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Biogenic amine production by *Lactobacillus*

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Aims: The aim of this work was to demonstrate that strains of *Lactobacillus* may be able to produce putrescine and agmatine from one of the major amino acids present in fruit juices and wine, arginine, and from amino acid-derived ornithine.

Methods and Results: Biogenic amines were determined by HPLC. Their production in the culture medium was similar under both microaerophilic and anaerobic conditions. The presence of Mn^{2+} had a minimal influence on the results, whereas the addition of pyridoxal phosphate increased amine production 10-fold. *Lactobacillus hilgardii* X_1B, isolated from wine, was able to degrade arginine by two pathways: arginine deiminase and arginine decarboxylase. The isolate was able to produce putrescine from ornithine and from agmatine. *Lactobacillus plantarum* strains N4 and N8, isolated from orange, utilized arginine via the arginine deiminase system. Only the N4 strain was able to produce putrescine from ornithine.

Conclusions: It has been demonstrated that *Lact. hilgardii* X_1B is able to produce the most important biogenic amine found in wine, putrescine, and also agmatine from arginine and ornithine, and that *Lactobacillus plantarum*, considered to be an innocuous spoilage micro-organism in fruit juices, is able to produce amines.

Significance and Impact of the Study: The results have significance in relation to food poisoning caused by beverages that have been contaminated with biogenic amines.

INTRODUCTION

Biogenic amines, low molecular weight organic bases, can be formed and degraded as a result of normal metabolic activity in animals, plants and micro-organisms. These amines occur in a wide variety of foods, such fish products, meat, cheese, wine and other fermented foods (Vidal-Carou *et al*. 1990; Izquierdo-Pulido *et al*. 1994). The amines are usually produced in foods by decarboxylation of amino acids (Halász *et al*. 1994). Amino acids are naturally present in grapes, representing 30–40% of the total wine nitrogen. Amine production has been associated with protective mechanisms of micro-organisms against an acidic environment (Vandekerckove 1977). Toxicological problems may result from the ingestion of foods containing relatively high levels of biogenic amines, and may provoke hypertensive crises in patients treated with monoaminoxidase inhibitors drugs (MAOI). Putrescine and agmatine have been described as potentiators that enhance the toxicity of histamine to humans by depressing histamine oxidation (Taylor 1986). In addition, putrescine and agmatine may originate carcinogenic nitrosamines in the presence of nitrites (Hotchkiss *et al*. 1977; Vandekerckove 1977). Halász *et al*. (1994) reported preliminary results suggesting that a positive correlation exists between the microflora and the putrescine concentration in vegetables.

Lactic acid bacteria able to grow in acidic fruit juice and wine are generally recognized as safe. However, the metabolic activity of the lactic acid bacteria involved may also give rise to the formation of biogenic amines. The potential of selected strains to produce the biogenic amines agmatine and putrescine from the arginine, and its amino acid derivative, ornithine, needs to be investigated.

Some lactic acid bacteria are known to degrade L-arginine (Manca de Nadra *et al*. 1981, 1982, 1986, 1988), one of the major amino acids found in orange and grape juices and wines. In addition, Manca de Nadra *et al*. (1997, 1999) reported that the protease produced by *Oenococcus oeni*, isolated from Argentinean wine, is effective on the

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nitrogenous macromolecular fraction of white and red wines, and that arginine is quantitatively one of the most important amino acids obtained by protease activity.

In previous papers, it was determined that arginine metabolism in *Lactobacillus plantarum* N4 and N8 strains isolated from orange juice (Arena *et al*. 1999a), and *Lactobacillus hilgardii* X_1B isolated form wine (Arena *et al*. 1999b), takes place via the Arginine Deiminase Pathway (ADI). The arginase–urease system was not detected.

Arginine could be decomposed to putrescine via citrulline and ornithine, or could be decarboxylated to agmatine, forming putrescine directly or via N-carbamoylputrescine.

In this study, the ability of *Lactobacillus* strains, isolated from orange and grape juices, to produce amines from arginine and ornithine precursors was examined. The pathway for putrescine and agmatine production was also investigated.

MATERIALS AND METHODS

Organisms

Lactobacillus hilgardii X_1B was isolated from Argentinean wine (Strasser de Saad and Manca de Nadra 1987). *Lactobacillus plantarum* strains N4 and N8 were isolated from orange (Arena *et al*. 1996).

Media, growth conditions and culture procedures

Moeller's decarboxylase medium was used as the basal medium (BM). The BM contained, in g l^{-1}: 5, peptone (Oxoid); 3, yeast extract (Oxoid); 1, glucose (Oxoid); 0·005, pyridoxal-5-phosphate (Sigma); and 0·016, bromocresol purple (Sigma) as the pH dye indicator.

Amino acid decarboxylation was tested by adding to BM 1 g l^{-1} of either L-arginine-hydrochloride (Sigma), or L(+)-ornithine-monohydrochloride (Merck). The effect of Mn^{2+} was tested by adding 0·03 g l^{-1} $MnSO_4$ (Sigma). The media were adjusted to pH 6·5 with NaOH and sterilized at 121°C for 15 min. Agmatine and putrescine were from Sigma.

The strains were pre-cultured in BM and then inoculated into experimental media at 5×10^7 cells ml^{-1}. These cultures were incubated statically at 30°C for 10 days under microaerophilic or anaerobic (by overlaying with paraffin) conditions.

Analytical methods

The concentration of amino acids was measured as described previously (Arena *et al*. 1999a,b). Biogenic amines were determined by a liquid chromatographic method as described by Eerola *et al*. (1993). Some additional changes were made, such as the inclusion of one more biogenic amine (agmatine). Agmatine eluted between cadaverine and histamine. The solvents used for the separation were solvent A: 0·1 M ammonium acetate and solvent B: acetonitrile. Solvent gradient conditions began with 50% B and ended with 90% B in 19 min. The total run time was 35 min including the washing time. Washing was essential to maintain column performance. The samples were derivatized prior to column injection as follows. Dansyl derivatives were generated with the addition of 100 μl 2N sodium hydroxide solution, 150 μl saturated sodium bicarbonate and dansyl chloride solution (5 mg dansyl chloride in 0·5 ml acetone). The reaction mixture was incubated at 40°C for 45 min. After incubation, the residual dansyl chloride was removed by addition of 50 μl ammonia. After 20 min, the sample was adjusted to 2·5 ml with acetonitrile and then filtered with a 0·22 μm filter. Chromatographic separation was performed on an O.D.S2 column (Phenomenex, Torrance, USA) 300 nm long $\times$ 4·6 nm i.d. The pre-column derivatization and the column apparatus were at 40°C. The flow rate was 1 ml min^{-1} and the column effluent was monitored at 254 nm using a Gilson 118 (Gilson Medical Electronics, Middleton, USA). 1,7-diaminoheptane was used as internal standard for quantification.

Statistical analysis

To validate the methods, the Student's Test was used. Three replicate determinations were carried out.

RESULTS

Biogenic amine production from arginine and ornithine by *Lact. hilgardii* X_1B and *Lact. plantarum* N4 and N8 strains is shown in Table 1. The concentration of amino acids has an effect on the overall formation of biogenic amines. A concentration of 1 g l^{-1} of amino acid was selected as in wine, amounts from 0·1 to 2·3 g l^{-1} are generally available for LAB metabolism. *Lactobacillus hilgardii* X_1B produced agmatine and putrescine from arginine and ornithine. *Lactobacillus plantarum* N4 and N8 strains did not produce agmatine from arginine or ornithine. Only the N4 strain was able to produce putrescine from arginine and ornithine at a lower concentration than that produced by *Lact. hilgardii* X_1B in the same conditions.

Table 2 shows the correlation between arginine consumption by *Lactobacillus* strains and citrulline, ornithine and biogenic amine excretion into the medium. During growth of *Lact. hilgardii* X_1B, arginine concentration declined while citrulline, ornithine and biogenic amines increased. After 10 days of incubation at 30°C, 85·4 and 90% of total arginine present in the medium was consumed under microaerophilic and anaerobic conditions, respectively. At the same time, citrulline and ornithine (via the ADI

Table 1 Biogenic amines production by *Lactobacillus* strains

Lactobacillus strains	BM + amino acids (1 g l^{-1})	Biogenic amines production (mg l^{-1})			
		Agmatine		Putrescine	
		Atmospheric conditions			
		Microaerophilic	Anaerobic	Microaerophilic	Anaerobic
Lact. hilgardii X_1B	Arginine	20·8*	33·7	216·2	244·7
	Ornithine	29·6	12·1	322·9	192·0
Lact. plantarum N4	Arginine	0	0	0·5	1·1
	Ornithine	0	0	10·6	6·4
Lact. plantarum N8	Arginine	0	0	0	0
	Ornithine	0	0	0	0

*At 10 days of incubation at 30°C. Means of three replicates, no significant difference ($P \leq 0{\cdot}05$).

Table 2 Arginine consumption and metabolites productions by *Lactobacillus* strains

Lactobacillus strains	Arginine consumption$^+$ (mmol l^{-1}) Atmospheric conditions		Metabolites production$^+$ (mmol l^{-1})							
			Citrulline		Ornithine		Agmatine		Putrescine	
	M*	A†	M	A	M	A	M	A	M	A
Lact. hilgardii X_1B	4·91	5·23	1·44	1·70	0·90	0·57	0·16	0·26	2·37	2·69
Lact. plantarum N4	1·69	1·75	0·82	0·74	0·72	0·88	0	0	0·01	0·01
Lact. plantarum N8	0·47	0·58	0·24	0·37	0·19	0·17	0	0	0	0

Arginine initial concentration 5·75 mmol l^{-1}. $^+$At 10 days of incubation at 30°C.
*M = microaerophilic; †A = anaerobic. Means of three replicates, no significant difference ($P \leq 0{\cdot}05$).

pathway), and agmatine and putrescine (by decarboxylation), were formed. Under microaerophilic conditions, 47·7% of the arginine consumed was transformed in amino acids and 51·5% was transformed into putrescine and agmatine; under anaerobic conditions, the percentage of recovery was 43·4 and 56·4 for amino acids and biogenic amines, respectively.

Lactobacillus plantarum N4 converted arginine into citrulline during which an NH_2 group was removed from arginine as a first step. Citrulline was next converted to ornithine, which then underwent decarboxylation to form putrescine. This strain consumed 29·4 and 30·4% of arginine under microaerophilic and anaerobic conditions, respectively; it was recovered as citrulline 48·5 and 42·3%, and ornithine 42·6 and 50·3% undern microaerophilic and anaerobic conditions, respectively. The recovery of putrescine was 0·6% under both conditions.

Of the initial arginine concentration, 10% was consumed by *Lact. plantarum* N8 strain, being completely converted into citrulline and ornithine. No biogenic amines were produced.

Table 3 shows the correlation between ornithine consumption by *Lactobacillus* strains and citrulline, arginine and

Table 3 Ornithine consumption and metabolites production by *Lactobacillus* strains

Lactobacillus strains	Ornithine consumption$^+$ (mmol l^{-1}) Atmospheric conditions		Metabolites production$^+$ (mmol l^{-1})							
			Citrulline		Ornithine		Agmatine		Putrescine	
	M*	A†	M	A	M	A	M	A	M	A
Lact. hilgardii X_1B	5·87	5·38	1·19	1·89	0·61	1·27	0·23	0·09	3·54	2·11
Lact. plantarum N4	2·71	2·48	1·17	1·34	0·86	0·87	0	0	0·12	0·07
Lact. plantarum N8	0·30	0·20	0·17	0·11	0·06	0·07	0	0	0	0

Ornithine initial concentration 7·55 mmol l^{-1}. $^+$At 10 days of incubation at 30°C.
*M = microaerophilic; †A = anaerobic. Means of three replicates, no significant difference ($P \leq 0{\cdot}05$).

biogenic amine excretion into the medium. After 10 days of incubation of *Lact. hilgardii* X_1B, ornithine concentration declined significantly while biogenic amines increased; 77 and 71·3% of the total ornithine present in the medium was consumed under microaerophilic and anaerobic conditions, respectively. At the same time, citrulline and arginine (anabolic system; Bringel *et al.* 1997), and agmatine and putrescine (by decarboxylation) were formed. Under microaerophilic conditions, 20·3% of ornithine consumed was transformed into citrulline and 10·4% was transformed into arginine; under anaerobic conditions, the percent of recovery was 35·1 and 23·6 for citrulline and arginine, respectively. The arginine produced from ornithine was the agmatine precursor. The production of biogenic amines from ornithine under microaerophilic and anaerobic conditions was 64·3 and 40·9%.

Lactobacillus plantarum N4 consumed 35·9 and 32·9% of ornithine under microaerophilic and anaerobic conditions, respectively, and was recovered as citrulline, 43·2 and 54%, arginine, 31·7 and 35%, and putrescine, 4·4 and 2·8%, under microaerophilic and anaerobic conditions, respectively.

Only 4 and 2·7% of the initial ornithine concentration was consumed by *Lact. plantarum* N8 strain under microaerophilic and anaerobic conditions, respectively. No biogenic amines were produced.

The results in the absence of pyridoxal phosphate show a small amount biogenic amine production possibly correlated with a pyridoxal phosphate contaminant in the yeast extract. The results were not modified by Mn^{2+} addition.

The amine-positive strain (*Lact. hilgardii* X_1B) differed from the amine-negative strain (*Lact. plantarum* N8) not only by producing amines but also, in the final pH of the medium. The final pH values of the media under the different conditions were approximately four units higher in the media inoculated with *Lact. hilgardii* X_1B than in the media inoculated with *Lact. plantarum* N8 (Table 4).

Table 4 Modification of pH media in different condition by *Lactobacillus* strains growth

Lactobacillus strains	BM + amino acids (1 g l^{-1})	Atmospheric conditions	
		Microaerophilic	Anaerobic
Lact. hilgardii X_1B	Arginine	8·05*	7·70
	Ornithine	7·23	7·38
Lact. plantarum N4	Arginine	5·62	5·50
	Ornithine	5·49	5·70
Lact. plantarum N8	Arginine	3·92	4·25
	Ornithine	4·04	4·39

Initial pH value: 6·50. *pH values after 10 days of incubation at 30°C. Means of three replicates, no significant difference ($P \leq 0{\cdot}05$).

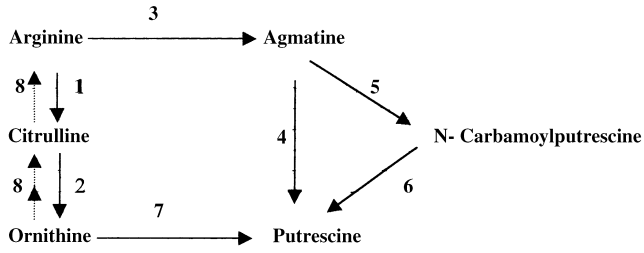

Fig. 1 Pathways of arginine metabolism in bacteria. (1) Arginine deiminase. (2) Ornithine transcarbamylase. (3) Arginine decarboxylase. (4) Agmatine deiminase. (5) Agmatinase. (6) N-Carbamoylputrescine hydrolase. (7) Ornithine decarboxylase. (8) Anabolic system

DISCUSSION

Biogenic amine formation is a protective mechanism for bacteria against acidic environments. According to Masson *et al.* (1996), pH measurement seems to give a satisfactory indication of biogenic amine production by LAB. *Lactobacillus hilgardii* X_1B and *Lact. plantarum* N4 strain may have adapted to the fruit juice environment, inducing the cells to protect themselves against the acidic environment by producing amines.

The capacity of *Lact. hilgardii* X_1B to produce biogenic amines has been demonstrated. Independently of microaerophilic or anaerobic conditions, *Lact. hilgardii* X_1B produces agmatine and putrescine in high concentrations. Putrescine is formed by the decarboxylation of either ornithine or arginine into agmatine, which is then converted into putrescine either directly or indirectly via carbamoylputrescine. *Lactobacillus hilgardii* X_1B produces 192–323 mg l^{-1} of putrescine under anaerobic or microaerophilic conditions respectively. Putrescine is the most abundant biogenic amine in wines. Amounts of 15–20 and 20–30 mg l^{-1} in white and red wines, respectively, are able to produce an important diminution in sensorial quality. Amines are present at higher concentrations in red wine than in white wine. It is thought that red wine has a great diversity of amine-producing micro-organisms such as LAB, due to the process of fermentation. Many authors have attributed the formation of amines to yeast and malolactic or other LAB (Baucom and Tabacchi 1986). Previously, Buteau *et al.* (1984) questioned the formation of biogenic amines to be the result of the controlled malolactic flora action; they attributed it mainly to the yeast action. Soufleros *et al.* (1998) suggest that during malolactic fermentation carried out by indigenous LAB, the concentration of biogenic amines increases. According to Andersson (1988), only small amounts of biogenic amines could be detected in fermentation products following inoculation of vegetables with *Lact. plantarum*. From the present results, it can be assumed that *Lact. plantarum* N8 did not represent a hazard for amine production in orange

juice, but *Lact. plantarum* N4 produced putrescine from arginine and ornithine (0·6–4·4% of turnover). Consistent with these results, Straub *et al*. (1995) reported that low amounts of putrescine were formed by a few strains of *Lact. plantarum* (1·0–4·4% of turnover). On the other hand, Leuschner and Hammes (1999) reported that *Lact. plantarum* isolated from natee is unable to produce putrescine.

In conclusion, *Lactobacillus* strains are considered to be generally safe. However, it has been demonstrated that *Lact. hilgardii* X_1B isolated from wine, and *Lact. plantarum* N4 strain usually considered as an innocuous spoilage organism in fruit juices, are able to produce biogenic amines from one of the major amino acids found in fruit juices and wine, arginine and its amino acid-derived ornithine.

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