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Arginine metabolism in wine *Lactobacillus plantarum*: *in vitro* activities of the enzymes arginine deiminase (ADI) and ornithine transcarbamilase (OTCase)

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Abstract - This work was carried out to determine the activity of enzymes involved in arginine metabolism in *Lactobacillus plantarum* isolated from wine and previously characterised at molecular level. The activity of the enzymes arginine deiminase and ornithine transcarbamylase was determined and citrulline and ornithine formed were analysed by HPLC analysis. Although the enzymatic activity was detected in all the strains analysed, a strong variability was observed between strains. *Lactobacillus plantarum* strain Lp60 is the strain with more possibilities to accumulate citrulline, precursor of the carcinogenic ethyl-carbamate, as showed by its high arginine deiminase activity and low ornithine transcarbamylase activity.

Key words*:* wine, arginine, *Lactobacillus plantarum,* arginine deiminase, ornithine transcarbamylase*.*

INTRODUCTION

Lactobacillus plantarum is a flexible and versatile species that is encountered in a variety of environmental niches, including fermented beverages (Beneduce *et al*., 2004). The ecological flexibility of *L. plantarum* is reflected by the observation that this species has one of the largest genomes known among lactic acid bacteria (LAB) (Kleerebezem *et al*., 2003; Molenaar *et al*., 2005). Although in wine *L. plantarum* is capable of malolactic fermentation, it usually contributes to production of undesirable substances such as biogenic amine and precursors of ethyl carbamate during and after winemaking and is therefore of general concern because of its spoilage nature (Lonvaud-Funel, 1999; Liu, 2002; Spano *et al*., 2004, 2006). Ethyl carbamate (or urethane), a well known animal carcinogen (Zimmerli and Schlatter, 1991) found in many fermented foods, including wine (Canas *et al*., 1994, Kodama *et al*., 1994), may be produced from precursors such as urea which is produced by yeasts, while citrulline and carbamyl phosphate are produced by LAB through the arginine deiminase (ADI) pathway (Liu and Pilone, 1998; Mira de Orduña *et al*., 2000, 2001; Liu, 2002; Spano *et al*., 2002). However, a positive effect of arginine on growth of wine lactic acid bacteria has been observed by several authors suggesting that arginine may facilitate growth of LAB in wine (Tonon and Lonvaud-Funel, 2000; Mira de Orduña *et al*., 2001). Moreover, arginine degradation in wine LAB may also play a role in adaptation to low pH (Lonvaud-Funel, 1999; Tonon and Lonvaud-Funel, 2000; Mira de Orduña *et al*., 2001; Cotter and Hill, 2003; Spano *et al*., 2004).

We previously reported the presence of genes (*arcABC*) coding for enzymes involved in the ADI pathway in wine *L. plantarum* (Spano *et al*., 2004, 2006). The high identities among arginine deiminase (ADI), ornithine transcarbamylase (OTCase) and carbamate kinase (CK) protein sequences between *Oenococcus oeni* and *L. plantarum* and the induction of *arcABC* genes by arginine suggested that the putative genes cloned controlled arginine catabolism in *L. plantarum*.

In this paper we report the activities of enzymes involved in arginine metabolism in wine *L. plantarum* and the effect of arginine on growth of *L. plantarum*.

MATERIALS AND METHODS

Strains*. Lactobacillus plantarum* strains Lp90, Lp65, Lp60, Lp61, Lp77 and Lp21 previously identified by Spano *et al*. (2004, 2006) isolated from red wine undergoing malolactic fermentation were used for enzymes analysis.

Growth and enzymes assay. *Lactobacillus plantarum* strains were grown in the basal medium (Arena and Manca de Nadra, 2001) containing the following, in g l^{-1}: 5, peptone (Oxoid); 3, yeast extract (Oxoid); 1, glucose (Britania 046, Buenos Aires, Argentina); 1, arginine (Sigma). After incubation at 30 °C for 24 h, the cells from the third subculture were harvested at the end of the logarithmic growth phase, and the activities of arginine deiminase and ornithine transcarbamylase, two enzymes of the ADI pathway, were determined.

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Cells were harvested by centrifugation at 10,000 *x g* for 15 min and the pellet was washed twice with 0.2 M sodium phosphate buffer, pH 6.5. Cells were then resuspended at 2.5% (w/v) in the same buffer for determination of arginine deiminase activity and in 0.2 M sodium acetate buffer, pH 5.8, for determination of OTCase. To prepare cell extracts, cells pellets were first resuspended in 10 ml of cold respective buffer and then passed four times through a French pressure cells. Cells debris was removed by centrifugation at 13,000 *x g* for 6 min, and the supernatant extract was used to assay the activities of ADI system enzyme. All operations were carried out at 4 °C.

Enzyme activity was determined according to the Oginsky method (Oginsky, 1955) with modifications. The composition of the reaction mixture for arginine deiminase determination was as follows: 0.5 ml of L-arginine-HCl (0.1 M) adjusted to pH 6.5, 0.2 ml of sodium phosphate buffer (0.2 M) pH 6.5, and 0.5 ml of cell free extract. One ml of supernatant was analysed for citrulline concentration. The reaction mixture for OTCase determination was as follows: 0.5 ml of L-citrulline-HCl (0.1 M), 1 ml of sodium acetate buffer (0.5 M) pH 5.8, and 0.5 ml of cell free extract. One ml of supernatant was analysed for ornithine concentration. The mixtures were incubated at 30 °C and samples were taken every 15 min; the reaction was stopped by the addition of 0.2 ml of perchloric acid (70%). Specific enzyme activity was defined as the amount of product (µmol) (citrulline and ornithine, for arginine deiminase and OTCase, respectively) formed per min and per microgram of protein.

Analytical methods. A reverse-phase high performance chromatography (RP-HPLC) using an ISCO system (ISCO, Lincoln, NE) and a fluorimeter model 121 (340 nm excitation filter and 425 nm emission filter) were used. A Waters Nova-pack C18 column, 3.9 x 150 mm, 4 µm particle size, was used for the stationary phase with a flow of 1.5 ml min^{-1}. Citrulline and ornithine were determined by HPLC method based in the technique proposed by Alberto *et al.* (2002), but the gradient was modified in order to obtain the best and faster results. Solvents used for the separation: A - methanol, 10 mM sodium phosphate buffer pH 7.3, and tetra-hydrofuran (19:80:1) and B - methanol and 10 mM sodium phosphate buffer pH 7.3 (80:20). Solvent gradient conditions were as follows: 6 min, 0% B; 10 min, 15% B; 8 min, 80% B and 5 min, 0% B. Protein was quantified using the Bradford method.

TABLE 1 - Specific activities of arginine deiminase and ornithine transcarbamylase in strains of *Lactobacillus plantarum* isolated from red wine. The data presented are the mean of three independent experiments with their standard deviation.

L. plantarum strains	Arginine deiminase*	Ornithine transcarbamylase**
Lp90	6.12 ± 0.16	3.32 ± 0.12
Lp77	4.94 ± 0.13	3.04 ± 0.12
Lp65	2.00 ± 0.21	0.41 ± 0.06
Lp61	6.06 ± 0.15	3.60 ± 0.19
Lp60	8.51 ± 0.18	1.89 ± 0.12
Lp21	4.71 ± 0.13	2.84 ± 0.10

* Activity expressed as (µmol citrulline/min/µg protein)
** Activity expressed as (µmol ornithine/min/µg protein)

Influence of arginine on growth of *Lactobacillus plantarum*. To evaluate the influence of arginine on growth of wine *L. plantarum*, strains Lp60 and Lp65 were inoculated into MRS broth (De Man *et al*., 1960) at 28 °C pH 6.8 for 24 h. Following this, 0.5 ml (OD_{600} = 2.6) of *L. plantarum* strains Lp60 and Lp65 were centrifuged (11,000 *x g*, 10 min) and diluted in 25 ml of a fresh Niven medium: 2 g l^{-1} of K_2HP_4, 0.1% (v/v) of Tween 80, 50 mg l^{-1} of $MnSO_4$, pH adjusted to 5, 2% (wt/v) of glucose (Curk *et al*., 1996) and supplemented with 0.4 g l^{-1} or 2 g l^{-1} of arginine. Aliquots were then removed and serial dilutions were plated on Niven media plus 15 g l^{-1} of agar and incubated at 28 °C for 72 h. *Lactobacillus plantarum* strains Lp60 and Lp65 inoculated in a Niven medium without arginine were used as negative control. All the experiments were repeated three times.

RESULTS AND DISCUSSION

Arginine metabolism in *Lactobacillus plantarum* isolated from red wine

Although the ADI pathway consists of three enzyme activities (ADI, OTCase, CK), due to the carbamylphosphate instability (Mira de Orduña *et al*., 2001; Arena *et al*., 2002; Arena and Manca de Nadra, 2005), only two (ADI and OTCase) were analysed in order to test the ability of wine *L. plantarum* to degrade arginine. The activity of enzymes ADI and OTCase was detected in all the strains analysed. The results presented in Table 1 show that all strains of *L. plantarum* were able to degrade arginine and citrulline through the ADI system. *Lactobacillus plantarum* strains Lp60 and Lp90 are the strains that form more citrulline and the strain Lp61 forms more ornithine via arginine deiminase system. Strain Lp65 is that with lower activities of both enzymes. The higher arginine deiminase activity is correlated with a higher OTCase activity with exception of the strain Lp60. This strain produces 8.51 µmol citrulline/min/µg protein and 1.89 µmol ornithine/min/µg protein. The variability observed between strains may suggest that production of citrulline and ornithine in wine *L. plantarum* is probably dependent on strain as previously been reported for some strains of *O. oeni* (Tonon *et al*., 2001; Divol *et al*., 2003). *Lactobacillus plantarum* Lp60 is the strain with more possibilities to accumulate citrulline, precursor of the carcinogenic ethyl-carbamate, in the medium as showed by its high arginine deiminase activity and low ornithine transcarbamylase activity. Arena and Manca de Nadra (2005) reported a correlation between citrulline production and ethyl-carbamate formation by *Lactobacillus hilgardii* isolated from wine. Moreover, the authors observed that in a strain of *O. oeni* the inability to metabolise arginine and the ability to consume the citrulline from the medium diminished the synthesis of ethyl carbamate in presence of ethanol.

Influence of arginine on growth of *Lactobacillus plantarum*

Significant differences in growth were observed between *L. plantarum* strains inoculated in Niven media supplemented or not with arginine (Fig. 1). After 20 h, counts of *L. plantarum* strain Lp60, increased from 2.7 x 10^2 CFU ml^{-1} to about 2.0 x 10^8 CFU ml^{-1} in Niven media with arginine (Fig. 1A). In contrast, the growth of *L. plantarum* strain Lp65 (the strain with the lowest enzymatic activity) was almost unaffected by arginine (≤ 0.3 log CFU ml^{-1}), as the same results

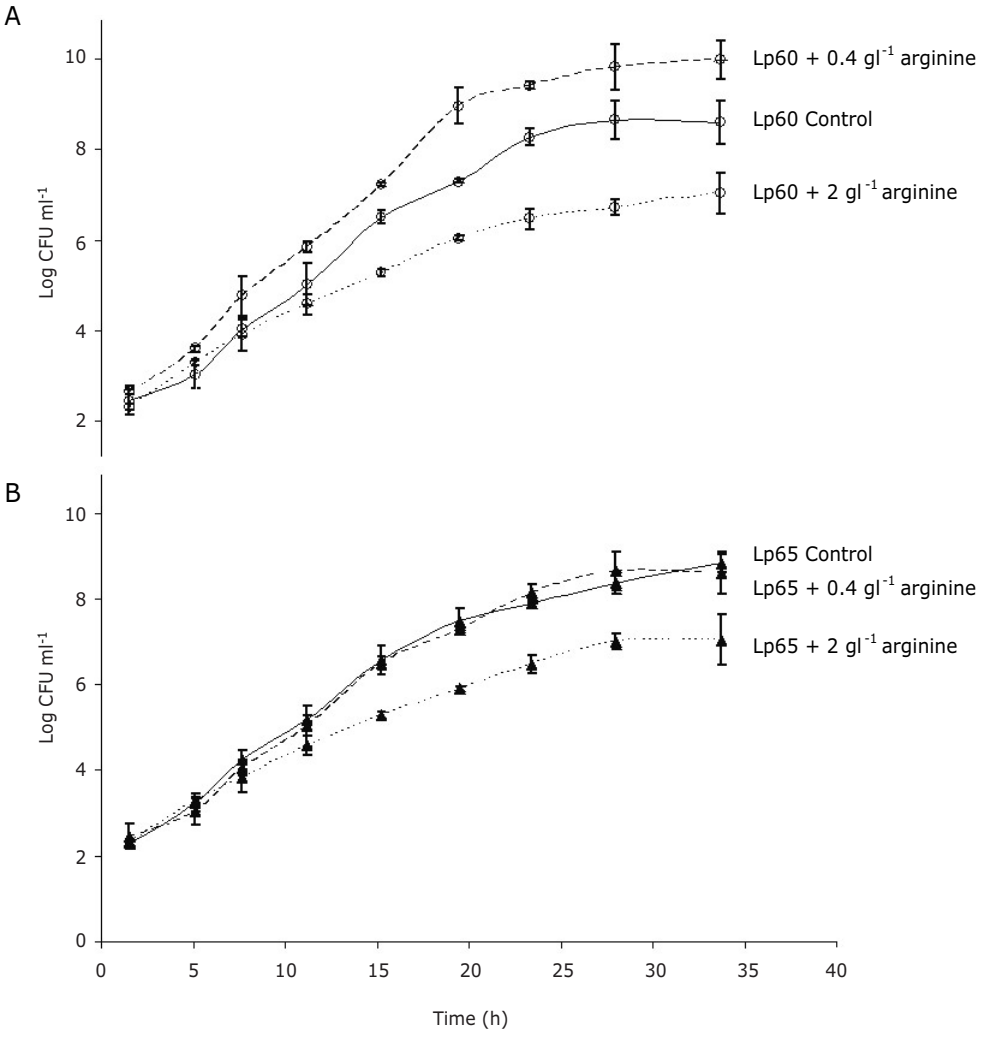

FIG. 1 - Effect of arginine on growth of *Lactobacillus plantarum* inoculated in Niven medium plus 0.4 g l^{-1} (----) or 2 g l^{-1} of arginine (.....) and in Niven medium without arginine (Control, solid line). A - *L. plantarum* strain Lp60 (○); B - *L. plantarum* strain Lp65 (▲). Growth was further monitored until the cells reached the stationary phase. The data presented are the mean of three independent experiments with their standard deviations indicated by vertical bars.

were observed in Niven media supplemented with or without arginine (Fig. 1B). At the end of the experiment, the CFU ml^{-1} recovered was about 2.0 log higher for the *L. plantarum* strain Lp60 inoculated in Niven medium supplemented with 0.4 g l^{-1} of arginine than the respective control, while no significant changes were observed for *L. plantarum* strain Lp65 (Fig. 1A). Interestingly, a reduction of growth (between 1.0 and 1.5 log CFU ml^{-1}) was noticed in both *L. plantarum* strains when the arginine concentration was increased from 0.4 g l^{-1} to 2 g l^{-1} (Fig. 1A and 1B).

The same results were observed when *L. plantarum* strains Lp65 and Lp60 were inoculated in basal media supplemented with 0.4 g l^{-1} of arginine instead of Niven medium (data not shown).

These findings suggest that the availability of arginine influenced positively the final biomass of wine *L. plantarum* strains able to metabolise it. However, growth of *L. plantarum* strains was apparently inhibited in Niven medium supplemented with 2 g l^{-1} of arginine, suggesting that arginine may acts as a growth inhibitor when arginine concentration surpasses a certain threshold. These results are in agreement with data already reported by several authors. Manca de Nadra *et al*. (1981) found growth inhibition of a *L. buchneri* strain by high concentrations (> 5 g l^{-1}) of arginine and the same result was reported for *Lactococcus lactis* subsp. *lactis* (Thompson, 1987). Moreover, growth of *O. oeni* strains has been reported to be inhibited by some concentrations of arginine (Thompson, 1987).

In conclusion, *L. plantarum* strains isolated from wine and harbouring genes involved in arginine catabolism are able, *in vitro*, to produce citrulline and ornithine from arginine. Arginine may have a positive effect on growth of *L. plantarum*. However, high concentrations of arginine reduce growth of wine *L. plantarum* as already reported for *O. oeni* and *Lactococcus lactis* subsp. *lactis* strains (Mira de Orduña *et al*., 2001) and competition with other amino acids by a common amino acid carrier has been proposed as inhibitory mechanism (Mira de Orduña *et al*., 2001).

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