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Comparative Survey in *Lactobacillus plantarum* of the Growth and Metabolism of Arginine and Citrulline in Different Media

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Arginine deiminase activity increased in the presence of arginine in *Lactobacillus plantarum* strains N4 and N8 isolated from orange. The influence of citrulline and ornithine on arginine deiminase and ornithine transcarbamylase activities was strain-dependent. The growth and arginine and citrulline metabolism of *L. plantarum* were studied in the presence of tomato juice. Its addition enhances the growth in both strains. The specific amino acids utilization was inversely proportional to the initial glucose concentration. Arginine and citrulline addition to basal medium exerted a stimulatory effect on the growth of N4 strain, and this effect was observed only with citrulline in strain N8. The magnitude of this effect was lower in the presence of tomato juice.

KEYWORDS: Arginine; citrulline; *Lactobacillus plantarum*

INTRODUCTION

Arginine is one of the substrates that, apart from carbohydrates, lactic acid bacteria can use as an additional energy source for growth (*1*−*3*). The ability to metabolize arginine is apparently distributed among the various groups of bacteria. Some homofermentative lactobacilli, isolated from fermented juices, do not degrade arginine (*4*, *5*). In contrast, arginine is degradable by a number of homofermentative lactobacilli from different sources, such as strains of *Lacotcoccus lactis* from cheese (*6*), *Lactobacillus plantarum* from fish (*7*), and *Lactobacillus sake* from meat (*8*). Arena et al. (*2*) demonstrated the ability of two strains of *L. plantarum* isolated from orange to degrade arginine and citrulline via arginine deiminase system (ADI).

The ADI system has three enzymatic reactions:

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

$$\text{citrulline} + P_i \xleftrightarrow{\text{ornithine transcarbamylase}} \text{ornithine} + \text{carbamyl-}P_i$$

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

This pathway encourages the development of spoilage of fruit juices by supplying an energy source and by protection against acid damage.

Some lactobacilli cannot metabolize arginine by the cells in the absence of a fermentable sugar (*9*−*12*). In *L. plantarum* strains N4 and N8, the metabolism of glucose and arginine or citrulline was concurrent (*2*).

Arena and Manca de Nadra (*13*) determined the ability of *L. plantarum* isolated from orange to produce biogenic amines from arginine and ornithine. The preceding citations suggest that the arginine catabolism by *Lactobacillus* spoilage of citrus can be of biological significance with industrial economical implications.

The purpose of this paper is to determine the metabolism of arginine and its amino acid derivate citrulline in strains of *L. plantarum* and spoilage of citrus products. We studied the levels and some properties of arginine deiminase and ornithine transcarbamylase, both enzymes involved in the ADI system, and the effect of different nutritional conditions on arginine and citrulline utilization.

MATERIAL AND METHODS

Organisms. *L. plantarum* strains N4 and N8 were isolated from orange peel of fresh orange.

Sequence Analysis. Searches in GenBank with the BLAST program (*14*) were performed to determine the closest known relatives of the partial 16S rDNA sequences obtained.

Nucleotide Sequence Accession Numbers. The 16S rDNA sequence for *L. plantarum* strains N4 and N8 were deposited in GenBank and assigned accession no. AY082883 and AY082884, respectively.

Culture Media. The basal medium (BM) contained the following, in g L^{-1}: 5, peptone (Oxoid L37); 3, yeast extract (Oxoid L21); 1, glucose (Britania 046, Buenos Aires, Argentina). Three media were prepared by the individual addition of 1 g L^{-1} of one of the following amino acids to the basal medium: L-arginine hydrochloride (Sigma A5131), L-citrulline (Sigma C7629), or l-ornithine monohydrochloride (Merck 6906). All media were adjusted to pH 6.5 with 1 mM KOH before sterilization in an autoclave at 121 °C during 20 min. The BME medium corresponds to BM medium containing 15% tomato juice.

Culture Procedures, Growth, and Sampling Regime. *L. plantarum* strains N4 and N8 were grown in BM medium. After incubation at 30 °C for 24 h, the cells from the third subculture were harvested by centrifugation for 30 min at 3000*g* at 4 °C, washed with sterile distilled water, and resuspended in sterile distilled water to $OD_{560nm} = 0.90$. This bacterial suspension was used to inoculate the experimental media

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at a rate of 2% (v/v). These cultures were incubated statically at 30 °C for 48 h, and the samples were taken every 6 h for growth and stored frozen (−18 °C) for subsequent chemical analyses.

Bacterial growth was determined by measurement of optical density at 560 nm using a Bausch and Lomb Spectronic 20 spectrophotometer (Bausch and Lomb, Inc., Rochester, NY). At the same time, the cells were counted on Petri dishes and expressed in colony-forming units (cfu). For this counting, MRS agar medium (*15*) at pH 6.0 and 48 h of incubation at 30 °C were used.

Analytical Methods. Determination of arginine concentration was based on the Sakaguchi reaction, using the method of Ceriotti and Spandrio (*16*). Citrulline concentration was analyzed with the procedure of Archibald (*17*) as modified by Spector and Jones (*18*). Ornithine was measured by colorimetric method with ninhydrin (*19*). Ammonia was determined by indophenol blue reaction (*20*). Glucose was analyzed by the glucose oxidase method (kits from Wiener, Rosario, Argentina).

Enzymes Assay. *L. plantarum* strains N8 and N4 were grown in the BM medium with and without 1 g L^{-1} arginine, citrulline, or ornithine. Cultures were harvested at the end of the logarithmic phase growth, and the activities of arginine deiminase and ornithine transcarbamylase (OTCase), two enzymes of the ADI pathway, were determined. Cells were washed with 0.2 M sodium phosphate buffer, pH 6.5. Centrifuged cells were resuspended in the same buffer to $OD_{560nm} = 0.55$ (3×10^9 cfu/mL) for determination of arginine deiminase activity and were washed and resuspended in 0.2 M sodium acetate buffer, pH 5.8, for the determination of OTCase activity. Activity was defined as the micrograms of products formed per hour per milliliter of cell suspension.

Both enzymatic activities were determined according to the Oginsky method (*21*). The reaction mixture for arginine deiminase determination had the following composition in a final volume of 3.6 mL: 0.4 mL of L-arginine−HCl (0.1 M) adjusted to pH 6.5; 1 mL of sodium phosphate buffer (0.2 M), pH 6.5; 0.6 mL of cell suspension, and 1.6 mL of distilled water. The mixture was incubated at 37 °C, and samples were taken every 15 min, stopping the reaction by the addition of 0.2 mL of perchloric acid (70%). The samples were centrifuged at 3000*g* for 30 min at 4 °C, and citrulline was determined in the supernatant.

The reaction mixture for OTCase determination had the following composition in a final volume of 3.6 mL: 1 mL of L-citrulline−HCl (0.1 M); 1 mL of sodium acetate buffer (0.5 M), pH 5.8; 1 mL of sodium arsenate (0.1 M), and 0.6 mL of cell suspension. The mixture was incubated at 37 °C, and samples were taken every 15 min, stopping the reaction by the addition of 0.2 mL of perchloric acid (70%). The samples were centrifuged at 3000*g* for 30 min at 4 °C, and ornithine was determined in the supernatant.

Statistical Analysis. The data were analyzed by the balanced ANOVA test. Variable means showing statistical significance were compared using Tukey' s test (Minitab Student R12).

RESULTS AND DISCUSSION

Arginine Deiminase and Ornithine Transcarbamylase Activities. In a previous paper, Arena et al. (*2*) demonstrated that *L. plantarum* strains N4 and N8 degraded arginine to citrulline, ornithine, and ammonia; that citrulline and ornithine were consumed; and that strain N4 utilized arginine and ornithine to a higher extent than strain N8.

We confirmed the activities of the enzymes involved in arginine and citrulline degradation. **Table 1** shows that arginine deiminase activity was higher in *L. plantarum* strain N4 than in strain N8 in BM. The activity increased 64 and 92% when the cells were grown in the presence of arginine for *L. plantarum* strains N4 and N8, respectively.

When the cells were grown in the presence of citrulline, the activity of the first enzyme of the ADI system decreased ∼10% with respect to BM for *L. plantarum* strain N4 and was not modified in *L. plantarum* strain N8. The arginine deiminase activity in cells cultured in the medium with ornithine decreased 9% in *L. plantarum* strain N4 and was not modified in strain N8. When *L. plantarum* strain N4 was grown in BM with added arginine, citrulline, or ornithine, the catabolic OTCase activity was not modified by arginine or citrulline and decreased 12% in the presence of ornithine. In the same conditions, the catabolic OTCase activity in *L. plantarum* stgrain N8 increased 12% in the presence of citrulline and was not modified by the other amino acids. The activities of ADI pathway enzymes varied considerably between strains of *L. plantarum* isolated from orange. The activity of arginine deiminase was lower in strain N8 than in strain N4. Arginine was catabolized to a very limited extent by *L. plantarum* strain N8. The activity levels detected in *L. plantarum* N4 were approximately similar to those found in *Lactobacillus leichmanii*, where the specific activities of arginine deiminase and OTCase were higher when arginine was added to the medium (*10*). The results obtained previously with *Lactobacillus buchneri* NCDO 110 were similar to the present results. The enzymes of the arginine dihydrolase pathway studied in *L. buchneri*, arginine deiminase and OTCase, appear in the presence or absence of added L-arginine or L-citrulline in the culture medium. The OTCase activity was not affected by the presence of arginine in the culture medium but was inhibited by its product, ornithine (*22*). In *Streptococcus faecalis* (*23*) an increase of arginine concentration up to 100 mM did not alter the differential rate of OTCase synthesis, and the addition to the medium of fermentation products, namely, ornithine and citrulline, was without effect on the formation of OTC.

Table 1. Specific Activities of Arginine Deiminase and Ornithine Transcarbamylase in *L. plantarum*[a]

	arginine deiminase (μg of citrulline/10^9 cells/h) × 10^2		OTCase (μg of ornithine/10^9 cells/h) × 10^2	
culture medium[b]	*L. plantarum* strain N4	*L. plantarum* strain N8	*L. plantarum* strain N4	*L. plantarum* strain N8
BM	26.7 ± 1.3a	4.9 ± 0.2d	37.3 ± 1.7f	42.4 ± 1.8h
BM + Arg	43.9 ± 2.2b	9.4 ± 0.7e	37.7 ± 1.8f	42.9 ± 2.0h
BM + Cit	22.1 ± 1.4c	5.3 ± 0.3d	37.6 ± 1.9f	47.6 ± 2.1i
BM + Orn	24.0 ± 1.2c	4.8 ± 0.4d	32.9 ± 1.9g	42.4 ± 1.8h

[a] Values are an average of three replicates. Means ± SD for the arginine deiminase enzyme with no common letters (a–e) differ significantly ($p < 0.05$). Means ± SD for the OTCase enzyme with no common letters (f–i) differ significantly ($p < 0.05$). [b] BM, basal medium; Arg, arginine; Cit, citrulline; Orn, ornithine; OTC, ornithine transcarbamylase. Initial concentrations (mmol L^{-1}): arginine, 5.74; citrulline, 5.75; ornithine 7.57.

Effect of Tomato Juice on *Lactobacillus* Growth. Table 2 shows a comparative study on the growth of two *L. plantarum* strains conducted in the same media with and without tomato juice, a common ingredient in media for culturing lactic acid bacteria (*24*). Tomato juice is a rich source of simple sugars, minerals, and vitamins B (*25*), and its addition to basal medium stimulated lactic acid bacteria (LAB) growth (*26*). At the optimal concentration of tomato juice (15%) for *L. plantarum* strains N4 and N8, glucose concentration increases from 5.5 to 20 mmol/L. The tomato juice stimulated the growth of *L. plantarum* strains N4 and N8 by 289 and 327%, respectively. The results of the present study confirm the data of Babu et al. (*27*), who reported that the addition of tomato juice to skimmed milk exerted a stimulatory effect on the growth of *Lactobacillus acidophilus* strains. Contrarily, Cogan et al. (*28*) reported that *Lactobacillus bulgaricus* growth was *stimulated* at low concentration (1 and 5%) and *inhibited* at high concentration (15%) of tomato juice. The requirement of a growth factor present in tomato juice by *Oenococcus oeni* distinguished it from other *Leuconostoc* spp. (*29*).

Arginine and citrulline are potential sources of energy for LAB. The question is whether the potential energy can be

Table 2. Effect of Tomato Juice, Arginine, and Citrulline on the Relative Growth of *L. plantarum* Strains[a]

L. plantarum strain	cell suspensions (cfu/mL × 10^9)					
	basal medium		basal medium + arginine		basal medium + citrulline	
	BM[b]	BME[b]	BM	BME	BM	BME
N4	3.16 ± 0.21	12.30 ± 0.66	5.01 ± 0.42	17.02 ± 1.23	6.03 ± 0.37	20.10 ± 0.96
N8	2.39 ± 0.27	10.20 ± 0.38	2.40 ± 0.28	10.71 ± 0.50	4.37 ± 0.25	20.02 ± 1.36

[a] Data are expressed as means ± standard deviations ($n = 3$). [b] BM, basal medium; BME, basal medium enrichment.

Table 3. Arginine Consumption by *L. plantarum*[a]

L. plantarum strain	arginine consumption[b] (mmol L^{-1} $cell^{-1}$) × 10^{11}		metabolite production[b] (mmol L^{-1} $cell^{-1}$) × 10^{11}					
			citrulline		ornithine		ammonia	
	BM[c]	BME[c]	BM	BME	BM	BME	BM	BME
N4	10.30 ± 0.56	7.61 ± 0.37	4.35 ± 0.23	4.24 ± 0.24	5.82 ± 0.23	3.00 ± 0.13	13.83 ± 0.83	8.10 ± 0.36
N8	3.89 ± 0.21	1.56 ± 0.09	1.94 ± 0.09	0.99 ± 0.04	1.94 ± 0.11	0.57 ± 0.03	5.83 ± 0.30	0.20 ± 0.12

[a] Data are expressed as means ± standard deviations ($n = 3$), no significant difference ($p < 0.05$). Initial arginine concentration = 5.70 mmol L^{-1}. [b] At 48 h of incubation at 30 °C. [c] BM, basal medium; BME, BM plus 15% tomato juice.

Table 4. Citrulline Specific Consumption by *L. plantarum*[a]

L. plantarum strain	citrulline consumption[b] (mmol L^{-1} $cell^{-1}$) × 10^{11}		metabolite production[b] (mmol L^{-1} $cell^{-1}$) × 10^{11}			
			ornithine		ammonia	
	BM[c]	BME[c]	BM	BME	BM	BME
N4	12.50 ± 0.48	7.98 ± 0.36	6.79 ± 0.25	4.42 ± 0.21	10.71 ± 0.53	7.24 ± 0.35
N8	15.50 ± 0.53	8.82 ± 0.42	5.44 ± 0.26	3.55 ± 0.21	13.59 ± 0.57	7.45 ± 0.37

[a] Data are expressed as means ± standard deviations ($n = 3$), no significant difference ($p < 0.05$). Initial citrulline concentration = 5.70 mmol L^{-1}. [b] At 48 h of incubation at 30 °C. [c] BM, basal medium; BME, basal medium plus 15% tomato juice.

coupled to cellular growth of LAB. The influence of arginine and citrulline in *L. plantarum* growth was examined in different media. Tomato juice added to the media was also used as a control to indicate the effect of arginine and citrulline on the microorganism growth. **Table 2** shows that at 48 h of incubation there was an increase in the growth (expressed as cfu per milliliter) of *L. plantarum* strain N4 by arginine and citrulline addition to the media and only by citrulline for *L. plantarum* strain N8. For strain N4, the growth in BME was improved 38 and 70% by the presence of arginine and citrulline, respectively. The observed enhancement in the presence of arginine and citrulline was lower than that observed in BM (59 and 91%, respectively). For strain N8 the addition of citrulline to BME stimulated the growth by 58%. This positive effect was lower than that observed in BM (83%). Arginine did not modify the growth. Manca de Nadra et al. (*1*) reported that in *L. buchneri* NCDO 110, the addition of 0.5% of arginine to $LAPT_{0.4}$ medium (*30*) with 0.05% glucose stimulated the growth by 80%, and in the same medium containing 0.5% of glucose the growth improved by 44% (*1*). The present results indicate that the stimulatory effect of arginine is higher when the sugar concentration is lower. This fact could be related with the diminution in the medium of one simple energy source such as glucose and the utilization of arginine as additional energy source.

As a consequence of the ammonia production, arginine and citrulline degradation also could be considered favorable to strain adaptation to acid environment.

Amino Acid Consumption. The effect of using arginine or citrulline by *L. plantarum* strains was studied in BM and BME (**Tables 3** and **4**). **Table 3** shows metabolite excretion from arginine degradation in different media. In BME arginine consumption by *L. plantarum* strains N4 and N8 was higher than that observed in BM. Taking into account the specific consumption of arginine as a function of cell number, in BM arginine utilization was 38 and 12% higher than in BME for strains N4 and N8, respectively. The citrulline produced from arginine was 13% higher in BME than in BM for both strains. The ornithine production was 17 and 13% lower in BM with respect to BME for *L. plantarum* strains N4 and N8, respectively. The utilization of arginine and citrulline as energy sources in the presence of fermentable carbohydrates was reported in *L. buchneri* (*9*, *31*), *Lactobacillus hilgardii* X_1B, and *O. oeni* m (*3*). Among lactic acid bacteria, *L. plantarum* is one of the most tolerant to low pH and high acidity (*32*). In the present study, this tolerance could be related to the ammonia formation from arginine in both strains (**Table 3**).

The consumption of citrulline by *L. plantarum* strains N4 and N8 increased with media enrichment. **Table 4** shows that the specific consumption of citrulline decreased 46 and 47% for *L. plantarum* strains N4 and N8 in BME with respect to BM. When the medium was supplied with tomato juice, the specific arginine and citrulline degradation by the ADI pathway diminished.

Citrulline production from arginine increases in relation with the medium tomato juice enrichment. The excess of ATP from sugar fermentation is anticipated to inhibit OTCase in *L. plantarum* strains N4 and N8, with the corresponding accumulation of citrulline in the media. Manca de Nadra et al. (*1*, *30*) demonstrated the inhibitory effect of ATP upon OTCase and carbamyl-phosphokinase activities in *L. buchneri* NCDO 110. Manca de Nadra's (*10*) investigation of *Lactobacillus leichmannii* ATCC 4797 demonstrated that arginine deiminase and OTCase were repressed by glucose.

Simon et al. (*23*) reported that supplementation with glucose considerably reduced the differential rate of ornithine carbamyltransferase formation in *S. faecalis.* Thompson et al. (*33*) demonstrated the ATP-mediated inhibition of OTC in dairy LAB. The results of the current study led to the conclusion that the recovery of arginine as citrulline increases in relation with the glucose added to the media.

The results confirmed that arginine and citrulline are metabolized via the ADI pathway by *L. plantarum* from orange. The ADI system is affected by the environmental conditions. Arginine stimulated the enzymatic activity in both strains N4 and N8, and the effect of citrulline and ornithine was strain-dependent. The specific amino acids utilization was inversely proportional to the medium enrichment.

As arginine is one of the major amino acids found in fruit juices, it can be concluded that the jeopardy of a contamination of the food product by *L. plantarum* would be maximized by a strain capable of hydrolyzing arginine and/or citrulline.

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