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Arginine catabolism and acid tolerance response in *Lactobacillus reuteri* isolated from sourdough

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Abstract

The physiological role of arginine degradation by the arginine deiminase (ADI) pathway and its relationship with the acid tolerance response (ATR) in *Lactobacillus reuteri* CRL 1098 cells, a lactic acid bacterium of sourdough origin, were studied. The activity of the ADI pathway enzymes (ADI, ornithine transcarbamoylase and carbamate kinase) in *L. reuteri* CRL 1098 grown in presence of arginine and the formation of ammonia ($1.39\,mmol\,l^{-1}$) and citrulline ($1.66\,mmol\,l^{-1}$) from arginine catabolism indicated the presence of the ADI pathway in this micro-organism. This system would be involved in the ATR developed by cells grown with arginine and adapted at pH 5.0. The results indicated that the ADI system is triggered either by the adaptation of exponentially growing cells at low pH or by energy depletion of the cells during the stationary phase. Results suggest that ADI is an important component of the ATR observed at pH 5.0 since cells at the exponential phase grown without arginine and further adapted and challenged without this amino acid remained sensitive to acid stress. The findings of this work provide information about the arginine catabolism by *L. reuteri*, which can contribute to protect this micro-organism in acid environments.

Keywords: Arginine catabolism; *Lactobacillus*; Acid tolerance; Sourdough

1. Introduction

A major problem for the deliberate use of micro-organisms in sourdoughs is their limited resistance to stress conditions occurring upon preparations, e.g. drying, heat and acid (Vogel et al., 1999). Inducible tolerance to acidic environments is recognized as an important survival strategy for many prokaryotic and eukaryotic cells, which also provides cross protection to stresses such as high temperature, oxidative damage and high osmolarity (Lorca and Font de Valdez, 2001; Lorca et al., 1998; Foster, 1999).

The arginine deiminase (ADI) pathway has been detected in several lactic acid bacteria (LAB) (Cunin et al., 1986). The protection of ADI pathway against damage caused by acidic environments depends on the pH rise associated with ammonia production (Marquis et al., 1987). The system is highly acid tolerant and can operate to produce NH_3 at pH values below the minimal for growth or glycolysis.

ADI pathway basically includes three enzymes: ADI, ornithine transcarbamoylase (OTC), and carbamate kinase (CK) which catalyse the following reactions:

$$\text{L-ARGININE} + H_2O \xrightarrow{ADI} \text{L-CITRULINE} + NH_3,$$

$$\text{L-CITRULINE} + Pi \xrightarrow{OTC} \text{L-ORNITHINE} + \text{carbamyl phosphate},$$

$$\text{carbamyl phosphate} + ADP \xrightarrow{CK} ATP + CO_2 + NH_3.$$

The ADI pathway has distinct functions in lactic acid bacteria (LAB) and related organisms: it is a potential source of energy that can be coupled to cellular growth (Tonon and Lonvaud-Funel, 2000), it is associated with a higher survival during the stationary phase (Champomier Vergès et al., 1999), it provides substrates for biosynthetic pathways as demonstrated for the synthesis of pyrimidines by *L. leichmanii* (Hutson and Downing, 1968). *L. reuteri*, an obligately heterofermentative lactobacillus, is closely related to or even identical with species found in the animal and human intestinal tract (Vogel et al., 1999; Casas et al., 1998). It is also

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frequently isolated from traditional and modern rye and wheat fermentations. In sourdough lactobacilli, ornithine produced from arginine catabolism can serve as a precursor for 2-acetyl-l-pyrroline, a roasty, popcorn-like smelling flavor impact compound of wheat bread crust (Schieberle, 1996).

The aim of this study was to determine the ability of *L. reuteri* CRL 1098 to catabolize arginine through the ADI pathway and the role this system plays protecting the cells against acid stress.

2. Materials and methods

2.1. Micro-organism and culture conditions

L. reuteri CRL 1098 of sourdough origin was obtained from the culture collection of Centro de Referencia para Lactobacilos (CERELA), Tucumán-Argentina. The strain was propagated in MRS broth (De Man et al., 1960) at 37°C for 12 h, the cells were harvested by centrifugation, washed twice with potassium phosphate buffer (pH 7.0) and subcultured (37°C for 12 h) three times in MAM (*M*) or MAM broth with arginine (0.3% w/v) (M_A) (Zúñiga et al., 1998) to induce the arginine catabolism.

2.2. Enzyme assays

The exponential-growth cells (6 h old culture) in M_A and *M* broths with glucose (0.05% or 0.5% w/v) were harvested by centrifugation at 7000*g* for 15 min at 4°C, washed twice with 10 mM potassium phosphate buffer (pH 7.0), resuspended in the same buffer, and disrupted with a French press (X-Presss Type X25 AB BIOX S-17523-Sweden). Cell extracts (supernatant) obtained by centrifugation (9000*g*, 15 min, 4°C) (International Equipment Company, model B-22 M centrifuge) of the disrupted cells were referred to as crude cell-free extracts (CE) and were used for enzymic assays.

Arginine deiminase (*ADI*): activity was assessed in terms of citrulline production from arginine. The standard assay mixture (2.5 ml) consisted in 50 mmol l^{-1} acetate buffer (pH 5.5); L-arginine, 3 mmol l^{-1} and CE (50 µl). The reaction was stopped after 60 min of incubation at 37°C by adding 0.5 ml 2 mol l^{-1} HCl, and the precipitate was removed by centrifugation. Citrulline was measured in the supernatant according to Archibald (1944) modified by Spector and Jones (1963). To 1 ml of supernatant, 1.5 ml of acid mixture (H_3PO_4/H_2SO_4, 3:1) and 250 µl diacetyl monoxime (2–3 butanedione monoxime) (1.5% w/v in ethanol 10% v/v) were added, mixed together and boiled under dark conditions for 30 min. After cooling for 10 min, the absorbance was measured at 460 nm. One enzymic activity unit (UE) was defined as the amount of enzyme needed to produce 1 µmol of citrulline per hour.

Ornithine transcarbamylase (*OTC*): activity was determined in terms of citrulline production from ornithine. The assay mixture (2.5 ml) contained 10 mmol l^{-1} phosphate potassium buffer (pH 7.0); L-ornithine, 1.0 mmol l^{-1}; ; carbamyl phosphate, 20 mmol l^{-1} and CE. After 30 min of incubation at 37°C, the reaction was stopped and citrulline was measured as described above for ADI. One UE is the amount of enzyme needed to produce 1 µmol of citrulline per minute.

Carbamate kinase (*CK*): activity was determined by measuring ammonia production. The assay mixture (1.0 ml) consisted in 50 mmol l^{-1} acetate buffer (pH 5.5); carbamyl phosphate, 1.33 mmol l^{-1}; ADP, 7.3 mmol l^{-1}; $MgCl_2$, 7.3 mmol l^{-1} and CE. It was equilibrated at room temperature for 10 min. After the addition of carbamyl phosphate, the mixture was incubated at 37°C for 10 min, and the enzymic reaction was initiated by the addition of CE. It was incubated at 37°C for 15 min. Ammonia was measured by enzymic methods (kit from Wiener Lab., Rosario, Argentina). One UE was the amount of enzyme needed to produce 1 µmol of ammonia per min.

The specific activity of all enzymes tested was defined as units of enzyme activity per milligram of protein used in the assays.

2.3. Effect of pH and temperature

The effect of pH on the enzyme activities was examined at 37°C in a pH range 3.5–9.0 using 50 mmol l^{-1} acetate buffer (pH 3.5–6.0), 10 mmol l^{-1} potassium phosphate buffer (pH 5.6–8.0) and 50 mmol l^{-1} Tris-HCl buffer (pH 7.0–9.0) as buffer solutions. The effect of temperature was determined at the optimum pH for each enzyme in a temperature range 20–75°C.

2.4. Protein determination

The protein concentration was determined according to Bradford (1976) using bovine serum albumin as standard.

2.5. Glucose determination

The glucose concentration was determined by the glucose oxidase method (kit from Wiener Lab., Rosario, Argentina).

2.6. Adaptation and challenge conditions

An overnight culture of *L. reuteri* CRL 1098 was harvested by centrifugation (7000*g* for 15 min at 4°C),

washed twice with 10 mM potassium phosphate buffer (pH 7.0), resuspended in the same buffer, inoculated in M or M_A broth until reaching an $OD_{580\,nm} = 0.3$ (exponential-growth phase). For the adaptation assay, the cells were harvested and suspended for 60 min in M or M_A media adjusted to pH 5.0 with lactic acid. For the challenge assay, the harvested cells were suspended in M or M_A media at pH 3.0 (adjusted with lactic acid) for 60 min at 37°C.

2.7. Evaluation of arginine during growth, adaptation (pH 5.0), and challenge (pH 3.0) conditions

Cells at the early exponential phase ($OD_{580\,nm} = 0.3$) grown in M or M_A media were adapted in M or M_A media at pH 5.0. Non-adapted (NA) cells were used as control. Adapted and NA cells were harvested by centrifugation and challenged by resuspension in M and M_A media at pH 3.0. Serial dilutions of each sample were plated in MRS agar (MRS broth plus 1.5% agar) by the plate dilution method and plates were incubated at 37°C for 72 h. Results were expressed as cfu ml^{-1} and the survival rate was expressed as N/N_0 where N is the cfu ml^{-1} after a given incubation time and N_0 is the cfu ml^{-1} at zero time (without acid shift).

2.8. Reproducibility

All experiments were performed in triplicate. The means of the data and standard deviation are presented.

3. Results

The activity of the ADI pathway enzymes was determined in crude CE of *L. reuteri* CRL 1098 grown in M and M_A media. The specific activities of ADI, OTC and CK were higher (34%, 33% and 40%, respectively) in M_A compared with those of cells grown in M broth, the optimal pH and temperature of the enzyme activities were pH 5.5, 65°C (ADI), pH 5.5, 37°C (CK) and pH 5.5–7.0, 40°C (OTC).

3.1. Effect of arginine on cell growth

The cultures in M_A medium showed a two-stage growth plot (Fig. 1a): the first one (up to 5 h of incubation) related to the glucose catabolism during which the pH dropped from 6.5 to 4.7, and the second one related to arginine utilization with a rise in pH due to ammonia production. These results would indicate the presence of ADI pathway in *L. reuteri* CRL 1098 supported by the activity of the enzymes ADI (4.7 UE mg^{-1}), OTC (1.6 UE mg^{-1}) and CK (42.0 UE mg^{-1}) in CE of *L. reuteri* as well as by the increase in citrulline and ammonia production (Fig. 1b). Urea was not detected, thus excluding the presence of arginine–urease pathway.

3.2. Effect of glucose on arginine catabolism

L. reuteri CRL 1098 was unable to utilize arginine in the absence of fermentable sugars. However, a partial inhibition of the ADI (47%), OTC (11%) and CK (48%) enzymes were observed at concentration of 0.5% w/v glucose compared to 0.05% w/v glucose (Table 1).

on the ADI induction, the cells were grown in M_A broth supplemented with different concentrations of glucose (0.05–1.0% w/v) in order to reach a final pH range from 6.4 to 4.3 after fermentation.

After glucose exhaustion, the cells entered into the stationary phase and the final pH began to increase in those cultures with low glucose concentrations (0.05–0.5%) (Fig. 2) indicating that the pH did not play a role for the ADI induction in stationary phase cells.

3.3. ADI and acid tolerance response (ATR)

The ability of arginine to trigger the ATR was determined at different stages during (A) the cell growth in M or M_A broth, (B) acid challenge at pH 3.0 in M or M_A medium and (C) adaptation of exponential-grown cell at pH 5.0 in M or M_A before challenge. For this purpose, the different stages were combined as follows: cells were grown separately in M and M_A media; then, each culture was NA or adapted in the presence (A_A) or absence of arginine (A), and finally each one challenged with and without arginine.

The results obtained are shown in Fig. 3 for the challenge in absence of arginine. The M-cells grown and adapted without arginine were as sensitive to acid stress as NA cells (3a), while the adaptation at pH 5.0 improved by 20 fold the viability of M_A cells (grown with arginine) after the challenge (3b). The survival rate of *L. reuteri* CRL 1098 was increased by the addition of arginine during challenge at pH 3.0 in all conditions assayed (Fig. 4). The adaptation of cells in presence of arginine increased by 7 and 15 fold the survival of M-cells and M_A-cells, respectively, compared to NA cells (Fig. 4a and b).

4. Discussion

The data presented in this paper indicate that *L. reuteri* CRL 1098 metabolizes arginine through the ADI pathway, which might be relevant for the survival of the micro-organism in the acid environment of sourdough. This is the first report about the arginine catabolism by ADI pathway in *L. reuteri,* a sourdough lactic acid bacterium.

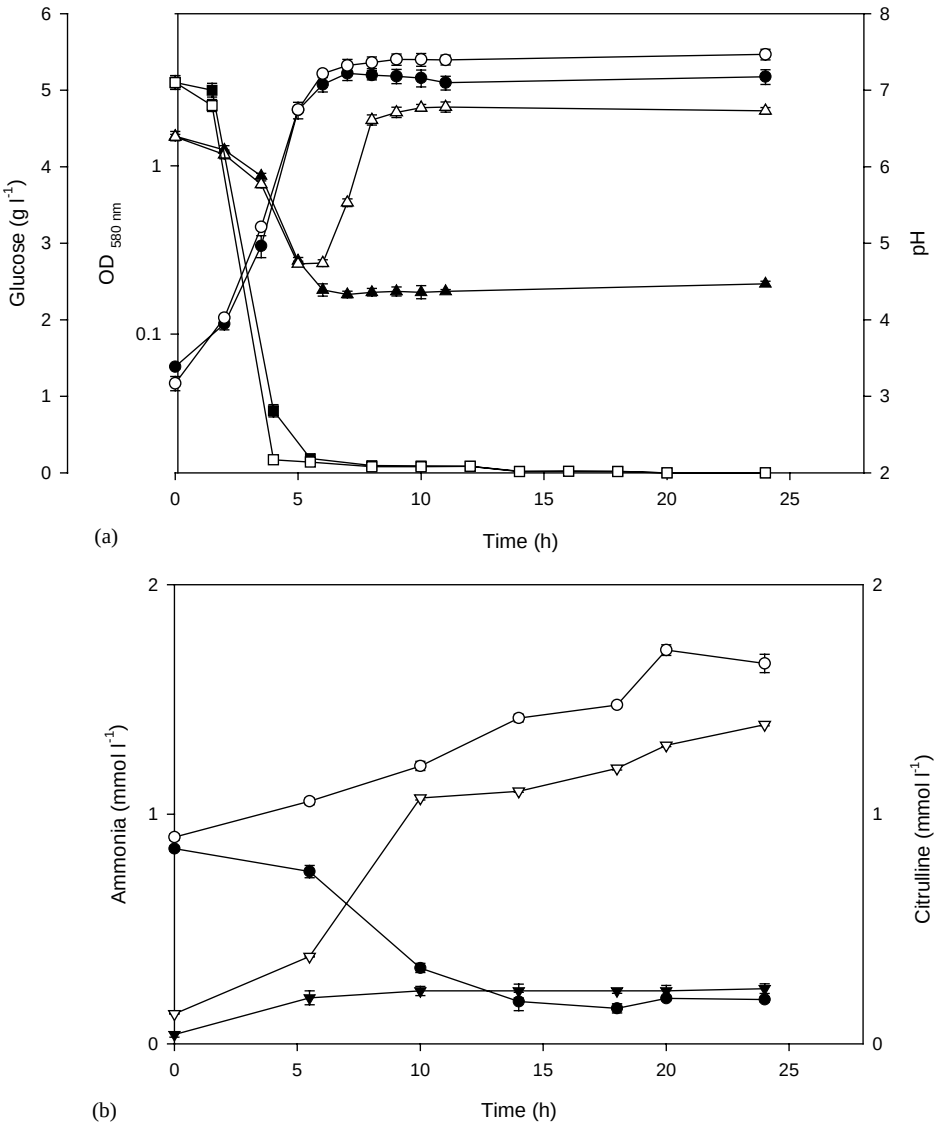

Fig. 1. (a) Effect of arginine on *L. reuteri* CRL 1098 growth. Cell growth (○) (●), culture pH (△) (▲) and glucose consumption (□) (■); (b) citrulline (○) (●) and ammonia (▽) (▼) production during *L. reuteri* growth. Cultures performed in *M* (closed symbols) and M_A (open symbols) media. (mean ± standard deviation determined in three independent experiments).

Table 1
Effect of glucose on the ADI system enzymes

Growth medium (M_A)	Specific activity (UE mg^{-1})		
	ADI	OTC	CK
Glucose 0.05%	8.9 ± 0.67	1.8 ± 0.07	80.0 ± 3.90
Glucose 0.5%	4.7 ± 0.50	1.6 ± 0.07	42.0 ± 3.7

The data are the averages ± standard deviation, determined in three independent experiments.

All enzymes (ADI, OTC and CK) of ADI pathway showed high activity in the temperature range 30–35°C, which is in agreement with the temperature used for sourdough fermentation, i.e. ADI enzyme had an optimal activity at 65°C, with 70–82% of the maximum activity at 30–35°C.

In *E. coli,* two acid resistance systems involving arginine decarboxylase and glutamate decarboxylase enzymes have been reported (Park et al., 1996; Bearson et al., 1997) but no direct role has been ascribed to decarboxylases in the ATR of lactobacilli. In *L. acidophilus,* the ATR appears to involve two basic steps: the induction of specialized pH–homeostasis systems involving membrane ATPases and the induction of acid shock proteins (Lorca et al., 1998; Lorca and Font de Valdez, 2001). Thus, in these LAB, the ADI system might be a secondary safety device as it has been described for oral bacteria (*Streptococcus sanguis* and *S. rattus*) (Casiano-Colón and Marquis, 1988). Results

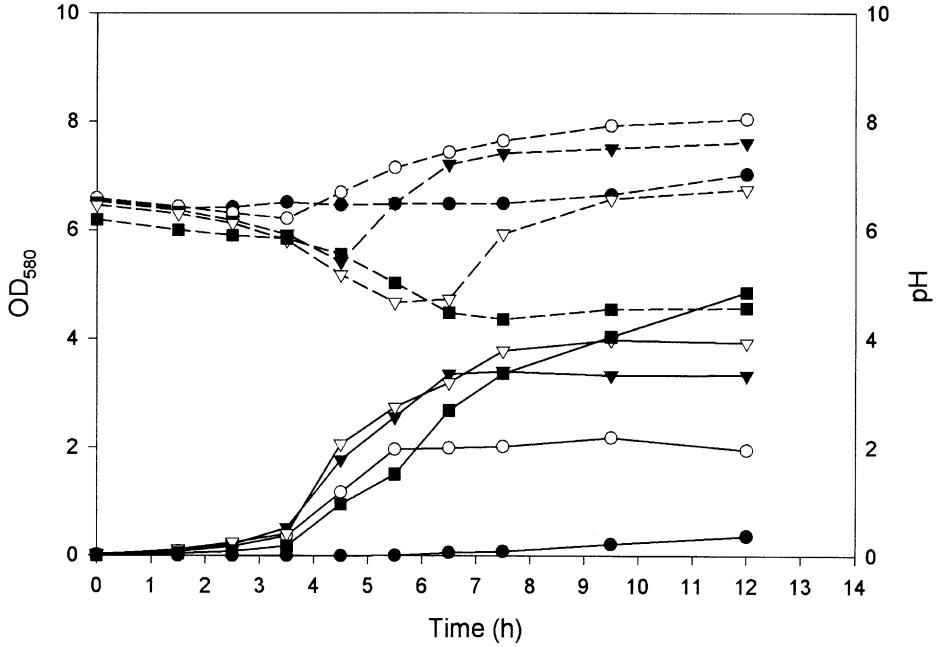

Fig. 2. Growth of *L. reuteri* CRL 1098 in M_A medium. Cell growth (—) and culture pH (- - - -) at different concentrations of glucose: 0% (●), 0.05% (○), 0.2% (▼), 0.5% (▽) and 1.0% (¦).

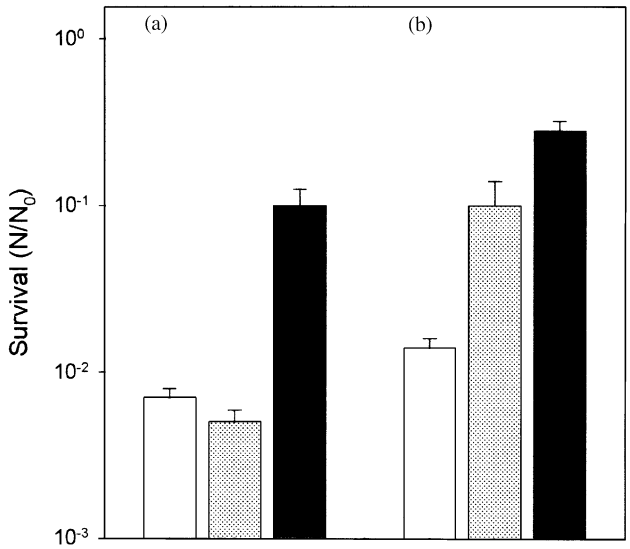

Fig. 3. Effect of pH 5.0-adaptation on the survival of *L. reuteri* during challenge at pH 3.0. Cultures grown without (a) and with arginine (b). NA cells (□), adapted without (■) and with (■)arginine. Survival was expressed as N/N_0, where N is the cfu ml^{-1} after a given incubation time and N_0: cfu ml^{-1} at zero time (without acid shift). (mean ± standard deviation determined in three independent experiments).

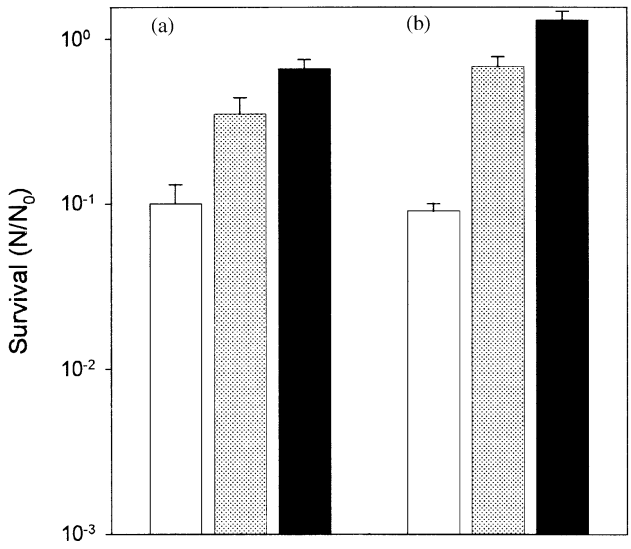

Fig. 4. Effect of arginine during challenge of *L. reuteri* at pH 3.0. For symbols see Fig. 3. Survival was expressed as N/N_0, where N is the cfu ml^{-1} after a given incubation time and N_0: cfu ml^{-1} at zero time (without acid shift).

obtained in *L. reuteri* CRL 1098 suggest that ADI is an important component of the ATR developed by the cells in the presence of arginine, a protective mechanism associated with ammonia production. Arginine should be present at least at one of the following levels: growth, adaptation at pH 5.0 or challenge at pH 3.0 to induce the ATR in the micro-organism. This observation is supported by the higher ADI, OTC and CK activities found in cells adapted with arginine compared to cells adapted without arginine (Table 2).

The utilization of arginine as an energy source for growth was showed in a variety of micro-organism (Tonon and Lonvaud-Funel, 2000; Crow and Thomas, 1982; Stuart et al., 1999; Simon et al., 1982; Montel and Champomier, 1987). The net gain of metabolic energy by ADI pathway is one ATP per arginine metabolized, since no metabolic energy is spent for the uptake of

Table 2
Effect of acid adaptation on the ADI pathway enzymes

Growth and adaptation conditions	Specific activity (UE mg^{-1})		
	ADI	OTC	CK
M	5.46±0.5	1.15±0.03	27±1.3
$M-A_A$	7.30±0.7	1.07±0.02	43±2.6
M_A	8.9±0.8	1.78±0.08	80±4.9
M_A-A	7.7±0.9	1.13±0.04	83±5.5
M_A-A_A	11.7±0.9	2.08±0.09	101±7.3

The data are the averages±standard deviation, determined in three independent experiments.

arginine or the excretion of ornithine (Poolman et al., 1987). In *L. reuteri* CRL 1098 the energy derived from arginine metabolism would be coupled to growth of this micro-organism.

The carbon source partially represses the activity of the ADI system enzymes (ADI, OTC and CK). The OTC enzyme was less sensible to repression by carbohydrate compared to the other two enzymes. Further studies are needed to determine the factors that regulate the OTC activity in *L. reuteri* CRL 1098. The OTC activity of *L. buchneri* NCDO 110 was non-affected by glucose in contrary that observed for ADI enzyme activity (Manca de Nadra et al., 1981). The synthesis of the ADI system enzymes (ADI, OTC and CK) was repressed by the carbon source in *S. lactis*. The regulation of the ADI pathway by ATP could be exerted not only on CK, for which ADP is a substrate and ATP a product, but possibly also on OTC. (Poolman et al., 1987). In *L. sake,* glucose repression on the ADI pathway has also been observed (Montel and Champomier, 1987). Northern blot analysis showed that the transcription of the arcA gene that encode for ADI in *L. sake* was clearly repressed by glucose. This mechanism involves the HPr and Ccpa protein, which binds on cre sequence, located upstream the operons, which will be regulated (Zúñiga et al., 1998).

In *L. reuteri* CRL 1098 the triggering factor for the ADI pathway would be the depletion in energy source rather than the pH attained by cultures at the stationary phase. In this sourdough micro-organism origin, the ADI pathway would provide ornithine, which is considered one of the key flavour precursors in the dough. Model experiments established that ornithine reacts in the presence of carbohydrates during baking generating significant amounts of 2-acetyl-l-pyrroline, a precursor for flavour compounds in the crust of wheat bread (Schieberle, 1990). Furthermore, the ADI system might be involved in the acid–base physiology of *L. reuteri* allowing the recovery of the cells from acid stress sufficiently severe to stop growth and glycolysis.

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