

RESEARCH ARTICLE



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Influence of wine-like conditions on arginine utilization by lactic acid bacteria

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Summary. Wine can contain trace amounts of ethyl carbamate (EC), a carcinogen formed when ethanol reacts with carbamyl compounds such as citrulline. EC is produced from arginine by lactic acid bacteria (LAB), e.g., *Lactobacillus* and *Pediococcus*. Although the amounts of EC in wine are usually negligible, over the last few years there has been a slight but steady increase, as climate change has increased temperatures and alcohol levels have become proportionately higher, both of which favor EC formation. In this study, resting cells of LAB were used to evaluate the effects of ethanol, glucose, malic acid, and low pH on the ability of non-oenococcal strains of these bacteria to degrade arginine and excrete citrulline. Malic acid was found to clearly inhibit arginine consumption in all strains. The relation between citrulline produced and arginine consumed was clearly higher in the presence of ethanol (10–12 %) and at low pH (3.0), which is consistent with both the decreased amount of ornithine produced from arginine and the reduction in arginine degradation. In *L. brevis* and *L. buchneri* strains isolated from wine and beer, respectively, the synthesis of citrulline from arginine was highest. [Int Microbiol 2011; 14(4):225-233]

Keywords: *Lactobacillus* · *Pediococcus* · arginine · ethyl carbamate · wine

Introduction

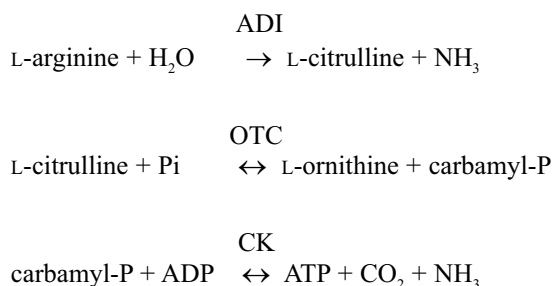
Wine, like most fermented foods and beverages [37], contains trace amounts of ethyl carbamate (EC) [21], also referred to as urethane. EC can bind covalently to DNA and is therefore a carcinogen to animals [26]. It is formed at low pH by the reaction between ethanol and *N*-carbamyl compounds, such as urea, citrulline, and carbamyl phosphate, with formation dependent on reactant concentrations [22]. As this reaction is favored by high temperatures [29], EC content

is higher in wines that have been stored for a long time under conditions in which the temperature has been poorly controlled [35]. Although EC concentrations in wine are usually negligible (< 10 µg/l), since 2002, in parallel with climate changes, a slight increase has been determined [20], since higher temperatures give rise to higher ethanol levels, both of which favor EC formation. This trend has evidenced the need for control and research on the mechanisms of EC formation.

Urea produced by yeast is the main potential EC precursor in wine, but lactic acid bacteria (LAB), mainly spoilage strains, can contribute to EC formation as well, due to their production of citrulline and carbamyl-P from arginine [2,12]. Furthermore, significant levels of EC found in some wines have been correlated with the former presence of LAB [35]. L-Arginine is one of the main amino acids in grapes and wine [11] and it is known to be degraded by some wine LAB [13]. Arginine catabolism by these LAB involves the arginine deiminase (ADI) pathway [14,18], which includes three

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enzymes, ADI (EC 3.5.3.6), ornithine transcarbamylase (EC 2.1.3.3, OTC), and carbamate kinase (EC 2.7.2.2, CK) [13], catalyzing the following reactions:



This pathway is thought to contribute positively to the growth and viability of LAB through ATP formation and the decrease in acidity caused by ammonium production [32]. Nevertheless, this has not been confirmed for *Oenococcus oeni*, the main malolactic bacteria in wine. In fact, arginine and citrulline do not stimulate the growth of some strains of this species in wine, in contrast to the growth of *Lactobacillus buchneri* [31]. In general, however, the degradation of arginine yields citrulline, which can react with ethanol to form EC. Moreover, the ADI pathway is sometimes indirectly related to the production of biogenic amines, specifically putrescine, which can be produced from ornithine by LAB [10,17].

Wine lactobacilli vary in their ability to degrade arginine. All heterofermentative lactobacilli are degradative [1,15]. In particular, *Lactobacillus hilgardii* plays a major role in fermented beverage spoilage, and those strains isolated from wine have been shown to degrade arginine [34,36]. Different strains of *Lactobacillus brevis* and *L. buchneri* isolated from wine have also been shown to degrade arginine [12].

While presumably facultative heterofermentative lactobacilli from wine are unable to degrade arginine [7,13], some *Lactobacillus plantarum* have been shown to consume arginine by means of the ADI pathway [1,28]. Likewise, homofermentative pediococci isolated from other fermented foods (beer, cheese, sausages) can degrade arginine [15], and we have found that some strains of *Pediococcus pentosaceus* isolated from wine also degrade this amino acid [1]. Although *O. oeni* is the main species responsible for malolactic fermentation (MLF) [9,38] and can degrade arginine [1,3,34], other LAB may proliferate during the early stages of MLF, or later if the conditions are propitious, such as when the acidity is low [16].

Some LAB, mainly *Pediococcus* and *Lactobacillus*, are also known to be spoilage microorganisms for beer [25].

Beer's acidic pH and ethanol conditions, similar to those of wine, are suitable for the production of EC precursors by these bacteria. Although the metabolic activity of LAB is influenced by ethanol, very little information is available regarding ethanol's influence on the activity of the arginine deiminase pathway in these LAB species, so such that their potential to degrade arginine in wine and beer is poorly understood.

The aim of this study was to determine the effects of ethanol, glucose, malic acid, and low pH values on the ability of LAB found mainly in wine to degrade arginine and to excrete citrulline, ornithine, and ammonia. We focused on those species considered responsible for spoiling wine (and beer), and therefore did not include *O. oeni*, the main species for MLF in wine. Moreover, arginine degradation and the metabolism and genetics of the ADI pathway are already well known in this species [4,12,15,19,31–33,39], and no effect of ethanol on arginine degradation has been reported [4], while pH values lower than 3.5 have been found to inhibit arginine consumption [30].

This study was based on LAB resting-cell experiments, in which the bacteria were grown in a complex medium and then prepared as highly concentrated cell suspensions in a defined medium for further analysis.

Materials and methods

Microorganisms and growth conditions. A pair of strains was used for each of the four different species of LAB frequently found in wine. Each pair consisted of the corresponding type culture strain (*Lactobacillus brevis* 4121^T, *Lactobacillus hilgardii* 4786^T, *Lactobacillus buchneri* 4111^T and *Pediococcus pentosaceus* 4695^T) and another strain isolated from wine (*L. brevis* 3824, *L. hilgardii* 4681 and *P. pentosaceus* 4214) or beer (*L. buchneri* 4674). All strains were from the CECT (Spanish Type Culture Collection, Valencia, Spain), except *L. brevis* 3824 and *P. pentosaceus* 4214, which were kindly contributed by S. Ferrer (Enolab, University of Valencia, Spain).

All strains were grown anaerobically at 27 °C in MRS medium [6] supplemented with 4 g DL-malic acid/l and 5 g D(-)-fructose/l. Bacterial growth was measured by determining the OD₆₀₀ of the bacterial suspension and by direct cell counts with a Neubauer chamber.

Culture condition and resting cells experiments. Each strain was grown anaerobically in 500 ml of MRS medium supplemented with 5 g L-arginine/l, at 27 °C, to the late-exponential/early-stationary phase. The cells were then harvested by centrifugation at 6000 ×g for 5 min at room temperature. The procedure for resting-cell experiments was designed based on that of Mira de Orduña et al. [18]. Cell pellets were resuspended in appropriate amounts of resting-cell buffer and transferred to small glass vials containing an aqueous solutions of 0.5 g arginine/l, at pH 3.6 (control assay, without ethanol or glucose or malic acid). The other conditions assayed with the same arginine concentration differed with respect to pH (3.0, 4.0, and 4.5), concentration of added ethanol (0, 5, 10, and 12 %), and amount of

added glucose (0.5, 3.0, and 5.0 g/l), or L-malic acid (0.5 and 2.0 g/l). Assays were also performed with increasing concentrations of arginine (1.5, 3.0, and 5.0 g/l) and the same control conditions. The glass vials were placed in a water bath (25 °C) and stirred gently. Samples were taken periodically, centrifuged at 13,000 ×g for 5 min, and the supernatants were frozen and kept at -20 °C until analyzed.

Analyses of amino acids and ammonia. An HPLC method based on one previously described [8,27] was adapted to improve the resolution of the three peaks of the amino acids of interest. The analyses were carried out on an Agilent 1100 series HPLC (Agilent Technologies, Wilmington, DE, USA) equipped with an automatic sampler system. The samples were filtered through a 0.45-µm membrane (Millipore) before injection. Two µl of each sample was mixed with 5 µl of borate buffer 0.4 M at pH 10.2, 1 µl of L-norvaline (internal standard), and 1 µl of the derivatization agent *o*-phthaldehyde-3-mercaptopropionic acid (OPA-3-MPA). One µl of this mixture was injected into a 4.0 × 250 mm ID column filled with Hypersil-ODS (Agilent Technology), with a 4 mm × 5 µm guard-column packed with the same phase.

Separation of the amino acids under study took 30 min at a flow rate of 1.5 ml/min. The mobile phase was composed of two different solvents: A and B. Solvent A was a mixture of 2.2 g of sodium acetate (Sigma), 220 µl triethanolamine (TEA, Sigma), and 6 ml tetrahydrofuran (Aldrich). After mixing, the pH was adjusted to 7.2 with 1 % acetic acid. Solvent B was a mixture of 1.8 g sodium acetate (Sigma), 320 ml of methanol (Panreac), and 400 ml of acetonitrile (Panreac), with the pH adjusted to 7.0 with 1 % acetic acid. The gradient was 100 % A for 7.5 min, 10 min with 15 % B, 1 min with 60 % B, 2 min more at 100 % B, and ending with 100 % A for 5 min, in order to prepare the column for the next sample. The analysis temperature was set at 40 °C.

Amino acids were detected using the retention time established for the individual amino acids and for a mixture thereof. The linearity of the peak areas for each amino acid was determined for different concentrations, ranging from 0 to 1000 µM. Calculations were based on the area under the

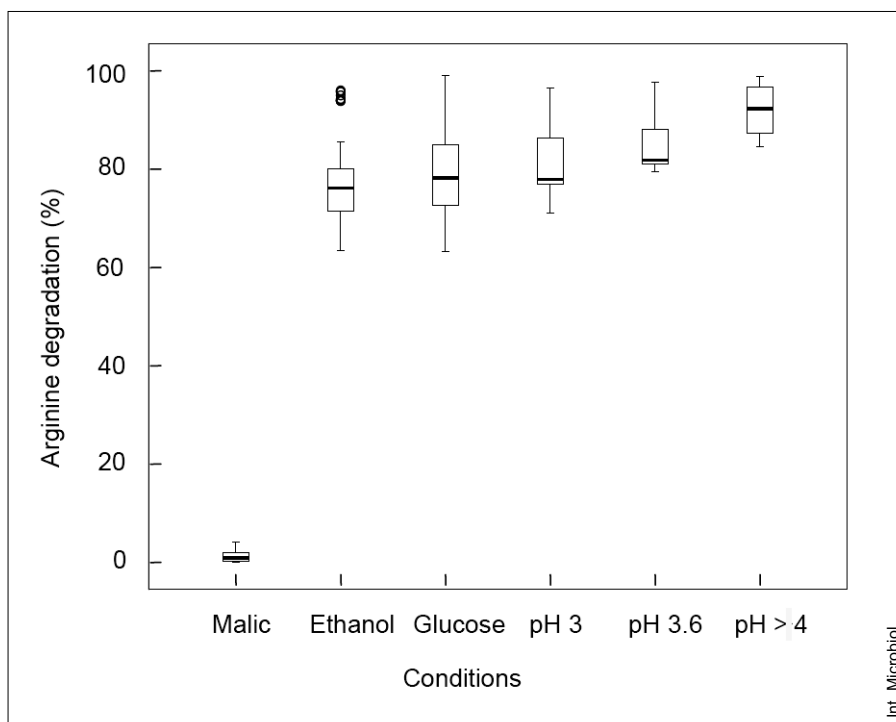
peak established for a given amino acid of known concentration and normalized with the internal standard. Ammonia was quantified with an enzymatic kit from Boehringer-Mannheim (Roche Pharma GmbH, Darmstadt, Germany).

Statistical analyses. Data univariate (ANOVA) and multivariate (PCA) analyses were conducted using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Variable means showing statistic significance were compared using Scheffé post-hoc comparisons at a significance level of 0.05, after testing the homogeneity of variance assumption between the various groups. Principal component analysis (PCA) with varimax rotation was performed for all the samples (408 samples: 4 species × 2 strains × 3 replicates × 17 conditions). The observed variables were the ratios between ornithine and arginine (Orn/Arg), between citrulline and arginine (Cit/Arg), and between ammonium and arginine (NH₄/Arg), as well as the percentage of arginine degradation (Arg %), and final pH. The experiments were performed in a laboratory that complies with ISO 9001 standards.

Results

Approximately 10⁹ cells per ml were obtained from the strains grown in MRS medium and harvested at OD 1.0 as determined by direct counts. Measurements of total arginine degradation are presented with respect to the specific group, defined according to the examined conditions, with the results for the different strains shown in the same group (Fig. 1). The data obtained under all the conditions are not shown, in order to facilitate a visual analysis of the results. Arginine (0.5 g/l) was almost fully degraded (70–100 %) in most of the

Fig. 1. Box-and-whiskers plot of the effect of different conditions on the degradation of arginine (0.5 g/l) for all assayed strains. The control assay was performed at pH 3.6. Malic: presence of L-malic acid (0.5 and 2.0 g/l) in the assay. Ethanol: presence (5, 10, and 12 %) in the assay. Glucose: presence (0.5; 3.0 and 5.0 g/l) in the assay. The assays plotted at pH 3 and pH > 4 (4 and 4.5) were without ethanol or glucose or malic acid. The line in the box is the median; the lower whisker is the minimal value found; the upper whisker the maximal value. Circles are the outliers with values greater than 1.5 times the spread outside.



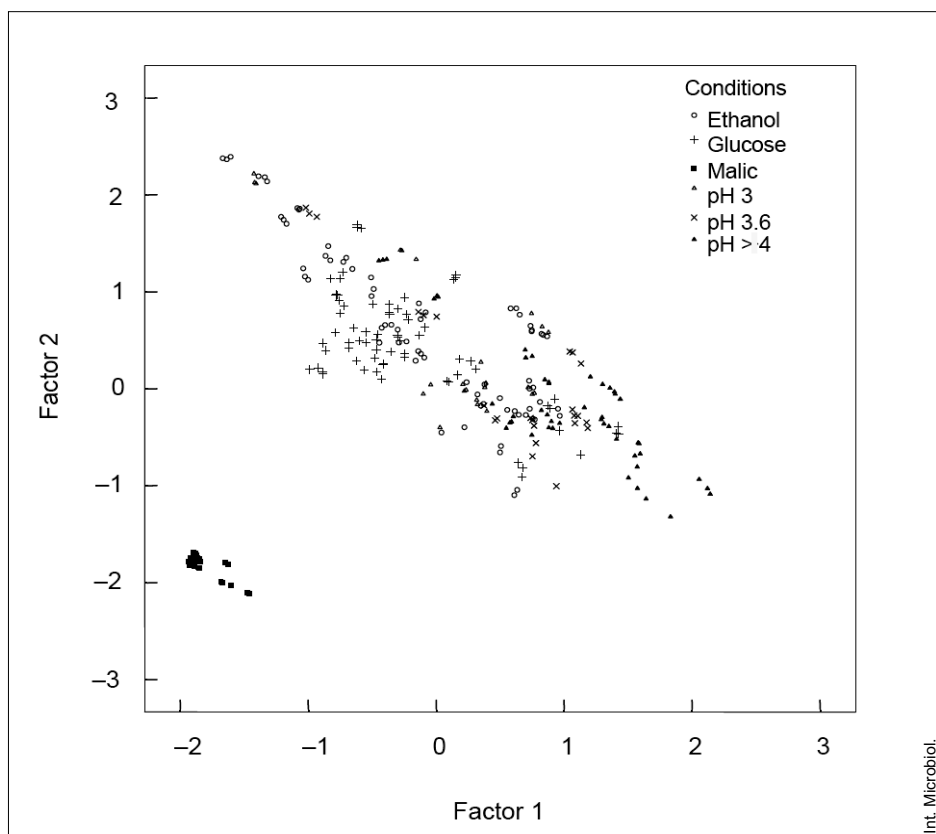


Fig. 2. Plot values of the first two factors (Factor 1 and Factor 2) of the PCA analysis according to the experimental conditions for the 408 samples. Kaiser-Meyer-Olkin (KMO): 0.803; variance of Factor 1: 40.7 %; variance of Factor 2: 22.4 %. Factor 1 positively correlated with Orn/Arg (0.930), Arg % (0.733) and final pH (0.719). Factor 2 positively correlated with Cit/Arg (0.710).

experiments. The strain with the highest levels of arginine consumption was, under most conditions, *L. hilgardii* 4681, with values exceeding 95 %.

Slightly higher consumption was observed for all the strains upon increasing pH whereas consumption was lower in the presence of ethanol. The presence of glucose also decreased arginine degradation. The only condition in which arginine was not consumed at all was in the presence of L-malic acid. When 0.5 or 2 g L-malic acid/l was added to the resting cells, the degradation of the initial 0.5 g arginine/l was nearly zero. When the initial arginine concentration was increased from 0.5 to either 1.5, 3, or 5 g/l (results not shown in figures), good consumption rates were noted for all the tested strains, with a few lower degradation values, progressively decreasing to 60 % at higher arginine levels, but also a higher total quantity of arginine consumed. Degradation rates were somewhat higher than those of the controls.

The amounts of citrulline, ornithine, and ammonium produced are expressed in relation to the arginine consumed, as determined by means of PCA (Fig. 2). The percentage of arginine degradation and the final pH obtained under different conditions were also used in the factorial analysis. Factor 1 explained 40.7 % of the variation and was marked by high

positive loadings for Orn/Arg, Arg %, and final pH. This result implies that higher levels of ornithine production led to higher final pH values. On the other hand, factor 2, which still explained 22.4 % of the variation, was marked by a high positive loading for Cit/Arg, which could be related to the high citrulline production by the strains. In relation to the third component (not shown), factor 3 explained 20.4 % of the total variation and was marked by a high positive loading for NH_4/Arg (0.900). To facilitate the interpretation of these results, the obtained scores were plotted by selecting the first two factors as axes (Fig. 2). As shown, the samples basically clustered in two groups. The first cluster (bottom left corner) represents the non-degradation of arginine in the presence of malic acid, regardless of the strain tested, and the second cluster represents the clear tendency of some species to alkalize the medium when ornithine (but not citrulline) was produced from arginine. Depending on the experimental conditions (ethanol, glucose, low pH values or increasing amounts of arginine), the strains used in this study showed different arginine-consuming behaviors.

We obtained values of Cit/Arg ranging from 10 % to 50 %, as shown in Fig. 3 for strain *L. hilgardii* 4786^T as an example. At higher pH, less citrulline was excreted and more

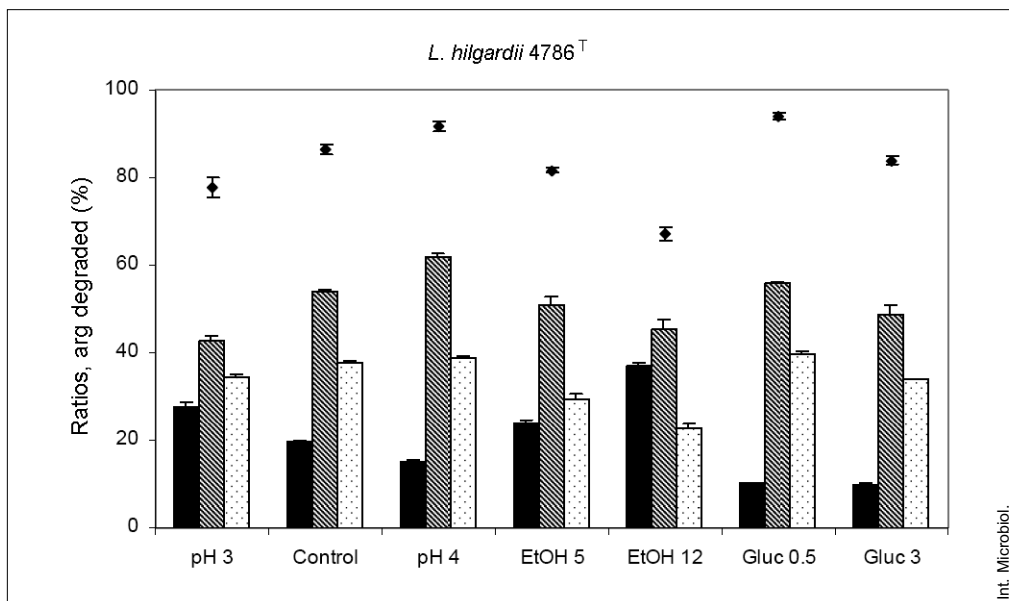


Fig. 3. Ratios of products from arginine degraded by *Lactobacillus hilgardii* 4786^T under different conditions: citrulline (black), ornithine (diagonal stripes), ammonium (x 2) (dotted). Percentages of degraded arginine are indicated with diamonds. Data represent the mean of three samples ± SD.

ornithine and ammonium were obtained. The ammonium /arginine ratio was expressed as 2 moles of NH₄⁺ per mole of arginine, because if all arginine were degraded then one mole of it would yield two moles of NH₄⁺.

A higher-level production of citrulline occurred when the quantity of ethanol was increased, which, in turn, led to lower values of Orn/Arg and NH₄⁺/Arg. The maximum ratio of Cit/Arg was obtained at 12 % ethanol. Increased glucose did

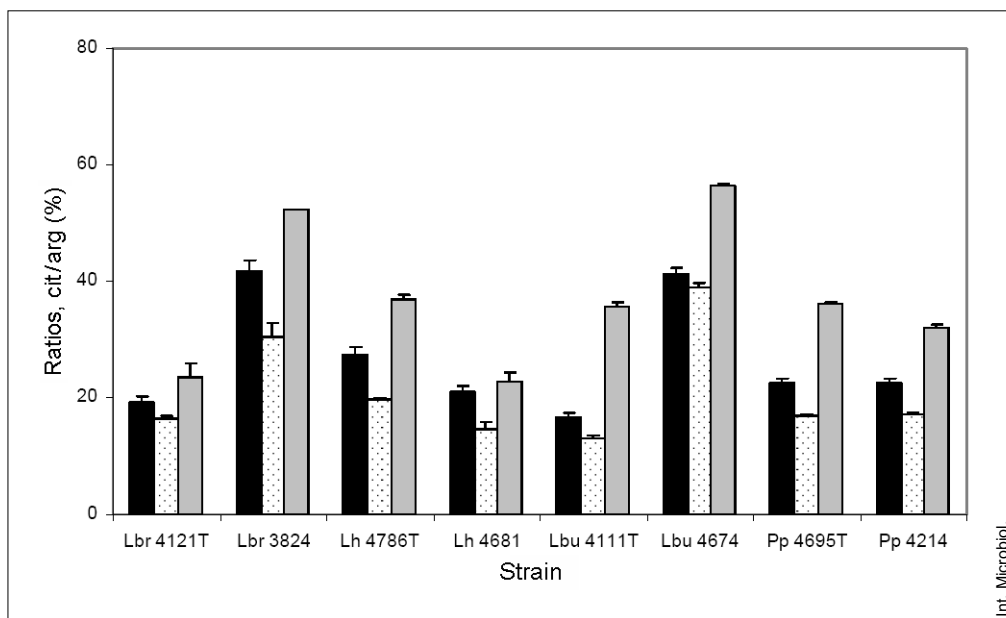


Fig. 4. Ratios of citrulline produced from arginine by different strains of LAB (see Materials and methods) under these conditions: without ethanol at pH 3.0 (black columns); without ethanol at pH 3.6 (control, dotted columns); without ethanol 12% at pH 3.6 (gray). Data represent the mean of three samples ± SD.

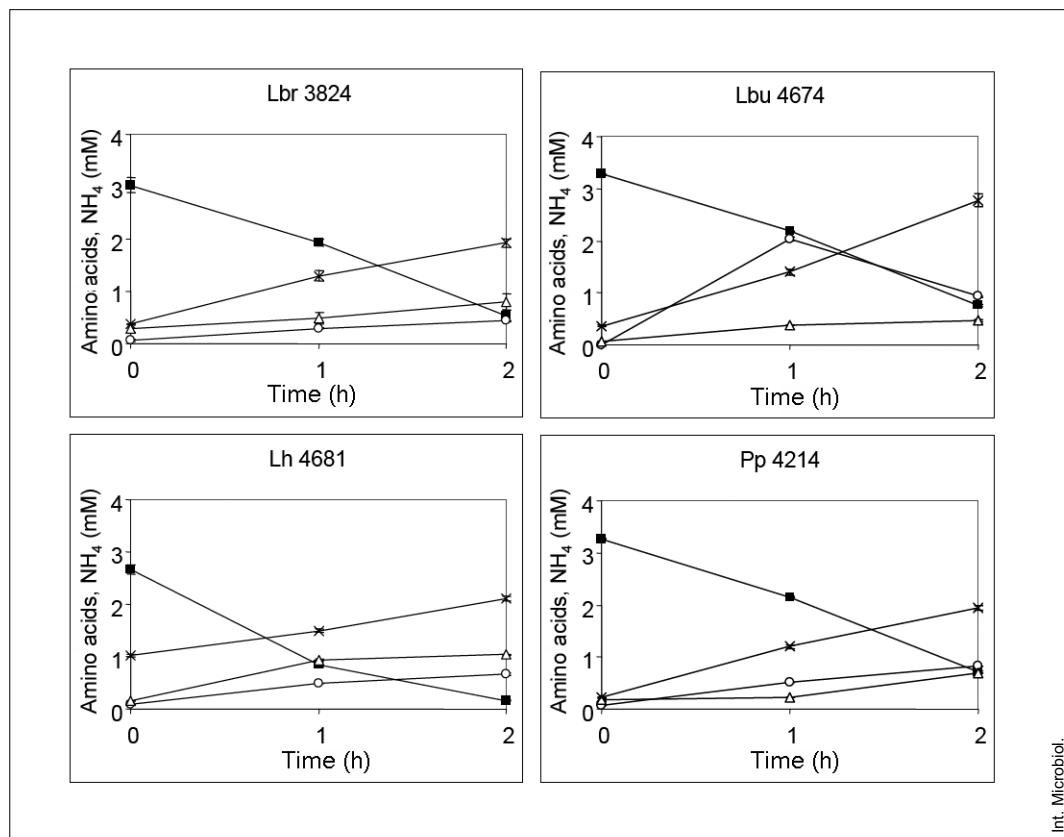


Fig. 5. Time evolution of arginine (solid squares), citrulline (empty circles), ornithine (triangles), and ammonium (crosses) in assays of resting cells and in the presence of 12 % ethanol. The strains used were *Lactobacillus brevis* 3824, *L. buchneri* 4674, *L. hilgardii* 4681 and *Pediococcus pentosaceus* 4214. Data represent the mean of three samples \pm SD.

not have as clear an effect as the other conditions, although there was a slight tendency towards lower values of Orn/Arg and H₄⁺/Arg. Thus theoretically, higher relative production of citrulline would be expected with increasing amounts of glucose.

In addition to these effects of pH, ethanol, glucose, and malic acid, the Cit/Arg values for all eight strains together (Fig. 4) was determined under the conditions that had the most significant effect, i.e., low pH and high ethanol, in order to compare all the strains. As Fig. 4 shows, strains *L. brevis* 3824 and *L. buchneri* 4674 produced the most citrulline in relation to arginine consumed. For all the strains, more citrulline was produced at pH 3 than at pH 3.6 (control), and in the presence of 12 % ethanol than in its absence.

A clear and logical relationship between a higher initial and higher final pH was found for all the strains (results not shown). Lower final pH values were recorded in the presence of higher quantities of ethanol, which correlates with the higher citrulline and lower ammonium production. When the percentage of glucose was higher, the final pH was lower,

consistent with the reduced production of ammonium in these assays. Generally, higher final pH values were observed for all the strains of *L. hilgardii* and *L. buchneri*.

In addition to the data presented above, corresponding to 2-h resting-cell experiments, analyses were conducted at 0 h and at 1 h in order to study the kinetics of the different compounds. In all cases, the kinetics showed a linear decrease in arginine and increases in citrulline, ornithine, and ammonium. As examples, Fig. 5 shows the results obtained for the four strains, one of each species, in the presence of 12 % ethanol. In some cases, as noted above for *L. buchneri* 4674, an initial increase of citrulline was followed by a subsequent decline in the last hour of the experiment.

The degradation rates for arginine were calculated based on the data obtained at 0 and 2 h. In the control assays, the values ranged from 19.96 μ M of arginine degraded per min for *L. brevis* 4121^T to 25.19 μ M per min for *L. hilgardii* 4681. The degradation rates were higher in the isolated strains than in the type strains. The rates were slightly lower than those of controls under conditions of lower pH or more ethanol, for all

strains. Thus, in the presence of 12 % ethanol, the rate of arginine degradation was 18.54 μM per min for *L. brevis* 4121^T and 20.89 μM per min for *L. hilgardii* 4681.

Discussion

The resting-cell experiments performed in this study, like those in other works [18], have several advantages: they avoid the presence of other compounds that could interfere with the experiment; they are an easy way to monitor degradation kinetics; and, compared to growth experiments, they can be established in very little time under conditions similar to those of wine. The strains used in this work were previously shown to degrade arginine when grown in MRS medium [1]. These strains were able to degrade 60–90 % of 5 g arginine/l in less than 2 h. All the strains of heterofermentative lactobacilli used in this study (*L. brevis*, *L. hilgardii* and *L. buchneri*) degraded almost all the arginine, as expected. The degradative ability of *P. pentosaceus* was also demonstrated, confirming the results obtained in growth medium [1].

The only condition under which arginine was not degraded was in the presence of malic acid. As shown in other works [19,30], malic acid degradation seems to take priority over arginine consumption for LAB. It may be that since MLF involves just one reaction, the consequent rapid production of ATP is more advantageous than the more complex and inducible ADI pathway of arginine utilization. From a technological point of view, MLF could be used to control the appearance of citrulline from arginine, as reported previously [30].

In spite of a low initial pH, arginine degradation increased during the assays, with final pH values ranging from 5.0 to 7.0 due to the production of ammonia from the degraded arginine. A similar increase in pH, by around 2.5 units, was recorded in assays with different initial pH values, ranging from 3.0 to 4.0, and a similar quantity of ammonia was accordingly produced.

Good inverse correlations between the values of Cit/Arg and Orn/Arg (Fig. 3, for *L. hilgardii* 4786^T) were found in the different assays. The former was higher when the second was somewhat lower, due to the conversion of citrulline to ornithine. The molar ratio of ammonium per arginine is usually higher (expressed as 2 \times in the figures) due to the appearance of two ammonium molecules for each one arginine (see the Introduction).

The relation between citrulline produced and arginine consumed ranged from 10 % to 50 %, depending on the strain and the conditions. In a previous work with resting cells, val-

ues around 5 % were reported [18], and in a more recent study of wine, the maximum was 4–5 % [30]. However, in yet other studies values of near 40 % were obtained for strains of *L. hilgardii* [34], and around 30–40 % for strains of *O. oeni* [24]. Thus, there seems to be great variability depending on the species and the different conditions.

These ratios can be used to estimate the contribution of citrulline to the EC precursor pool from a given amount of initial arginine, as suggested by Mira de Orduña et al. [19]. Of all the different conditions assayed, those that clearly resulted in higher rates of citrulline production compared to arginine consumption in all the strains (Fig. 4) included the presence of ethanol. The Cit/Arg value increased significantly when the ethanol concentration increased from 0 (control) to 5, 10, and 12 %. At 12 % ethanol, the average increase in the ratio in all the strains was ca. 85 %, with values ranging from 44 % (*L. brevis* 4214^T or *L. buchneri* 4674) to 173 % in *L. buchneri* 4111^T. Regarding the lower pH, a comparison of the control (pH 3.6) with the assays carried out at pH 3.0 showed that the average increase in the Cit/Arg value was 29 %, ranging from 6 % for *L. buchneri* 4674 to 43 % for *L. hilgardii* 4681.

Regarding the effect of ethanol, the higher Cit/Arg value recorded here is consistent with the decreased Orn/Arg value. There seemed to be less conversion of citrulline to ornithine, accompanied by a decreased incorporation of arginine. The exposure of cells to ethanol usually results in an increase in their permeability and the concomitant loss of intracellular material [5]. Therefore, it may have been the case that the cells were less efficient at retaining citrulline, such that it could easily escape.

The higher value of Cit/Arg at low pH (3.0) is also consistent with reduced arginine degradation. The release of ammonium from the arginine-deiminase reaction, yielding citrulline, would allow the cells to compensate for the external acidity.

The results obtained for the different species and strains tested (Fig. 4) clearly show that strains of *L. brevis* and *L. buchneri* isolated from wine and beer, respectively, produced higher yields of citrulline in relation to arginine consumed, more than the type culture strains, which were isolated from other environments. Therefore, an increased ability to produce citrulline might characterize strains genetically accustomed to ethanol environments.

In most cases citrulline production was linear, although in some strains, particularly *L. buchneri* 4674, a period of increased synthesis was followed by a decline. This can be interpreted as due to the excretion of citrulline, which was

then reabsorbed by the cells and transformed into ornithine and ammonia, as other authors have also postulated [12,18].

In most of the assays, there was a balance between degraded arginine and the sum of the amount of citrulline, ornithine, and ammonia produced. However, this was not the case with *L. hilgardii* 4681, for which the sum of these products was less than the amount of arginine consumed. This might be explained by the transformation of ornithine into putrescine by ornithine decarboxylase, as described in *O. oeni* [17] and in other LAB [23].

In conclusion, in this work we were able to demonstrate the effects of both ethanol and low pH on a higher relative production of citrulline compared to arginine consumed in some species of LAB related to wine and beer. In addition, the yields were found to depend on both the species and the strain. Since these species are mainly related to spoilage, possible contaminations must be prevented with well-controlled aseptic processes and through the use of starters that ensure correct alcohol fermentation and of MLF, if required. This should prevent the appearance of citrulline, and thus a potential source of EC.

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Competing interests: None declared.

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