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Wine composition plays an important role in the control of carcinogenic precursor formation by *Lactobacillus hilgardii* X_1B

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Abstract

BACKGROUND: *Lactobacillus hilgardii*, a wine lactic acid bacterium, is able to use arginine, through the arginine deiminase pathway with the formation of citrulline, a precursor of the carcinogen ethyl carbamate. The influence of different Argentine wine varieties (Merlot, Cabernet Sauvignon and Malbec), on bacterial growth and arginine metabolism was examined. Furthermore, the effect of different components normally present in wines on the enzyme activities of the arginine deiminase system was determined.

RESULTS: Malbec wine under all conditions assayed (33, 50 and 100% supplemented wine : basal media) showed higher arginine consumption and citrulline production than the other wines, as well as the highest bacterial growth and survival of *Lactobacillus hilgardii* X_1B. Glucose and L-malic inhibited both arginine deiminase enzymes while fructose and citric acid only inhibited arginine deiminase. The red wines assayed in this study had different composition, and this is an explanation for the different behavior of the bacterium.

CONCLUSION: The highest citrulline production in Malbec wine could be correlated with its lower concentrations of glucose, fructose, citric and phenolic acid than the other wines. Therefore, a wine with lower concentration of these sugars and acids could be dangerous due to the formation of ethyl carbamate precursors.
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Keywords: wine; arginine; lactic acid bacterium; grape variety

INTRODUCTION

Arginine is one of the substrates, apart from carbohydrates, that can be used as an additional energy source for the growth by lactic acid bacteria (LAB).[1–3] In wine, arginine is quantitatively one of the most prevalent amino acids.

Considering bacterial growth and metabolism, degradation of arginine could be beneficial to some degree. Its degradation by LAB through the arginine deiminase pathway (ADI) allows the development of spoilage by supplying an energy source (ATP) and, due to the formation of ammonia, provides protection against possible damage caused by acids.[2–4]

The ADI system comprises the following enzymatic reactions:

$$\text{arginine} + H_2O \xrightarrow{\text{arginine deiminase}} \text{citrulline} + NH_3$$

$$\text{citirulline} + P_i \xleftrightarrow{\text{ornithine transcarbamylase}} \text{oirnithine} + \text{carmamoyl-}P_i$$

$$\text{carbamyl-}P_i + ADP \xleftrightarrow{\text{carbamate kinase}} ATP + NH_3 + CO_2$$

On the other hand, this pathway could be dangerous due to the production of citrulline and carbamoyl phosphate. These products can react by a spontaneous chemical reaction with ethanol present in wine, to form ethyl carbamate (EC), an animal carcinogen.[2,4–8] Most fermented foods and beverages, including wine, contain trace amounts of EC (also known as urethane).[9]

Lactobacillus hilgardii is a very common heterofermentative LAB found in wine.[10] *L. hilgardii* X_1B isolated from Argentine wine produces citrulline from arginine.[3] Previous studies have shown that *L. hilgardii* X_1B is able to produce citrulline in the presence of different concentrations of ethanol and, consequently, it results in an increase in the formation of EC in the medium.[11] Fermented beverages are complex systems with a wide range of factors that affect the metabolic activity of LAB. Factors that have been observed to affect the wine bacterial metabolism are temperature, pH, alcohol content, organic acids,

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sugars and phenolic compounds and the time of bacterial survival.[7,12–17]

The purpose of this paper is to determine the influence of different varieties of Argentine wine on arginine metabolism and growth of *L. hilgardii* and to correlate this with the composition of the wines and the effectors of the ADI system.

EXPERIMENTAL

Organisms

L. hilgardii X_1B was isolated from Argentine wine.[18]

Media

Three different varieties of Argentine red wines, Merlot, Malbec and Cabernet Sauvignon, from the same winery (Mendoza city) were used for a comparative study. Each wine variety was supplemented (supplemented wine) with the following components: 1 g L^{-1} glucose (Cicarelli, •XX, Argentina), 2 g L^{-1} peptone (Britania, •XX, Argentina) and 2 g L^{-1} yeast extract (Britania). The basal medium used as control (BM) contained 1 g L^{-1} glucose, 2 g L^{-1} peptone and 2 g L^{-1} yeast extract.

Different experimental media were prepared by mixing different supplemented wine concentrations (0, 33, 50 and 100%, v/v) with the BM. Either arginine or citrulline was added to the different media at a final concentration of 2 g L^{-1}.

Growth conditions and culture procedure

Prior to the assays microorganisms were grown in BM, supplemented with 5% ethanol at pH 3.8, without agitation, until late exponential growth phase. After incubation at 30 °C for 24 h, the cells from the third sub-culture were harvested by centrifugation (1700 × *g* for 20 min at 4 °C), washed with sterile distilled water, and resuspended in BM to $OD_{560\ nm} = 0.90$. This bacterial suspension was used to inoculate the experimental media at a rate of 5% (v/v). These cultures were incubated in darkness at 30 °C for 15 days without shaking.

Growth measurement

Bacterial viability was determined by direct counting of colony-forming units (cfu mL^{-1}) determined by plating 0.1 mL of inoculated media on MRS medium.[19]

Analytical methods

Arginine and citrulline were determined by high-performance liquid chromatography.[20] Ammonia was determined by the indophenol blue reaction.[21] L-Malic and citric acids were determined using commercial enzymatic kit from Boehringer (Mannhein, Germany), glucose was assayed by the glucose oxidase method from Wiener (Lab. Rosario, Argentina), and fructose was assayed according to the Roe method.[22] Protein was quantified by Bradford's method.[23]

Total phenolic content was obtained by colorimetry using the Folin–Ciocalteu reagent[24] and the results were expressed in mg L^{-1} gallic acid equivalents. The quantitative precipitation of flavonoids by formaldehyde in strongly acid solution followed by determination of the residual phenols (Folin–Ciocalteu) gave the non-flavonoids. When this value was subtracted from that obtained for total phenols the difference is a measure of the flavonoid content.[25,26]

Assay for the enzyme activity

L. hilgardii X_1B was grown in BM medium supplemented with either arginine (1 g L^{-1}) to determine arginine deiminase activity or citrulline (1 g L^{-1}) to determine ornithine transcarbamylase (OTCase) activity. Cultures were harvested at the end of the logarithmic growth phase, and the activity of arginine deiminase and OTCase, two enzymes of the ADI pathway, was determined. Cells were harvested by centrifugation at 4000 × *g* for 15 min and the pellet was washed twice with 0.2 mol L^{-1} 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 mol L^{-1} sodium acetate buffer, pH 5.8, for determination of OTCase. Prior to the assay for enzyme activity the cells were ruptured in a French press and a cell-free extract was obtained by centrifugation at 5000 × *g* for 10 min. All operations were carried out at 4 °C.

Enzyme activity was determined according to the Oginsky method.[27] The composition of the reaction mixture for arginine deiminase determination was as follows: 0.4 mL of L-arginine HCl (0.1 mol L^{-1}) adjusted to pH 6.5; 1 mL of sodium phosphate buffer (0.2 mol L^{-1}), pH 6.5; 0.6 mL of cell-free extract and 1.6 mL of distilled water. The reaction mixture for OTCase determination was as follows: 1 mL of L-citrulline HCl (0.1 mol L^{-1}); 1 mL of sodium acetate buffer (0.5 mol L^{-1}), pH 5.8; 1 mL of sodium arsenate (0.1 mol L^{-1}) and 0.6 mL of cell-free extract. Both mixtures were incubated at 37 °C and samples were taken every 15 min; the reaction was stopped by the addition of 0.2 mL perchloric acid (70%). The samples were centrifuged at 4000 × *g* for 30 min at 4 °C, and the product (citrulline or ornithine) was determined in the supernatant.

For both enzymes the specific buffer was supplemented with different glucose, fructose, L-malic, and citric concentrations (0, 0.5, 1.0, 2.0 and 3.0 g L^{-1}). Only for arginine deiminase its metabolic product, citrulline, was also added to the reaction buffer at different concentrations. Enzyme activity was defined as the amount of product (mmol) formed per minute and per microgram of protein.

Statistical analysis

Minitab Statistical Software (Minitab Inc., •XX, USA) was used. Four replicates were used for each sample, and each experiment was performed three times. The data are presented as mean ± standard deviation.

RESULTS

The effect of wine varieties on the growth of *L. hilgardii* X_1B

Bacterial growth diminished with increasing wine concentration. At a concentration of 33% of supplemented wines maximal bacterial growth (3 days) and survival rate (15 days) were reduced, in relation to media without wine, from 7.68 to 7.45, 6.53 and 6.54 Log cfu mL^{-1} and from 5.50 to 2.80, 2.00 and 2.50 Log cfu mL^{-1}, for Merlot, Cabernet Sauvignon and Malbec wines, respectively (Fig. 1A–C).

At a concentration of 50% supplemented wines, the maximal growth rate was 6.80, 6.72 and 6.45 Log cfu mL^{-1} and the survival after 15 days of incubation was 2.23, 0.45 and 1.20 Log cfu mL^{-1} in the presence of Merlot, Cabernet Sauvignon and Malbec wines, respectively (Fig. 1A–C).

In the case of 100% supplemented Merlot wine (without amino acids), no growth was observed and the survival rate

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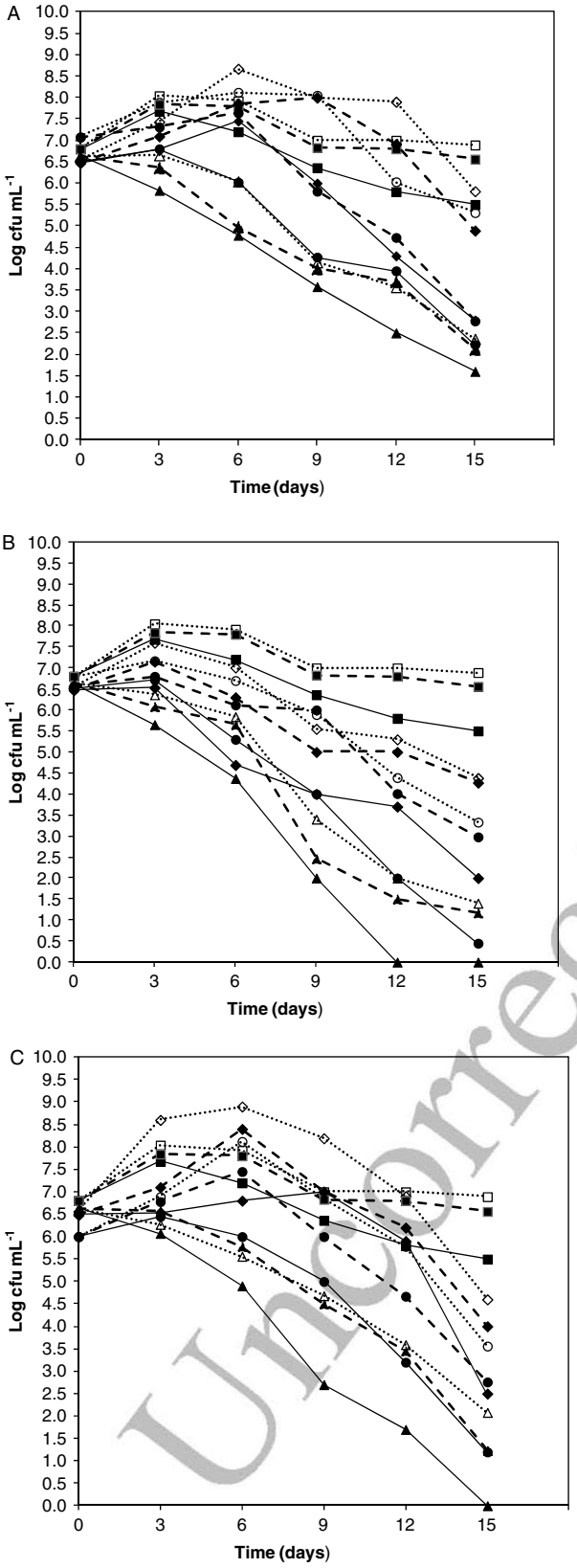

Figure 1. Viability of *Lactobacillus hilgardii* X_1B in media with 0 (■, □), 33 (♦, ◊), 50 (●, ○) and 100% (▲, △) concentrations of Merlot (A), Cabernet Sauvignon (B) and Malbec (C) supplemented wines in the presence of either arginine (broken lines and empty symbols) or citrulline (broken lines and solid symbols). Control without arginine or citrulline (continuous lines and solid symbols). Data are expressed as means, $n = 4$). Relative standard deviation (RSD) less than 6.00%, $P = 0.05$.

was 1.6 Log cfu mL^{-1} after 15 days incubation (Fig. 1A). In 100% supplemented Cabernet Sauvignon and Malbec media without the addition of amino acids neither growth nor survival was observed after 15 days of incubation (Fig. 1B and C). Survival was observed until 9 days (2.00 Log cfu mL^{-1}) for Cabernet Sauvignon and 12 days of incubation (1.70 Log cfu mL^{-1}) for Malbec wine.

The effect of wine varieties in the amino acid supplemented media on the growth of *L. hilgardii* X_1B

The addition of arginine or citrulline, but mainly arginine, to the different media increased bacterial growth. In fact, in BM the presence of arginine increased 0.36 Log cfu mL^{-1} the bacterial growth rate and the supplementation with citrulline increased 0.16 Log cfu mL^{-1} the bacterial growth rate.

In the presence of arginine the maximal growth rate increased 1.22 and 1.32 Log cfu mL^{-1} in BM plus 33 and 50% Merlot wine, respectively (Fig. 1A). Addition of citrulline to the media produced a stimulatory effect on maximal bacterial growth of 0.55 and 0.84 Log cfu mL^{-1} in BM plus 33 and 50% Merlot wine, respectively (Fig. 1A). In the case of 100% supplemented Merlot wine with the addition of arginine and citrulline the survival rate was 2.36 and 2.12 Log cfu mL^{-1}, respectively.

Figure 1B shows that the presence of arginine increased the maximal growth rate 1.07 and 0.45 Log cfu mL^{-1} in BM plus 33 and 50% Cabernet Sauvignon, respectively. Addition of citrulline to the same media produced a growth stimulatory effect of 0.62 and 0.08 Log cfu mL^{-1} in BM plus 33 and 50% Cabernet Sauvignon, respectively. In 100% supplemented Cabernet Sauvignon *L. hilgardii* X_1B was able to survive after 15 days of incubation with 1.40 and 1.17 Log cfu mL^{-1} in the presence of arginine and citrulline, respectively.

It can be seen from Fig. 1C that the enrichment with arginine stimulated the maximal growth rate 1.90 and 1.40 Log cfu mL^{-1} in BM plus 33 and 50% Malbec wine, respectively. Addition of citrulline to these media produced a less pronounced effect than with arginine; an increase of 1.90 and 1.00 Log cfu mL^{-1} was observed in BM plus 33 and 50% Malbec, respectively. In the presence of either amino acid in 100% supplemented Malbec wine, *L. hilgardii* X_1B was able to survive after 15 days of incubation with 2.08 and 1.23 Log cfu mL^{-1} for arginine and citrulline, respectively.

Arginine consumption, and citrulline and ammonia production

In the media supplemented with arginine the amino acid utilization decreased with increasing wine concentration. After 15 days of incubation in BM, arginine degradation was 9.21 mmol L^{-1}, whereas in Merlot at concentrations of 33, 50 and 100%, consumption was 6.79, 3.07 and 1.40 mmol L^{-1}, respectively (Fig. 2A). Arginine utilization in Cabernet Sauvignon at concentrations of 33, 50 and 100% was 7.07, 3.17 and 1.30 mmol L^{-1}, respectively (Fig. 2B) and 7.89, 3.40 and 2.60 mmol L^{-1} at concentrations of 33, 50 and 100% of Malbec wine, respectively (Fig. 2C).

The production of citrulline, an EC precursor, was well correlated with the consumption of arginine present in the media. After 15 days of incubation, 30% of the arginine consumed was recovered as citrulline in BM. In Merlot, the arginine recovered as citrulline was 12, 8 and 11% at concentrations of 33, 50 and 100%, respectively. Calculations of the molar ratio of citrulline formed from arginine utilized yielded values of 14, 11 and 9% for 33, 50 and 100% Cabernet Sauvignon, and 15, 24 and 18% for 33, 50 and 100% Malbec, respectively.

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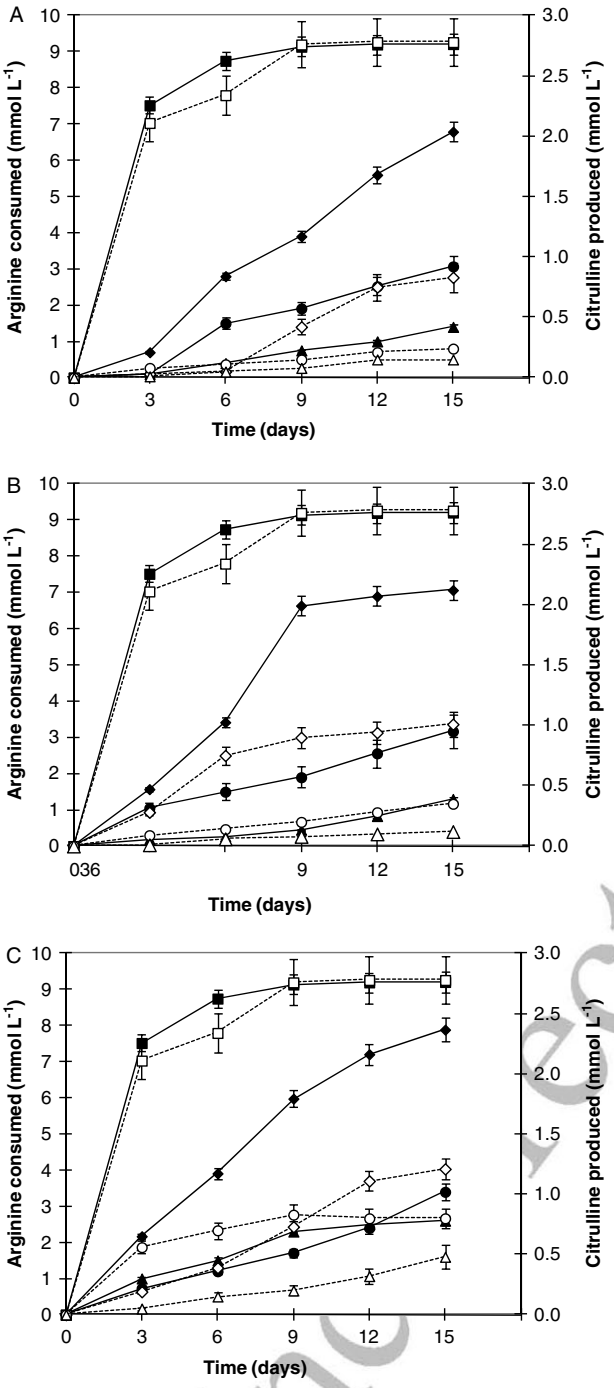

Figure 2. Arginine consumption (continuous lines and solid symbols) and citrulline production (broken lines and empty symbols) in BM (■, □), BM + 33% wine (♦, ◊), BM + 50% wine (●, ○) and in 100% wine (▲, △). Merlot (A), Cabernet Sauvignon (B) and Malbec (C) wines. Data are expressed as means ± standard deviation ($n = 4$) $P = 0.05$.

The ammonia produced in BM supplemented with arginine was 14.07 mmol L^{-1}. On a molar basis, the amount of ammonia released accounted for 1.53 of the arginine consumed; this result is well correlated with the ADI system. In the presence of wine the molar ratio of ammonia formed from arginine was 1.28, 1.60 and 1.21 for 33, 50 and 100% of Merlot wine; 1.19, 1.64 and 1.53 for 33, 50 and 100% of Cabernet Sauvignon, and 1.47, 186 and 1.90 for 33, 50 and 100% of Malbec, respectively.

Citrulline consumption and ammonia production

The consumption of citrulline is desirable in order to diminish the possibility of EC formation. In a previous study we demonstrated that the citrulline consumption decreased the amount of EC formed.[11] In the media supplemented with citrulline the amino acid degradation at the end of the incubation in BM was 3.10 mmol L^{-1} in all the cases the addition of wine to the medium reduced the consumption of citrulline. Merlot wine at a concentration of 33, 50 and 100% showed at 15 days of incubation a citrulline consumption of 1.74, 0.28 and 0.07 mmol L^{-1}, respectively. Citrulline consumption in Cabernet Sauvignon at concentrations of 33, 50 and 100% was 2.90, 0.50 and 0.08 mmol L^{-1}, respectively. The citrulline utilization in Malbec at concentrations of 33, 50 and 100% was 2.00, 0.20 and 0.09, respectively ●(Fig. 3).

At 15 days of incubation the ammonia formed as consequence of the citrulline consumed via the ADI pathway was 84% in BM, and in Merlot at a concentration of 33, 50 and 100% this percentage was 69, 36 and 57, respectively. In Cabernet Sauvignon at the same concentrations the results were 53, 40 and 50%, respectively. For Malbec the percentages were 57, 55 and 67%, respectively. The results indicate that nearly all the citrulline was transformed into ammonia.

pH modifications

The production of ammonia from arginine (2 moles per mole of totally consumed arginine) and from citrulline (1 mole per mole of totally consumed citrulline) is important because this component is able to modify the pH of the medium.

The final pH of BM was 3.40 without supplement of amino acids, and 4.70 and 3.75 in the presence of arginine and citrulline, respectively. For the combination of BM and 33% Merlot without addition of amino acids and with the addition of either arginine or citrulline, the final pH was 3.50, 4.10 and 3.90, respectively. At a Merlot concentration of 50% the final pH was 3.60, 4.00 and 3.80, respectively, and in 100% supplemented Merlot wine media the final pH was 3.10, 3.30 and 3.30, respectively. When BM was combined with Cabernet Sauvignon without addition of amino acids and with the addition of either arginine or citrulline the final pH was 3.70, 4.00 and 3.80 (33% Cabernet Sauvignon + BM), 3.68, 3.95 and 3.78 (50% Cabernet Sauvignon + BM) and 3.12, 3.40, 3.30 (100% Cabernet Sauvignon), respectively. Thirty-three, 50 and 100% of Malbec wines showed final pH values higher in presence of arginine (4.30, 4.10 and 3.60, respectively) and citrulline (3.80, 3.78 and 3.30, respectively) than without the amino acids supplementation (3.70, 3.68 and 3.12, respectively).

Production of ammonia from arginine is important. The pH increased in all the media supplemented with the amino acids with respect to the pH observed under the same conditions but without amino acids. Although glucose fermentation would normally lead to a decrease in pH, the production of ammonia from the amino acids would explain the higher final pH values. Furthermore, the variation in pH is another indicator that the ADI system was still functional in 100% supplemented wines.

Arginine deiminase and ornithine transcarbamylase activities

Table 1 shows the influence of different concentrations of glucose, fructose, malic acid, citric acid and citrulline on arginine deiminase and OTCase activity. Addition of 2.0 and 3.0 g L^{-1} of glucose to the reaction buffer produced a significant decrease in arginine

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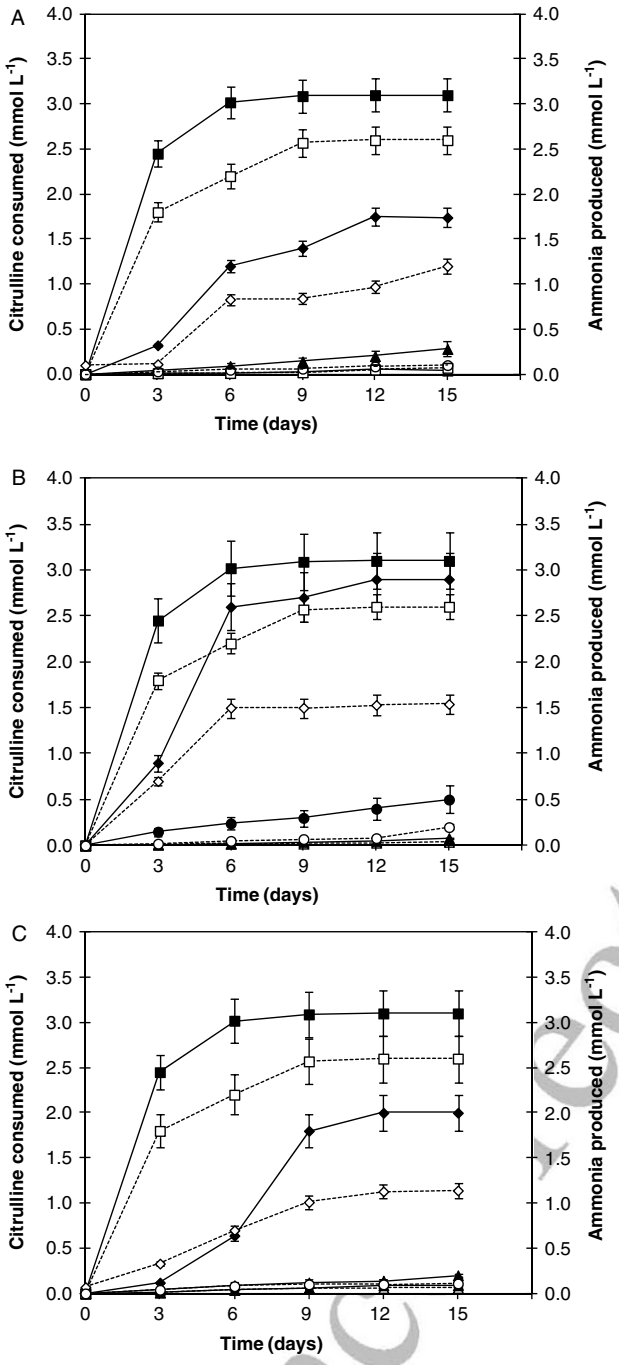

Figure 3. Citrulline consumption (continuous lines and solid symbols) and ammonia production (broken lines and empty symbols) in BM (■, □), BM + 33% wine (♦, ◇), BM + 50% wine (●, ○) and in 100% wine (▲, △). Merlot (A), Cabernet Sauvignon (B) and Malbec (C) wines. Data are expressed as means ± standard deviation ($n = 4$) $P = 0.05$.

deiminase activity (18 and 21%, respectively). Addition of 0.5 and 1.0 g L^{-1} did not modify the enzymatic activity. OTCase activity was reduced by 12 and 18% only after addition of 2.0 and 3.0 g L^{-1} of glucose, respectively.

When fructose was added to the reaction buffer, all the concentrations assayed (0.5, 1.0, 2.0 and 3.0 g L^{-1}) showed a significant inhibitory effect on arginine deiminase activity (13, 18, 29 and 37%, respectively). However, the effect on OTCase was not statistically significant.

The presence of 0.5, 1.0, 2.0 and 3.0 g L^{-1} of L-malic acid in the reaction buffer produced a decrease of 58, 63, 66 and 71%, respectively on arginine deiminase activity. However, significant inhibition of OTCase activity was only observed at 3 g L^{-1} of L-malic acid (17%).

Citric acid in the reaction mixture produced an inhibitory effect higher than 50% on arginine deiminase enzyme at all concentrations assayed and no modified the activity of OTCase. When citrulline, product of arginine deiminase enzyme, was added to the reaction buffer the arginine deiminase activity decreased proportionally to this amino acid concentration, suggesting that citrulline regulate its own synthesis.

Composition of different wines

We determined the chemical composition (Table 2) of the different wines to determine a relationship between the effects of different wines on arginine metabolism and its amounts of sugars and acids, effectors of the ADI system. Malbec wine has lower glucose, fructose, citric and phenolic acids concentrations, which result inhibitory for arginine deiminase enzyme, than the other wine varieties.

DISCUSSION AND CONCLUSIONS

In previous studies, the degradation of arginine into citrulline, ornithine and ammonia by *L. hilgardii* X_1B in the absence[2] and in the presence of ethanol[11] was demonstrated; furthermore the genes encoding the enzymes of the ADI system were described.[28] In this paper, the influence of three different varieties of Argentine wine on arginine metabolism and growth of *L. hilgardii* X_1B and the activity of the enzymes involved in arginine and citrulline degradation was determined as well as the influence of different components normally present in wines. According to Etièvant *et al.*,[29] concentrations of wine acids other than malic and lactic appeared to be related more to area of production than to grape variety. For this reason we used different wine varieties from the same region.

Degradation of arginine and excretion of citrulline concurred with increasing biomass, but the amino acid utilization and the bacterial growth are influenced by the wine varieties. At 15 days of incubation, in Malbec wine media supplemented with arginine at all conditions assayed (33, 50 and 100%) we observed higher survival, arginine consumption and citrulline production than the other wines.

Mira de Orduña *et al.*[30] have stated that 20 mg L^{-1} of citrulline would yield 30 µg L^{-1} EC after 3 years of storage at 15 °C. In the presence of ethanol, *L. hilgardii* X_1B has the potential to contribute to EC formation, whereas citrulline utilization by *Oenococcus oeni* in the presence of ethanol would contribute to diminish the formation of EC.[11] In our study, citrulline formation by LAB was 26, 21 and 84 mg L^{-1} from arginine in 100% supplemented Merlot, Cabernet Sauvignon and Malbec wines, respectively. These results suggest a possible relationship between the wine composition and the bacterial metabolism of arginine. Consequently, a possible spoilage or starter culture with the ADI pathway could be more dangerous in Malbec wine that in Merlot or Cabernet Sauvignon due to the major formation of EC precursors in this wine.

Uthurry *et al.*[31] have reported an increased concentration of EC after malolactic fermentation, irrespective of the bacteria used or the prevailing physicochemical conditions and observed that an

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Table 1. Effect of the energy sources present in wine on the arginine deiminase system enzymes

	Enzyme activity (mmol min^{-1} µg protein) × 10^2								
	Arginine deiminase					Ornithine transcarbamylase			
Concentration (g L^{-1})	Glucose	Fructose	L-Malic acid	Citric acid	Citrulline	Glucose	Fructose	L-Malic acid	Citric acid
0	3.8 ± 0.2	3.8 ± 0.2	3.8 ± 0.2	3.8 ± 0.2	3.8 ± 0.2	3.6 ± 0.2	3.6 ± 0.2	3.6 ± 0.2	3.6 ± 0.2
0.5	3.8 ± 0.3	3.3 ± 0.2	1.6 ± 0.3	1.8 ± 0.3	2.8 ± 0.3	3.6 ± 0.2	3.6 ± 0.3	3.6 ± 0.3	3.6 ± 0.3
1.0	3.7 ± 0.2	3.1 ± 0.2	1.4 ± 0.4	1.7 ± 0.3	2.6 ± 0.3	3.4 ± 0.3	3.5 ± 0.2	3.6 ± 0.3	3.6 ± 0.3
2.0	3.1 ± 0.2	2.7 ± 0.3	1.3 ± 0.3	1.6 ± 0.3	2.2 ± 0.3	3.2 ± 0.2	3.4 ± 0.2	3.2 ± 0.2	3.5 ± 0.3
3.0	3.0 ± 0.1	2.4 ± 0.2	1.1 ± 0.4	1.4 ± 0.3	1.6 ± 0.3	3.0 ± 0.1	3.3 ± 0.2	3.0 ± 0.1	3.4 ± 0.2

Enzyme activity was defined as the amount (mmol) of product formed (citrulline and ornithine) per min and per microgram of protein.
Enzyme activity of arginine deiminase and ornithine transcarbamylase after addition of glucose, fructose, L-malic acid, citric acid or citrulline to the reaction medium at concentrations of 0.5, 1.0, 2 and 3 g L^{-1}.
Results are the mean ± standard deviation.

Table 2. Composition of the wines

Variety	Glucose (g L^{-1})	Fructose (g L^{-1})	Citric acid (g L^{-1})	L-Malic acid (g L^{-1})	Total phenolics (mg GAE L^{-1})	Flavonoids (mg GAE L^{-1})	Phenolic acids (mg GAE L^{-1})
Merlot	0.69 ± 0.06	0.95 ± 0.06	0.89 ± 0.05	4.90 ± 0.06	2718 ± 115	1826 ± 98	892 ± 64
Cabernet Sauvignon	0.65 ± 0.08	0.82 ± 0.05	0.67 ± 0.06	5.20 ± 0.05	2390 ± 100	1750 ± 74	640 ± 30
Malbec	0.50 ± 0.05	0.66 ± 0.04	0.48 ± 0.03	5.30 ± 0.06	2452 ± 123	2008 ± 50	444 ± 80

GAE, gallic acid equivalents.

O. oeni strain was able to produce more EC than *L. hilgardii* in Cabernet Sauvignon but not in Tempranillo wine.

Our results showed that citric acid, fructose and citrulline inhibited degradation of arginine, and that L-malic acid and glucose inhibited degradation of both arginine and citrulline. Liu *et al.*[5] observed that when fructose was omitted from the culture medium production of ammonia from arginine increased, and Hiraoka *et al.*[32] reported that fructose seemed to reduce activity of the ADI pathway in *Streptococcus mitis*. Our results are in accordance with these observations, as the inhibitory effect of fructose for the first enzyme of the ADI system, arginine deiminase, was higher than that observed for glucose at the same concentrations. This suggests more a specific effect of fructose than an inhibitory effect due to the production of energy by sugars.

It has been suggested that if malo-lactic fermentation is desired, winemakers should either use commercial strains that do not produce high levels of citrulline or monitor the juice for citrulline contents after fermentation. It has been shown that the metabolic activity of wine LAB leads to the production of EC precursors in wine.[33] *O. oeni* seemed to inhibit arginine degradation when malic acid was present in the culture medium,[30] which agrees with our observations, because arginine deiminase activity decreased significantly in the presence of L-malic acid in the reaction medium. The fact that the presence of malic acid inhibits formation of citrulline, suggests that if the LAB metabolism is inhibited immediately when the malolactic fermentation finish, the formation of EC could diminish.

On the other hand, phenolic compounds or polyphenols are natural constituents of grapes and wines. These compounds are very important since they are responsible for many of the organoleptic properties of wines, especially color and astringency.[34] Alberto *et al.*[17] found that protocatechiuc and gallic acids, at the concentrations normally present in wine, could inhibit the activity of the ADI pathway.

The red wines assayed in this study had different composition, and this is a possible explanation for the wine influence on the amino acids metabolism observed.

There is good correlation between increased degradation of arginine in Malbec wine compared to other wines and its lower concentrations of glucose, fructose, citric and phenolic acids, which inhibit the arginine deiminase enzyme. Therefore, a wine with lower concentration of these sugars and acids would be dangerous from a hygienic point of view in relation to the formation of EC precursors.

From the results of this work it can be concluded that the ADI pathway in *L. hilgardii* X_1B was still active in the supplemented wine media. Thus, the presence of arginine or citrulline in the medium could affect bacterial survival and the production of EC, but, as a matter of fact, the different wine varieties also affect bacterial metabolism.

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