins and membranes in environments with low water or high-temperature activity and inhibits aggregation during protein folding (Da Silveira et al. 2002; 2003; Kumar et al. 1998; Liang et al. 2013; Samuel et al. 2000; Wen-ying and Zhen-kui 2013). These observations

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suggest that intracellular L-proline could play a crucial role in reducing ethanol stress by preventing protein denaturation and membrane disorder. *Oenococcus oeni* and *Lactobacillus plantarum* are the two LAB species responsible for malolactic fermentation (MLF) during the winemaking process. MLF occurs after alcoholic fermentation and may be either spontaneous, carried out by indigenous LAB or induced by commercial MLF starter cultures (Iorizzo et al. 2016). MLF consists in the conversion of L-malic acid into L-lactic acid, reducing the acidity of the wine and improving its microbiological stability and organoleptic characteristics of wine. Although MLF is desirable because of the benefits it confers to wine, it is often difficult to perform due to various stress factors that inhibit the activity of lactic bacteria: high ethanol concentration, low pH, SO₂, medium-chain fatty acids, some phenolic compounds. Increased membrane fluidity, which causes cofactor leakage and loss of membrane potential, has long been documented as a cause for decreased cell growth during exposure to ethanol. The increased membrane fluidity is not the sole cause of growth inhibition and that alcohols may also denature proteins within the membrane and cytosol, adversely affecting metabolism and decreasing cell growth. (Betteridge et al. 2015; Huffer et al. 2011). Therefore, a better knowledge of stress tolerance and the adaptation mechanisms of the malolactic bacterium *O. oeni* is essential to improve the efficiency of the malolactic starter in wine (Chu-Ky et al. 2005). One major adaptation involves mechanisms that counteract the increase in membrane fluidity at high ethanol concentrations (Betteridge et al. 2018; Liu et al. 2014; Maitre et al. 2014; Martos et al. 2007; Marielle and Sarrah 2017; Silveira et al. 2004). The availability of oenological starter with good technological properties and able to survive in stressful conditions, it can help improve the microbiological, physico-chemical and organoleptic characteristics of wines (Lombardi et al. 2018; Testa et al. 2014; 2020). The objective of this study was to understand the correlation between L-proline, accumulated in the growing cell, and the alcoholic tolerance and the MLF performance of *O. oeni* in synthetic wine.

Materials and methods

Bacterial strains and growth condition

Two commercial freeze-dried *O. oeni* strains: XXL plus (Probiotec, Villanova sull'Arda PC, Italy) and PN4 (Lallemand Inc., Montreal, Canada), were used. Before using the strains, they were rehydrated as reported by the manufacture and grown at 28 °C in *Leuconostoc oenos* medium (LOM) having the following composition::

10.0 g/L casein peptone, 5.0 g/L yeast extract, 10.0 g/L glucose, 5.0 g/L fructose, 0.20 g/L MgSO₄, 0.05 g/L MnSO₄, 3.50 g/L (NH₄)₃C₆H₅O₇, 1.00 mL/L tween 80, 0.50 g/L cysteine-HCl (Sigma Aldrich). After sterilization of the medium, the tomato juice (10%) was added by filtration (Millipore, 0.45 µm) and the pH adjusted to 4.5 using HCl 1 N.

Bacterial inoculum

Bacterial cultures of 24 h in LOM medium, were centrifuged at 9300×g for 5 min at 4 °C, washed twice with physiological solution (0.9% NaCl) and inoculated (10⁸ CFU/mL) in 50 mL of LOM with and without the addition of 50 mM of L-proline (Sigma Aldrich), and incubated at 28 °C for 24 h. The L-proline was added at the medium by filtration (Millipore, 0.20 µm). These preadapted cultures were used as inoculum for subsequent tests.

L-proline and total proteins assay

For intracellular L-proline extraction, 1 mL of overnight culture in LOM medium, was centrifuged at 9300×g at 4 °C, pellet was washed twice with physiological solution (0.9% NaCl) and pellet re-suspended in ethanol solution at 40% (v/v), vortexed, sonicated for 20 min at 4 °C and incubated overnight at − 20 °C. The L-proline assay, was performed by colorimetric determination as reported by Carillo and Gibon (2011) using a BioSpectrometer basic (Eppendorf). All the reagents used for the experiment were by Sigma Aldrich. As control was used LOM medium inoculated with the two *O. oeni* strains without the L-proline addition. L-proline was expressed as nmol/µg of total proteins. The total protein analysis was performed as reported by Bradford (1976) with some modification, using a microplate reader (Thermo fisher multiskan FC). As standard was used Bovine Serum Albumin (BSA, Sigma Aldrich). The proteins were expressed as µg/mL. Samples concentration, both for total proteins and L-proline, has been referred to respectively calibration curves. All the determinations were in triplicate.

Ethanol tolerance test

For this study, the preadapted strains in LOM medium, with and without the addition of L-proline, were inoculated in the same medium containing different ethanol concentrations (%v/v) as reported: (a) 0% (control); (b) 6%; (c) 10%; (d) 12%; (e) 14%. The pH of the culture media was adjusted at 4.5. The ethanol was added after the sterilization of the medium by filtration (Millipore, 0.20 µm). Viable cell count was assessed after inoculation at 0 h, 4 h, 24 h, 30 h, 48 h and after that every 2 days for a total of

12 days, by plating onto LOM agar incubated for 72 h at 28 °C in anaerobic conditions. Data was expressed as CFU/mL and all the determinations were in triplicate.

MLF performance in synthetic wine medium

The MLF was performed in synthetic wine (SW) having the following composition: 5 g/L tartaric acid, 0.6 g/L acetic acid, 3.5 g/L L-malic acid, 2.0 g/L glucose, 2.0 g/L fructose, 2.0 g/L yeasts extract, 0.2 g/L NaCl, 1.0 g/L NH₄Cl, 2.0 g/L K₂HPO₄, 0.2 g/L MgSO₄ 7H₂O, 0.05 g/L MgSO₄, pH 3.5 at different ethanol concentration 12% (v/v) and 13% (v/v). The different conditions were the following: (a) PN4 + 12% (v/v); (b) XXL + 12% (v/v); (c) PN4 + 13% (v/v); (d) XXL + 13% (v/v). The negative control was represented by SW without any bacterial inoculation. The preadapted strains have been made to grow as reported above and inoculated in SW (10⁷ CFU/mL). The viable cell count was determined during the MLF by plating onto LOM agar and incubation for 72 h at 28 °C in anaerobic conditions. The L-malic acid assay (g/L) was performed at 0 h, 1 h, 5 h, 12 h, 24 h, 48 h, 72 h and after 5, 7, 14, 21, 30 and 40 days, using an enzymatic kit (Megazyme, USA). All the determinations were in triplicate.

Statistical analysis

All data are expressed as means ± SD of three measurements. Microbial count levels and chemical parameters were analyzed by a General Linear Model based on ANOVA (IBM SPSS Statistics 21).

Results and discussion

L-proline assay

In the LOM medium without L-proline, for both the strains, L-proline value was equal to 0.04 nmol/μg of total proteins. In the LOM medium with the L-proline addition, after overnight incubation, the value found was 9.3 nmol/μg for XXL strain and 12.6 nmol/μg for PN4 strain. The level of L-proline derived from LOM medium is very low, so data show that there was an intracellular accumulation of exogenous L-proline added in to the culture medium. The PN4 strain accumulated higher levels of L-proline compared with XXL strain and compared with the control. As reported from some authors, intracellular L-proline can protect the cells against stress preserving the integrity of the plasma membrane and can stimulate cell growth (Baliarda et al. 2003). In fact, the purpose of this work was to understand first of all if the *O. oeni* strains were able to

carry exogenous L-proline inside, and this data was confirmed with the results obtained. Further research are necessary to identify the mechanisms by which L-proline interacts with membrane phospholipids and proteins and understand how L-proline increases ethanol tolerance. Thus, L-proline might serve as a protective agent for industrial microorganisms.

Response to ethanol stress

The results of bacterial tolerance at different ethanol concentrations have been reported in Fig. 1. In the test without the addition of ethanol, for both the strains, the preadaptation with L-proline in growth phase did not influence the microbial kinetics and after 12 days of incubation the bacterial concentration was around 10⁶ CFU/mL. Instead, with the addition 6% (v/v), 10% (v/v), 12% (v/v) and 14% (v/v) of ethanol, the behavior of the two strains was different if preadapted with or without proline. The L-proline could play a protective role on cellular membranes and therefore on their vitality, so it may have such possible effects on cell properties and composition. In particular in test with 6% (v/v) of ethanol, PN4 strain after 9 days of incubation showed a different concentration of viable cells when preadapted with proline (4.9×10^6 CFU/mL) compared to when preadapted without proline (4.4×10^5 CFU/mL); in test with XXL strain, the concentration of viable cells was very similar, respectively 5.6×10^6 CFU/mL and 2.4×10^6 CFU/mL. In the test with 10% (v/v) of ethanol, the situation was different. In fact, after 9 days the vitality was similar in the two treatments for PN4 strain (4.3×10^5 CFU/mL with proline and 1.5×10^5 CFU/mL without proline), instead for XXL strain grown with proline showed a cell density of 1.8×10^6 CFU/mL and without L-proline of 4.2×10^5 CFU/mL. In the test with 12% (v/v) of ethanol, after 9 days for PN4 the concentration of viable cells, with and without L-proline preadaptation, was respectively of 6.0×10^5 CFU/mL and 6.1×10^4 CFU/mL; for XXL strain instead the concentration of viable cells was very similar: 3.0×10^5 CFU/mL and 3.6×10^5 CFU/mL with and without L-proline preadaptation respectively. In the test with 14% (v/v) of ethanol, after 9 days for PN4 strain the concentration of viable cells has been of 1.9×10^4 CFU/mL with L-proline and 1.6×10^3 CFU/mL without L-proline; for XXL strain the concentration of viable cells was been respectively of 5.0×10^4 CFU/mL and 1.0×10^3 CFU/mL with and without L-proline preadaptation. Based on the results obtained, the presence of exogenous L-proline improved the growth performance of both bacterial strains. As reported by some authors, L-proline is a known key amino acid in bacteria, fungi and plants, it acts as a protection against stress preserving the integrity of the plasma membrane, preventing

Fig. 1 Survival of *O. oeni* PN4 and XXL strains, preadapted with and without L-proline (P), in LOM medium. Experimental conditions: **a** 0% (v/v), **b** 6% (v/v), **c** 10% (v/v), **d** 12% (v/v), **e** 14% (v/v). *XXL*

solid line (filled triangle), *XXL* + *P* dash line (filled circle), *PN4* solid line (filled diamond), *PN4* + *P* dash line (filled square). Error bars indicate standard deviations ($n = 3$)

protein aggregation (Samuel et al. 2000), acting as a co-substrate for metabolism (Takagi et al. 2000) and accumulating as a compatible intracellular solute (Hoffmann et al. 2012). It has been shown that the exogenous addition of L-proline and betaine as osmoprotectors, on the LAB species, had stimulating properties in particular on the growth of *Pediococcus pentosaceus*, *L. plantarum* and *Lactococcus lactis* grown in hyperosmotic conditions, moreover, the accumulation of molecules with osmoprotective properties has been considered a potential solution also to stabilize frozen or freeze-dried starter cultures used in the food industry as LAB. As for the function of L-proline as a protector of stress, preserving the integrity of the plasma membrane, this is very important especially for malolactic bacteria because they work in a complex matrix such as wine, and in particular alcohol toxicity is one of the stresses more common because this molecule can cause cell membrane rupture, causing growth inhibition and cell death. Intracellular concentrations of L-proline increase when some species of LAB (*L. plantarum*, *L. lactis* and *Tetragenococcus halophilus*) are grown under conditions

of high osmotic pressure. Some amino acids reduced the impact of osmotic stress on growth when they were added exogenously to LAB cultured in chemically defined media (Papadimitriou et al. 2016). Osmotically active compounds like L-proline, serve a dual function in osmoregulation. In addition to maintaining a stable cell turgor, they also exert protective effects on biomolecules in vitro during stressful challenges. Various osmotically active compounds, such as L-proline and betaine, protect soluble and peripherally membrane-bound proteins from the denaturing effects of modifications in intracellular ionic strength and water availability. Osmotically active compounds can interact with proteins in several different ways, such as preferential exclusion, water replacement, hydration forces, and vitrification of sugars.

Malolactic fermentation assay

Figure 2 shows malolactic activity (g/L L-malic acid) in synthetic wine medium at 12% and 13% ethanol concentrations. In the test with 12% (v/v) ethanol, the MLF was

Fig. 2 Malolactic activity of *O. oeni* PN4 and XXL strains, preadapted with and without L-proline (P) and inoculated in synthetic wine (SW) with 12% or 13% ethanol. XXL solid line (filled triangle),

XXL + P dash line (filled circle), PN4 solid line (filled diamond), PN4 + P dash line (filled square). Error bars indicate standard deviation (n = 3)

regular, very fast and there were very few differences in vitality and performance of the two bacteria grown either without or with the addition of exogenous proline. In the wine inoculated with PN4 starter, when grown without exogenous proline, after 24 h of incubation, the L-malic acid content was of 0.58 g/L; and when grown in the presence of exogenous proline the L-malic acid content was of 0.23 g/L, for XXL strain after 24 h respectively of 0.52 g/L and 0.19 g/L. In all tests, in synthetic wine with 12% (v/v) ethanol, the bacterial density of the two starters during the MLF, remained constantly around 10^7 CFU/mL. In the test with 13% (v/v) of ethanol the situation was different; in fact the treatment with L-proline had an evident effect on the malolactic performance of the tested strains. With the inoculum of PN4 strain, grown without exogenous L-proline, after 21 and 30 days the L-malic acid content was respectively of 1.90 g/L and of 1.50 g/L; instead with the inoculum of PN4 strain, preadapted with L-proline, L-malic acid content was of 0.90 g/L after 21 days and of 0.01 g/L after 30 days; with the inoculum of XXL strain grown without L-proline after 21 and 40 days the L-malic acid content, in the synthetic wines, was respectively of 2.10 g/L and 0.50 g/L; instead, with the inoculum of same strain, preadapted with L-proline, after 21 and 40 days the L-malic acid content was respectively of 1.50 g/L and 0.09 g/L. As demonstrated by the results

obtained, that when the bacteria were grown in the presence of proline, in particular in that with 13% of ethanol, it had a noticeable effect, in fact, both strains completed the MLF. Instead when the starter bacteria have grown without addition of exogenous proline, the MFL has been slower and incomplete and also cell viability was lower. After 30 days the bacterial density of the two starters, when grown without proline, was about 10^5 CFU/mL, instead when grown with proline the cell density was about 10^6 CFU/mL.

In the control test without bacterial inoculum, the concentration of L-malic acid was unchanged (3.5 g/L), so there was no interference and no contamination. Surely the presence of L-proline in a stressful environment such as a synthetic wine with 13% (v/v) ethanol has had a protective action against its negative action on the cell membrane, consequently preserving its integrity and therefore the vitality of the cells (Margalef-Català et al. 2016).

Conclusion

O. oeni is one of the LAB species responsible for MLF, a secondary fermentation that plays an important role in winemaking processes. But the harsh compositional characteristics of the must and the wine conditions of the wine

such as low pH, high ethanol content, changes in cellar temperatures can hinder bacterial growth in wine. Due to the, lactic acid bacteria often have difficulty in carrying out MLF. A limiting factor is the alcohol content which affects the vitality of malolactic bacteria. The results illustrated in our work showed that, during the growth phase, *O. oeni* was able to use the proline added to the culture medium, improving its ability to tolerate ethyl alcohol and its performance (speed and duration) in carrying out of MLF. Further scientific investigations will be carried out to inquire about the mechanisms of transport of proline inside the cell and the physical–chemical modalities with which it manages to improve the performance of *O. oeni* during the MLF in the wines. It would be important to the mechanisms of exogenous L-proline transport within the *O. oeni* cell and what role this amino acid plays in the mechanisms of homeoviscous adaptation. From these assumptions, further research will be carried out to identify the mechanisms by which osmoprotectors like L-proline can interact with membrane phospholipids and proteins of malolactic bacteria and understand how L-proline can increase ethanol tolerance and so improve the malolactic activity. The use of malolactic starters is widely recommended. Besides, another important aspect that should be examined is the strategy of inoculation, as well as the preparation and preadaptation of the culture starter (Succi et al. 2017).

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