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Influence of ethanol and low pH on arginine and citrulline metabolism in lactic acid bacteria from wine

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Abstract

The aim of this work was to study the effects of ethanol on cell growth and arginine and citrulline metabolism in two heterofermentative lactic acid bacteria from wine, and to determine their possible association with the formation of ethyl carbamate (EC), a carcinogenic compound. *Lactobacillus hilgardii* X_1B is able to utilize arginine and citrulline, while *Oenococcus oeni* m can only use citrulline, a precursor of EC. Growth of both microorganisms was partially inhibited by 10 and 15% (v/v) ethanol. Specific arginine consumption by *L. hilgardii* increased when the pH value diminished from 6.5 to 3.8, but was not affected by an increasing ethanol concentration. However, the ethanol concentration affected the specific citrulline consumption of both microorganisms. Arginine metabolism by *L. hilgardii* X_1B increased the amount of citrulline, thus allowing production of EC in the medium. Citrulline utilization by both microorganisms, at all pH values studied, indirectly inhibited the formation of EC; indeed, one of the precursors had practically disappeared after 48 h of incubation. Due to its ability to form precursors, *L. hilgardii* X_1B has the potential to contribute to EC formation, whereas citrulline utilization by *O. oeni* m in the presence of ethanol may contribute to diminishing the formation of EC. Rapid degradation of citrulline in the presence of ethanol by *O. oeni* m is important from a toxicological point of view, because it is important to keep the EC levels as low as possible.
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1. Introduction

During the growth of malolactic acid bacteria, the formation of additional fermentation products and conversion of certain naturally occurring components in wines have generally been of only secondary interest. However, these secondary products, formed as an indirect result of malolactic fermentation or by spoilage organisms, may affect wine quality and possibly the health of the consumer.

Amino acids may serve as energy sources for certain lactic acid bacteria. Most strains of *Oenococcus oeni* isolated from Italian wines degraded arginine and excreted citrulline in synthetic medium [10]. Arena et al. [1] demonstrated that two *O. oeni* strains from wine, m and X_2L, were not able to use arginine, and that *Lactobacillus hilgardii* X_1B utilized arginine via the arginine deiminase system (ADI), forming ATP, NH_3, CO_2, ornithine and citrulline. They also showed that citrulline was utilized by *L. hilgardii* X_1B and *O. oeni* m in the absence of ethanol. Arena et al. [3] sequenced and cloned a gene cluster of *L. hilgardii* X_1B encoding enzymes involved in the ADI pathway and this represented the first example of cloning and heterologous expression of a *L. hilgardii* gene.

The metabolic activity of LAB is known to be influenced by ethanol concentrations [20], but no information is available on the ethanol influence on the activity of the arginine deiminase pathway in wine LAB.

One of the major concerns in arginine and citrulline metabolism by wine LAB is their association with the formation of ethyl carbamate (EC). EC precursors can be formed by yeasts [12,17,18] or bacteria [1,13]. Several pos-

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sible sources for the formation of EC have been proposed. This formation is a spontaneous chemical reaction involving ethanol and a compound that contains a carbamoyl group such as urea, citrulline or carbamyl phosphate. A variety of concentrations of EC have been found in wines [19]. Criteria related to consumer concern regarding environmental health issues include the demand for low-alcohol wine and ethyl-carbamate-free beverages. Detection of EC in wines has prompted the selection of strains producing no, or only traces of, ethyl carbamate precursors.

The aim of this paper was to study the effects of ethanol and pH on growth and arginine and citrulline metabolism in two heterofermentative LAB from wine and to determine their possible association with the formation of EC.

2. Materials and methods

2.1. Organisms

L. hilgardii X_1B and *O. oeni* m were isolated from Argentine wines [14,22].

2.2. Media, growth conditions and culture procedures

The basal medium (BM) contained in $g\,l^{-1}$: peptone, 5; yeast extract, 3; glucose, 1 and 15% (v/v) tomato juice. $1\,g\,l^{-1}$ L-arginine-hydrochloride or L-citrulline was added to the BM. The pH was adjusted to 3.8 and to the optimal pH values for the growth of each microorganism: 6.5 for *L. hilgardii* X_1B and 4.8 for *O. oeni* m, before sterilization in an autoclave at 121 °C for 20 min. Absolute ethanol was added to final concentrations of 10 and 15% (v/v), respectively. The control media and the media with 10% (v/v) of ethanol were diluted to 15% (v/v) with distilled sterile water in order to obtain the same dilution of nutrients in all media.

The strains were precultured in BM and then inoculated into experimental media at a concentration of 5×10^7 cells ml^{-1}. Cultures were incubated statically at 30 °C for 10 days under microaerophilic conditions. Microaerophilic growth was conducted in two-thirds-full capped tubes. Samples were taken every 6 h for growth measurement and stored at −18 °C for subsequent analyses.

2.3. Growth measurement

Bacterial growth was determined spectrophotometrically (OD_{560}) and by direct cell counts.

2.4. Analytical methods

Arginine, citrulline, ornithine and ammonia were determined colorimetrically [7,8,21,23]. Glucose was analyzed by the glucose oxidase method.

Potential EC was defined as the amount of EC in sample supernatant of cultures after 10 days of incubation formed during heating at 80 °C for 48 h. For EC determinations the samples were twenty- and sixtyfold concentrated and analyzed according to the AOAC Official Methods for Analysis of alcoholic beverages [6], using a capillary HP-20 M column.

The control medium for EC formation was the same medium without inoculation and processed under the same conditions.

2.5. Statistical analysis

The data were analyzed by the balanced ANOVA test. Variable means showing statistical significance were compared using Tukey' s test (Minitab Student R14).

3. Results

3.1. Effect of arginine and citrulline on L. hilgardii and O. oeni growth in the presence of ethanol

The influence of amino acids on bacterial growth in the presence of ethanol was assayed both at the average pH of wine and at the optimal growth pH of each microorganism.

Fig. 1 shows that addition of 15% ethanol to control media without amino acids reduced *L. hilgardii* X_1B growth from 9.69 to 9.42 log CFUs ml^{-1} (from 4.90^9 to 2.63^9 cells ml^{-1}) at initial pH 6.5, and from 7.62 to 7.47 log CFUs ml^{-1} (from 4.17×10^7 to 2.95×10^7 cells ml^{-1}) at an initial pH of 3.8.

Addition of 15% ethanol to the same medium supplemented with arginine or citrulline also reduced cell growth at both initial pH values. At pH 6.5 growth diminished from 3.09×10^{10} to 1.48×10^{10} cells ml^{-1} and from 8.7×10^{10} to 7.08×10^{10} cells ml^{-1} in the presence of arginine and citrulline, respectively.

At low pH 15% ethanol reduced the number of cells from 2.24×10^9 to 7.94×10^8 cells ml^{-1} with arginine and from 2.00×10^9 to 7.24×10^8 cells ml^{-1} with citrulline.

On the other hand, addition of arginine or citrulline to the medium stimulated growth of *L. hilgardii* X_1B under all conditions. In the absence of ethanol and at pH 6.5 growth increased from 9.69 to 10.49 and 9.94 log CFUs ml^{-1} in the presence of arginine and citrulline, respectively. Addition of 10 and 15% ethanol enhanced growth from 9.65 to 10.26 and 9.88 log CFUs ml^{-1} and from 9.42 to 10.17 and 9.85 log CFUs ml^{-1} in the presence of arginine and citrulline, respectively.

In those media with an initial pH of 3.8 growth increased by the addition of arginine and citrulline from 7.62 to 8.35 and 8.30 log CFUs ml^{-1}, respectively, and without ethanol. In the presence of 10% ethanol growth was enhanced from 7.51 to 8.16 and to 8.06 log CFUs ml^{-1} after addition of arginine and citrulline, respectively. In the media with 15% ethanol growth was stimulated from 7.47 to 7.9 and 7.86 log

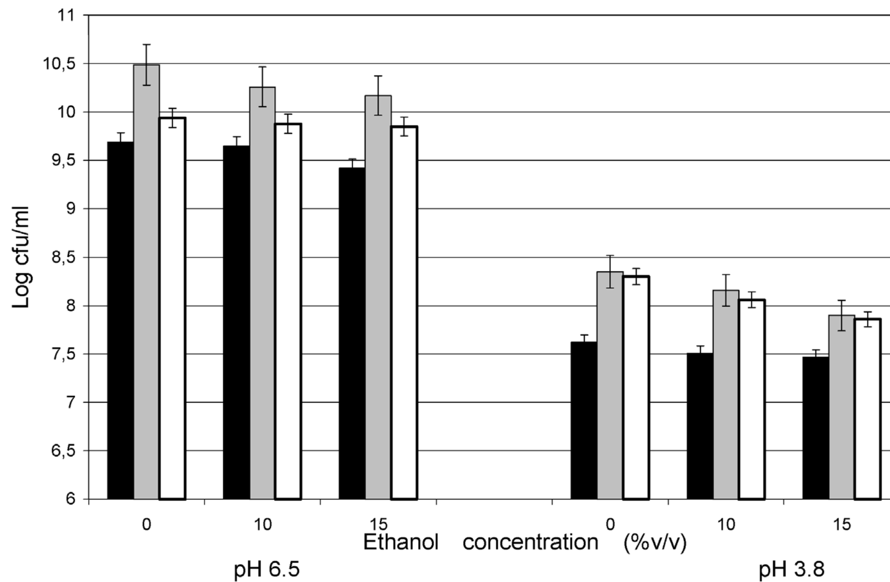

Fig. 1. Growth of *L. hilgardii* X_1B in BM (■), and BM added with 1 $g\,l^{-1}$ arginine (■) or citrulline (□) in the presence of 0, 10 and 15% (v/v) ethanol at pH 6.5 and 3.8 after 10 days of incubation at 30 °C. Data are expressed as means ± standard deviation ($n = 4$) $P \leqslant 0.05$.

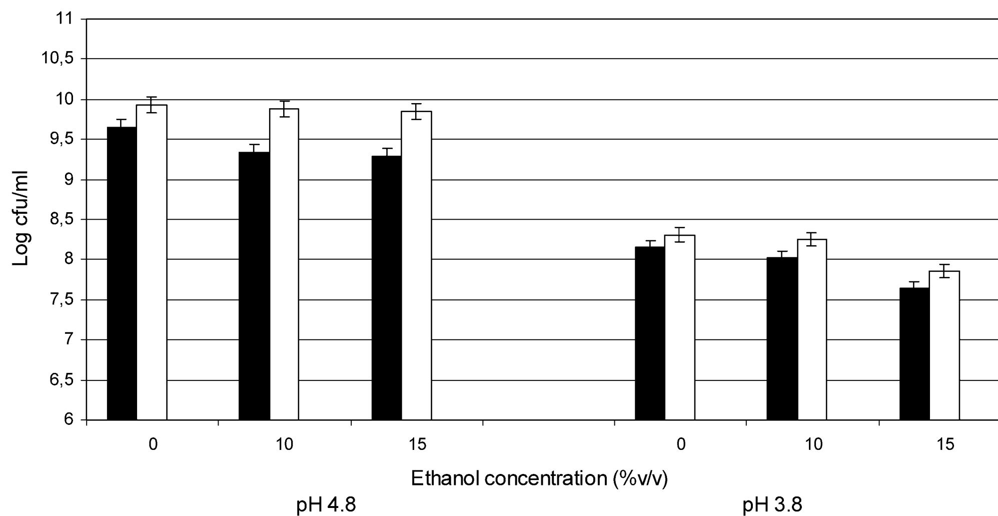

Fig. 2. Growth of *O. oeni* m in BM medium (■) and BM added with 1 $g\,l^{-1}$ citrulline (□) at pH 4.8 and pH 3.8 in the presence of 0, 10 and 15% ethanol after 10 days of incubation at 30 °C. Data are expressed as means ± standard deviation ($n = 4$) $P \leqslant 0.05$.

CFUs ml^{-1} in the presence of arginine and citrulline, respectively.

The effect of the initial pH was significant. In general, growth in basal medium at pH 6.5 was two logarithmic units higher than that at pH 3.8.

Fig. 2 shows that growth of *O. oeni* m at pH 4.8 was inhibited by 10 and 15% ethanol.

The inhibitory effect of ethanol (15%) in basal medium reduced the log CFUs ml^{-1} from 9.65 to 9.29 at an initial pH of 4.8 and from 8.15 to 7.65 at pH 3.8. In BM supplemented with citrulline the inhibitory effect of ethanol reduced the log CFUs ml^{-1} from 9.93 to 9.85 at a high initial pH and from 8.31 to 7.86 at a low initial pH.

Addition of 1 $g\,l^{-1}$ L-citrulline to the medium stimulated growth at all ethanol concentrations. At pH 3.8 (Fig. 2) growth was lower than at pH 4.8, but addition of citrulline showed the same stimulatory effect.

A reduction in the initial pH produced an inhibitory effect that was more pronounced for *L. hilgardii* (a decrease of 2.07 log CFUs ml^{-1}) than for *O. oeni* (a decrease of 1.50 log CFUs ml^{-1}).

3.2. Influence of ethanol and pH on arginine catabolism

Utilization of arginine by *L. hilgardii* X_1B was examined at three ethanol concentrations and at two pH values (Table 1).

Consumption of arginine diminished with decreasing pH values of the medium. Taking into account the number of cells, specific arginine consumption in $mmol\,l^{-1}$ at pH 3.8

Table 1
Arginine utilization and production of metabolites by *L. hilgardii* in the presence of ethanol

Ethanol (%)*	Final biomass (10^8 CFUs ml^{-1})		Arginine utilization ($mmol\,l^{-1}$)		Metabolite production ($mmol\,l^{-1}$)					
					Citrulline		Ornithine		Ammonia	
	pH 6.5	pH 3.8	pH 6.5	pH 3.8	pH 6.5	pH 3.8	pH 6.5	pH 3.8	pH 6.5	pH 3.8
0	310 ± 12	2.14 ± 0.16	5.19 ± 0.31	4.04 ± 0.36	1.02 ± 0.05	0.85 ± 0.10	1.33 ± 0.07	0.89 ± 0.06	9.26 ± 0.56	6.03 ± 0.45
10	181 ± 9	1.46 ± 0.12	3.05 ± 0.15	2.02 ± 0.21	0.60 ± 0.03	0.55 ± 0.08	0.68 ± 0.04	0.33 ± 0.03	5.22 ± 0.27	2.73 ± 0.39
15	150 ± 8	0.79 ± 0.09	2.50 ± 0.15	1.15 ± 0.19	0.46 ± 0.02	0.39 ± 0.06	0.57 ± 0.02	0.19 ± 0.02	4.44 ± 0.21	1.96 ± 0.32

* Ethanol was added to BM containing 5.55 $mmol\,l^{-1}$ arginine. Determinations were carried out after 48 h of incubation at 30 °C. Data are expressed as means ± standard deviation ($n = 6$) $P \leqslant 0.05$.

Table 2
Citrulline utilization and production of metabolites by *L. hilgardii* X_1B in the presence of ethanol

Ethanol (%)*	Final biomass (10^8 CFUs ml^{-1})		Citrulline utilization ($mmol\,l^{-1}$)		Metabolite production ($mmol\,l^{-1}$)			
					Ornithine		Ammonia	
	pH 6.5	pH 3.8	pH 6.5	pH 3.8	pH 6.5	pH 3.8	pH 6.5	pH 3.8
0	280 ± 13	1.34 ± 0.10	1.80 ± 0.08	0.69 ± 0.09	0.83 ± 0.04	0.39 ± 0.03	1.59 ± 0.05	0.62 ± 0.03
10	170 ± 7	0.67 ± 0.03	0.96 ± 0.04	0.28 ± 0.03	0.43 ± 0.03	0.17 ± 0.02	0.78 ± 0.03	0.28 ± 0.02
15	142 ± 5	0.43 ± 0.02	0.77 ± 0.03	0.19 ± 0.02	0.33 ± 0.02	0.09 ± 0.01	0.61 ± 0.03	0.18 ± 0.01

* Ethanol was added to BM containing 5.69 $mmol\,l^{-1}$ citrulline. Determinations were carried out after 48 h of incubation at 30 °C. Data are expressed as means ± standard deviation ($n = 6$) $P \leqslant 0.05$.

was two orders higher than that at pH 6.5. The corresponding values for the molar ratio of ammonia formed to total arginine utilized were: 1.78 and 1.49; 1.71 and 1.35; 1.78 and 1.70, for 0, 10 and 15% ethanol, and at a pH 6.5 and 3.8, respectively.

A low pH, a stress factor for growth, did not inhibit the strain's ability to consume arginine present in the medium, a property that could be related to the release of ammonia. In those media with an initial pH of 3.8 the final pH was higher than the initial pH (4.05, 4.00 and 4.15 for the media with 0, 10 and 15% ethanol, respectively). In those media with an initial pH of 6.50 a decrease in the final pH was observed. This reduction was 0.32 (±0.03) and 0.21 (±0.04) unit of pH lower in the media with addition of arginine with respect to BM with 10 and 15% ethanol, respectively.

The specific arginine consumption at pH 6.5 was 1.67 ± 0.15, 1.69 ± 0.16 and 1.66 ± 0.10 $mmol\,l^{-1} \times 10^{10}$ cells for 0, 10 and 15% ethanol, respectively. At pH 3.8 the specific arginine consumption was 189 ± 15, 138 ± 15 and 146 ± 12 $mmol\,l^{-1} \times 10^{10}$ cells for 0, 10 and 15% ethanol, respectively. A decrease in pH from 6.5 to 3.8 increased the specific arginine consumption two orders. Only at a low pH did the presence of ethanol diminish the specific arginine consumption.

When arginine was present in the medium the cell number increased (Fig. 1). The molar growth yield (Y arginine) was expressed as: the increase in the number of cells with respect to the BM without arginine divided by the arginine consumed ($mmol\,l^{-1}$) $\times 10^8$. The Y arginine could be influenced by ethanol and pH. At an initial pH of 6.5, the Y arginine $\pm\delta$Y was: 50.3 ± 3.08; 44.7 ± 3.92 and 49.5 ± 3.77 in the presence of 0, 10 and 15% ethanol, respectively. At an initial pH of 3.8, the Y arginine was 0.42 ± 0.04; 0.56 ± 0.08 and 0.43 ± 0.10 with 0, 10 and 15% ethanol, respectively. The test for error propagation showed a significant difference only at different pH, but not for the ethanol concentrations at the same pH value.

3.3. Influence of ethanol and pH on citrulline consumption

Utilization of citrulline by *L. hilgardii* X_1B and *O. oeni* m was examined at two different ethanol concentrations. After 48 h of incubation, production of ornithine and ammonia corresponded well with the degradation of citrulline in both microorganisms at all pH values under study (Tables 2 and 3). Recovery of the citrulline degraded by *L. hilgardii* X_1B into ammonia was 88 and 90; 81 and 100; and 79 and 95% for 0, 10 and 15% ethanol at pH 6.5 and 3.8, respectively (Table 2). The minor recovery as ornithine is in accordance with the ability of the strain to degrade ornithine [1,2]. Recovery of citrulline degraded by *O. oeni* m into ammonia was 95 and 88, 93 and 80, 91 and 88% for 0, 10 and 15% ethanol at pH 4.8 and 3.8, respectively. The citrulline recovered as ornithine was 98 and 92, 93 and 87, 94 and 75% for 0, 10 and 15% ethanol at pH 4.8 and 3.8, respectively (Table 3).

In those media with an initial pH of 3.8 and utilization of citrulline by *L. hilgardii* with production of ammonia after 10 days of incubation, final pH values increased from 3.40 to 3.85, from 3.72 to 3.91 and from 3.70 to 3.95 in the presence of 0, 10 and 15% ethanol, respectively. In medium with an initial pH of 6.5 in the presence of citrulline the final pH was 0.28 (±0.04); 0.20 (±0.03) and 0.16 (±0.03) units of pH lower than the BM with 0, 10 and 15% ethanol.

When citrulline was added to the BM the number of cells of *L. hilgardii* increased (Fig. 2). The molar growth yield

Table 3
Citrulline utilization and production of metabolites by *O. oeni* in the presence of ethanol

Ethanol (%)*	Final biomass (10^8 CFUs ml^{-1})		Citrulline utilization ($mmol\,l^{-1}$)		Metabolite production ($mmol\,l^{-1}$)			
					Ornithine		Ammonia	
	pH 4.8	pH 3.8	pH 4.8	pH 3.8	pH 4.8	pH 3.8	pH 4.8	pH 3.8
0	87 ± 5	2.06 ± 0.04	0.44 ± 0.02	0.24 ± 0.01	0.43 ± 0.02	0.22 ± 0.01	0.42 ± 0.02	0.21 ± 0.02
10	76 ± 3	1.76 ± 0.05	0.35 ± 0.01	0.15 ± 0.01	0.33 ± 0.01	0.13 ± 0.01	0.32 ± 0.01	0.12 ± 0.03
15	72 ± 2	0.73 ± 0.06	0.33 ± 0.01	0.08 ± 0.01	0.31 ± 0.02	0.06 ± 0.01	0.30 ± 0.01	0.07 ± 0.01

* Ethanol was added to BM containing 5.69 $mmol\,l^{-1}$ citrulline. Determinations were carried out after 48 h of incubation at 30 °C. Data are expressed as means ± standard deviation ($n = 6$) $P \leqslant 0.05$.

(Y citrulline) was expressed as: the increase in the number of cells with respect to the same media without citrulline divided by the amino acid consumed $(mmol\,l^{-1})^{-1} \times 10^8$. At initial pH 6.5, the Y citrulline by *L. hilgardii* was 128 ± 8; 130 ± 13 and 150 ± 7 with 0, 10 and 15 ethanol, respectively. At initial pH 3.8, the Y citrulline was: 1.34 ± 0.20; 1.24 ± 0.94 and 0.71 ± 0.38 in the presence of 0, 10 and 15 ethanol, respectively.

A reduction in pH from the optimal pH value for *L. hilgardii* growth (pH. 6.5) to the usual pH of wine (3.8) diminished the molar growth yield for arginine and citrulline 120- and 96-fold, 80- and 105-fold and 115- and 211-fold with 0, 10 and 15% ethanol, respectively, thus indicating an important inhibitory effect of the pH on cell growth and amino acid metabolism.

The specific citrulline consumption by *L. hilgardii* at pH 6.5 was 0.64 ± 0.04, 0.57 ± 0.03, and 0.54 ± 0.02 $mmol\,l^{-1} \times 10^{10}$ cells for 0, 10 and 15% ethanol, respectively. At pH 3.8 the specific citrulline consumption was 51.5 ± 3.3, 41.8 ± 4.5, and 44.2 ± 5.0 $mmol\,l^{-1} \times 10^{10}$ cells for 0, 10 and 15% ethanol, respectively. The specific amino acid consumption by *L. hilgardii* was increased two orders by a decrease in pH. In all cases, the presence of ethanol and a decrease in pH diminished the specific consumption of citrulline.

In media with a lower initial pH the metabolism of *O. oeni* increased the final pH with respect to that observed in basal media. The values were 3.42, 3.60 and 3.65 for basal media and 3.87, 3.90 and 3.83 for media with addition of citrulline, with 0, 10 and 15% ethanol, respectively. In media with an initial pH of 4.8, citrulline showed the same stimulatory effect on the final pH.

The specific citrulline consumption by *O. oeni* at pH 4.8 was 0.51 ± 0.02, 0.43 ± 0.03, and 0.42 ± 0.02 $mmol\,l^{-1} \times 10^{10}$ cells for 0, 10 and 15% ethanol, respectively. At pH 3.8 the specific citrulline consumption was 11.7 ± 1.2, 8.6 ± 1.3, and 11.0 ± 1.4 $mmol\,l^{-1} \times 10^{10}$ cells for 0, 10 and 15% ethanol, respectively. A decrease in pH significantly increased the specific amino acid consumption. Specific consumption of citrulline by *O. oeni* diminished in the presence of ethanol except for 15% ethanol at pH 3.8

When citrulline was added to the BM the number of cells of *O. oeni* increased (Fig. 2). The molar growth yield (Y citrulline) was expressed as: the increase in the number of cells with respect to the same media without citrulline divided by the amino acid consumed $(mmol\,l^{-1})^{-1} \times 10^8$. At initial pH 4.8 the Y citrulline by *O. oeni* was 91.8 ± 9; 154.0 ± 14.6; 155 ± 8. At initial pH 3.8 Y citrulline for the same strain was: 2.63 ± 0.16; 4.87 ± 0.39; 3.50 ± 0.69 with 0, 10 and 15% ethanol, respectively. A reduction of one unit of the initial pH for this strain reduced the molar growth yield for citrulline 35-, 32- and 44-fold, thus indicating an inhibitory effect of low pH on growth and amino acid metabolism.

In addition, the presence of 10% ethanol in the media increased the Y citrulline at pH 4.8 nearly 1.7-fold and the Y citrulline at pH 3.8 1.33-fold.

Table 4
Ethyl carbamate formation from arginine metabolism by *L. hilgardii*

Ethanol (%)*	Ethyl carbamate formation ($ng\,ml^{-1}$)	
	Control medium	*L. hilgardii* X_1B
0	1.02 ± 0.11	3.90 ± 0.29
10	2.89 ± 0.21	16.97 ± 1.17
15	3.45 ± 0.27	20.89 ± 1.36

* Ethanol was added to BM containing 1 $g\,l^{-1}$ arginine. Control: BM containing 1 $g\,l^{-1}$ arginine without inoculation. Potential EC formation was determined after 30 days of incubation at 30 °C. Data are expressed as means ± standard deviation ($n = 3$) $P \leqslant 0.05$.

3.4. Influence of arginine metabolism on ethyl-carbamate formation

To elucidate whether EC formation is influenced by citrulline formed by *L. hilgardii* X_1B metabolism, its concentration in the medium was determined at different concentrations of ethanol.

The amount of ethyl-carbamate in all the media studied without inoculation was not significant. Secretion of citrulline into the medium by *L. hilgardii* X_1B resulted in the formation of 17 and 21 $ng\,ml^{-1}$ of EC in the presence of 10 and 15% ethanol, respectively, at pH 3.8 (Table 4). An increase in ethanol in the medium from 10 to 15% corresponded to an increase of 23% in ethyl carbamate.

3.5. Influence of citrulline metabolism on ethyl-carbamate formation

Citrulline in juice and wines varies from less than 1 to 55 $mg\,l^{-1}$ [19]. Table 3 illustrates that at pH 3.8 *O. oeni* m

Table 5
Ethyl carbamate formation from citrulline by *L. hilgardii* and *O. oeni*

BM + citrulline ($50\ mg\,l^{-1}$), ethanol (%)*	Ethyl carbamate ($ng\,ml^{-1}$)		
	Control	*L. hilgardii* X_1B	*O. oeni* m
10	16.24 ± 1.01	13.56 ± 1.06	11.06 ± 0.75
15	18.67 ± 1.12	14.31 ± 0.81	12.79 ± 0.72

* Ethanol was added to BM containing $50\ mg\,l^{-1}$ citrulline. Control: without inoculation. Potential EC formation was determined after 10 days of incubation at 30 °C. Data are expressed as means ± standard deviation ($n = 3$) $P \leqslant 0.05$.

degraded $0.15\ mmol\,l^{-1}$ ($26\ mg\,l^{-1}$) citrulline in the presence of 10% ethanol and $0.08\ mmol\,l^{-1}$ ($14\ mg\,l^{-1}$) with 15% ethanol. Almost all the citrulline consumed was recovered as ammonia, the final product of the ADI system in both cases. Table 5 shows a reduction of 32% in EC formation by *O. oeni* m in the presence of 10 and 15% ethanol. Utilization of citrulline by *L. hilgardii* X_1B also reduced formation of EC (Table 5).

4. Discussion

Arginine and citrulline are substrates that can be used to keep LAB viable, and the ability of the strains studied to utilize these amino acids in the presence of ethanol is an important property in surviving adverse conditions.

Arginine stimulated growth of *L. hilgardii* under all conditions studied, and was partially recovered as citrulline and ornithine. This result agrees with the ability of *L. hilgardii* X_1B to degrade citrulline and ornithine, as previously demonstrated [1]. In addition, Tonon and Lonvaud-Funel [25] reported that all *L. hilgardii* strains studied have the ability to degrade arginine via the ADI system. Although lactic acid bacteria effectively degraded arginine, this led to only a moderate pH increase because arginine degradation favored formation of acid from sugar [16].

The total amount of arginine utilized decreased with increasing ethanol concentrations. The higher specific consumption at pH 3.8 than at 6.5 could be explained by considering the inhibitory effect of the low pH on growth. The arginine used at low pH probably serves more as energy for survival than for growth. The amount of maintenance energy required at low pH is high [11]. Stuart et al. [24] reported, for *Lactococcus lactis,* that arginine provided metabolic products and energy that allowed cells to survive.

When pH decreased, arginine utilization increased; this result is in agreement with previous reports [16,25]. However, specific arginine consumption was not modified by the increase in ethanol, indicating that ethanol only affected bacterial growth.

With respect to citrulline utilization, both microorganisms studied were able to degrade it in the presence of ethanol. Ethanol has an inhibitory effect on the specific utilization of citrulline by *L. hilgardii* X_1B and *O. oeni* m.

We observed higher specific citrulline consumption at lower pH. Tonon and Lonvaud-Funel [25] have reported that more citrulline was used at pH 4.5 than 6.0, and that the increase in the final pH was more significant at pH 4.5, with an increase of one pH unit.

According to the maximum concentration of EC suggested in wine, $15\ ng\,ml^{-1}$ in the USA and $30\ ng\,ml^{-1}$ in Canada, the citrulline produced by *L. hilgardii* through metabolism of arginine and its subsequent conversion into EC may account for an important amount of this compound.

EC was detected in table wine at concentrations between 0 and $102\ ng\,ml^{-1}$ [5]. Six percent of Canadian red wines tested were above the permitted level [9]. Azevedo et al. [4] reported that citrulline is apparently the main EC precursor produced by *L. hilgardii* strains in spoiled, fortified wine. The metabolism of arginine by *Pediococcus halophillus* was thought to be the source of an EC precursor in soy sauce [15]. Ethanol concentrations increased EC formation in our analysis. Uthurry et al. [26] showed that ethanol was not directly correlated with the amount of EC in Spanish wines.

Citrulline utilization by *L. hilgardii* X_1B is correlated with a decrease in the formation of EC. However, the amount of EC formed through production of the EC precursor from arginine was higher than the reduction observed from citrulline utilization.

Degradation of citrulline and fast degradation of carbamoyl phosphate to form ATP and ammonia by *O. oeni* may have toxicological implications, and therefore it could be important to keep EC levels in wines as low as possible [19].

From these results, it can be concluded that *L. hilgardii* X_1B has the potential to contribute to EC formation through the production of citrulline, whereas citrulline utilization by *O. oeni* m in the presence of ethanol could possibly avoid the formation of EC.

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