Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Effect of biosynthetic intermediates and citrate on the phenyllactic and hydroxyphenyllactic acids production by *Lactobacillus plantarum* CRL 778

A.M. Dallagnol[1], C.A.N. Catalán[2], M.I. Mercado[2], G. Font de Valdez[1,3] and G.C. Rollán[1]

1 Centro de Referencia para Lactobacilos (CERELA-CONICET), S. M. de Tucumán, Argentina
2 Instituto de Química del Noroeste Argentino (INQUINOA-CONICET) y Facultad de Bioquímica, Química y Farmacia de la Universidad Nacional de Tucumán (UNT), S. M. de Tucumán, Argentina
3 Cátedra de Microbiología Superior, Facultad de Bioquímica, Química y Farmacia de la Universidad Nacional de Tucumán (UNT), S. M. Tucumán, Argentina

Keywords
citrate, glutamate, glutamate dehydrogenase, hydroxyphenyllactic acid, *Lactobacillus plantarum*, phenyllactic acid.

Correspondence
Graciela C. Rollán, Centro de Referencia para Lactobacilos (CERELA-CONICET), Chacabuco 145 (4000), S. M. de Tucumán, Argentina.
E-mail: rollan@cerela.org.ar

2011/0672: received 20 April 2011, revised 30 August 2011, accepted 18 September 2011

doi:10.1111/j.1365-2672.2011.05159.x

Abstract

Aim: To evaluate the influence of biosynthetic precursors, intermediates and electron acceptors on the production of antifungal compounds [phenyllactic acid (PLA) and hydroxyphenyllactic acid (OH-PLA)] by *Lactobacillus plantarum* CRL 778, a strain isolated from home-made sourdough.

Methods and Results: Growth of fermentative activity and antifungal compounds production by *Lact. plantarum* CRL 778 were evaluated in a chemically defined medium (CDM) supplemented with biosynthetic precursors [phenylalanine (Phe), tyrosine (Tyr)], intermediates [glutamate (Glu), alpha-ketoglutarate (α-KG)] and electron acceptors [citrate (Cit)]. Results showed that the highest PLA production (0·26 mmol l^{-1}), the main antifungal compound produced by *Lact. plantarum* CRL 778, occurred when greater concentrations of Phe than Tyr were present. Both PLA and OH-PLA yields were increased 2-folds when Cit was combined with α-KG instead of Glu at similar Tyr/Phe molar ratio. Similarly, glutamate dehydrogenase (GDH) activity was significantly ($P < 0·01$) stimulated by α-KG and Cit in Glu-free medium.

Conclusion: Phe was the major stimulant for PLA formation; however, Cit could increase both PLA and OH-PLA synthesis by *Lact. plantarum* CRL 778 probably due to an increase in oxidized NAD^+. This effect, as well as the GDH activity, was enhanced by α-KG and down regulated by Glu.

Significance and Impact of the Study: This is the first study where the role of Glu and GDH activity in the PLA and OH-PLA synthesis was evidenced in sourdough lactic acid bacteria (LAB) using a CDM. These results contribute to the knowledge on the antifungal compounds production by sourdough LAB with potential applications on the baked goods.

Introduction

The antimould activity of lactic acid bacteria (LAB) in sourdough has been generally attributed to the presence of organic acids, particularly lactic and acetic acids (Spicher 1983; Rocken 1996). In recent years, different studies have shown that other low-molecular-weight bacterial compounds are also involved such as cyclo (L-Phe–L-Pro), cyclo (L-Phe–*trans*-4-OH-L-Pro), phenyllactic acid (PLA), hydroxyphenyllactic acid (OH-PLA), propionic acid, cumaric acid, phenylpropanic acid, 2-methylcinnmic acid, salicylic acid and sodium decanoate (Lavermicocca *et al.* 2000; Ström *et al.* 2002; Zhang *et al.* 2010; Ryan *et al.* 2011). These antifungal substances are produced in low amounts that do not reach the minimum concentration necessary to inhibit the fungal growth (Vermeulen *et al.* 2006; Ryan *et al.* 2009). Thus, the antifungal activity of LAB is a synergic effect among different compounds,

which is enhanced by the fermentation end products (lactic and acetic acids) (Corsetti *et al.* 1998; Schnürer and Magnusson 2005). In the last decade, Lavermicocca *et al.* (2000) reported first the production of PLA and OH-PLA by *Lactobacillus plantarum* B21 that was effective against the main genera that affect the baked goods: *Penicillum*, *Aspergillus* and *Fusarium*. Further studies showed that PLA is more effective than OH-PLA and a synergistic effect takes place when it is combined with acetic or lactic acid (Lavermicocca *et al.* 2003).

The synthesis of PLA and OH-PLA in LAB results from the catabolism of phenylalanine (Phe) and tyrosine (Tyr), respectively (Fig. 1) (McSweeney and Sousa 2000; Yvon and Rijnen 2001). In *Lactococcus lactis*, the transamination reaction is the first catabolic step of Phe and Tyr and is initiated by an aromatic aminotransferase (AAT) that is also active with tryptophan, methionine and leucine (Yvon *et al.* 1997; Rijnen *et al.* 1999). This enzyme catalyses the transference of ammonium from an R-amino group to a keto acid acceptor being alpha-ketoglutarate (α-KG) the favourite acceptor in most LAB. Because of this, the bioavailability of α-KG becomes a limiting factor for all transamination reactions and amino acid catabolism (Yvon *et al.* 1998; Rijnen *et al.* 2000). The amino acid degradation in LAB may be increased by glutamate dehydrogenase (GDH), enzyme that catalyses the reversible oxidative deamination of glutamate (Glu) to α-KG and ammonium (Rijnen *et al.* 2000; Tanous *et al.* 2002). Thus, the α-KG formation through GDH activity would be an indirect way to increase PLA and OH-PLA formation by antifungal LAB strains. The GDH activity is strongly influenced by the redox state of the cell. Compounds able to act as alternative electron acceptors, such as citrate (Cit) or fructose (Fru) might increase the formation of NAD^+ and $NADP^+$, thus stimulating the Phe and Tyr catabolism through GDH activity. The addition of exogenous α-KG increased the PLA synthesis by the negative GDH strain *Lact. plantarum* TMW1.468, while the addition of Cit or fructose together with α-KG or Glu was necessary for *Lact. sanfranciscensis* DSM2045, a positive GDH strain (Vermeulen *et al.* 2006).

In a previous work, the production of PLA by *Lact. plantarum* CRL 778 isolated from sourdough was reported (Gerez *et al.* 2009). Further studies revealed that the strain also produces OH-PLA. To optimize the antifungal activity of *Lact. plantarum* CRL 778, the present work addresses the effect of different compounds (precursors, intermediate and electron acceptors) on the biosynthesis of PLA and OH-PLA by *Lact. plantarum* CRL 778 using a chemically defined medium (CDM). The GDH activity, growth and organic acid production of the cells were also evaluated.

Materials and methods

Micro-organism and growth medium

Lactobacillus plantarum CRL 778 was isolated from homemade wheat dough and belongs to the Culture Collection (CRL) of Centro de Referencia para Lactobacilos (CERELA), Argentina. Overnight (16 h) cultures of CRL 778 strain were harvested by centrifugation (8609 ***g***, 10 min), washed twice with sterile potassium phosphate buffer 0·1 mol l^{-1} (pH 6·5) and suspended in the same buffer. The cell suspension (3×10^9 CFU ml^{-1}) was used to inoculate (1%, v/v) a CDM containing (% w/v) the following: glucose, 1·0; KH_2PO_4, 0·3; K_2HPO_4, 0·3; sodium acetate, 0·5; hydrogen ammonium citrate, 0·1; $MgSO_4.7H_2O$, 0·02;

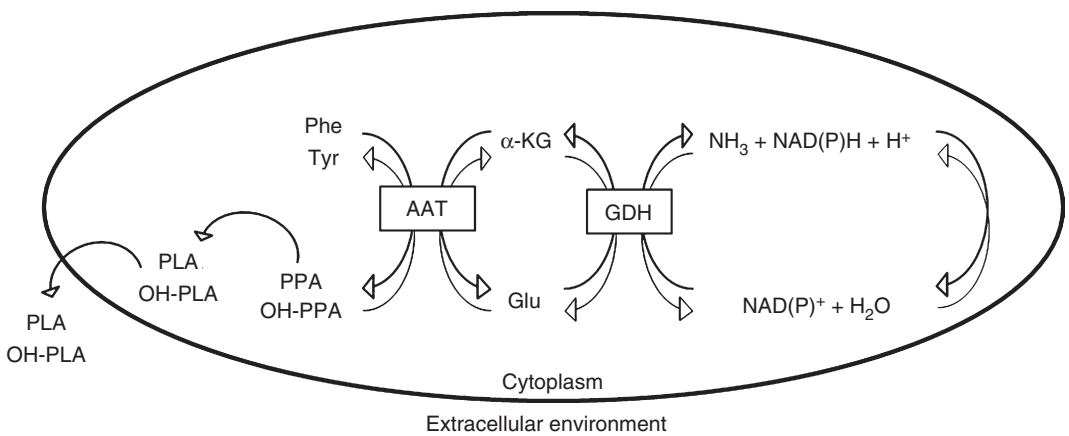


Figure 1 Metabolic pathway involved in phenyllactic acid (PLA) and hydroxyphenyllactic acid (OH-PLA) synthesis in glutamate dehydrogenase (GDH) positive lactic acid bacteria strains. Abreviations: phenylalanine (Phe), tyrosine (Tyr), aromatic aminotransferase, phenylpyruvic acid (PPA), hydroxyphenylpyruvic acid (OH-PPA), phenyllactic acid (PLA), hydroxyphenyllactic acid, (OH-PLA), alpha-ketoglutarate, glutamate (Glu), GDH.

L-alanine, 0·01; L-arginine, 0·01; L-asparagine, 0·02; L-aspartic acid, 0·02; L-cysteine, 0·02; L-glutamine, 0·02; L-glutamic acid, 0·02; glycine, 0·01; L-histidine, 0·01; L-isoleucine, 0·01; L-leucine, 0·01; L-lysine, 0·01; L-methionine, 0·01; L-phenylalanine, 0·01; L-proline, 0·01; L-serine, 0·01; L-threonine, 0·01; L-tryptophan, 0·01; L-tyrosine, 0·01; L-valine, 0·01; uracil, 0·001; guanine, 0·001; adenine, 0·001; xanthine, 0·001; nicotinic acid, 0·0001; calcium pantothenate, 0·0001; pyridoxal, 0·0002; riboflavin, 0·0001; orotic acid, 0·005; folic acid, 0·0001; vitamin B12, 0·0001; thiamine, 0·0001; biotin, 0·001; *p*-aminobenzoic acid, 0·001 and Tween 80, 0·1. This medium was adapted from that described by Pescuma *et al.* (2007). The CDM (pH 6·5) was prepared from sterile concentrated stock solutions, stored at 4°C, except for the cysteine solution that was freshly prepared. Stock solutions were sterilized by filtration (0·20-μm pore size) (Sartorius AG, Gottingen, Germany). The behaviour of *Lact. plantarum* CRL 778 in CDM was evaluated at 30°C for 72 h. At different time intervals (0, 12, 24, 48 and 72 h), samples were withdrawn to determine cell viability, Phe and Tyr consumption and PLA and OH-PLA production.

To assess the effect of precursors (Phe, Tyr), biosynthetic intermediates (Glu, α-KG) and electron acceptors (Cit) on the growth and PLA and OH-PLA synthesis by *Lact. plantarum* CRL 778, different assays were carried out by adding or omitting of the mentioned compounds in CDM (Table 1). Each culture was grown at 30°C for 24 h, and samples were taken to evaluate cell viability, maximum growth rate, antifungal compounds synthesis, GDH activity and organic acid production. The CDM without modifications was taken as control.

Growth determinations

The growth was determined by both optical densities at 580 nm and cell viability. A microplate reader spectrophotometer (VERSAmax; Molecular Devices, Sunnyvale, CA, USA) was used to obtain growth plots at 30°C and to determine the maximum specific growth rates (μ_{max}, h^{-1}). This was calculated by linear regression (indicated by the correlation coefficient R^2) from the plots of $\ln(OD_f/OD_i)$ versus time, where OD_i and OD_f are the initial and final value of the exponential growth phase. Cell viability was determined by the plate dilution method in MRS broth (De Man *et al.* 1960) plus 1.3% w/v agar from cultures. Plates were incubated for 48 h, and the results were expressed as log CFU per ml.

Aromatic amino acid and antifungal compounds determinations

Phenylalanine, Tyr, PLA and OH-PLA determinations were performed by HPLC according to Valerio *et al.* (2004) with modifications. The cell-free supernatants filtered (0·45 μm filters, Ministart high flow; Sartorius) were injected (20 μl) in a RP C18 column (250 × 4·6 mm, 5 μm particles) (Grace Davison Discovery Sciences, RW Grace & Co., Deerfield, CT, USA) and eluted under the following solvent system: solvent A, water plus 0·05%

Table 1 Fermentations conditions of *Lactobacillus plantarum* CRL 778 in chemically defined medium modified as follow: (i) different Phe and Tyr molar ratio; (ii) addition or omission of Glu, α-KG and Cit; (iii) omission of acetate (Ac)

		Precursors (% p/v)		Intermediaries, electron acceptors (% p/v)			
Assay	Conditions	Phe	Tyr	Glu	α-KG	Cit	Ac
Control	1 (Control)	0·01 (0·60)*	0·01 (0·55)	0·02	–	0·10	0·50
1	2 (Phe/Tyr)	0·03 (1·80)	0·03 (1·65)	0·02	–	0·10	0·50
	3 (Phe/Tyr)	0·05 (3·00)	0·05 (2·75)	0·02	–	0·10	0·50
	4 (Phe/Tyr)	0·05 (3·00)	0·01 (0·55)	0·02	–	0·10	0·50
	5 (Phe/Tyr)	0·01 (0·60)	0·05 (2·75)	0·02	–	0·10	0·50
2	6 (no supplemented)	0·01	0·01	–	–	–	0·50
	7 (+Glu)	0·01	0·01	0·02	–	–	0·50
	8 (+α-KG)	0·01	0·01	–	0·02	–	0·50
	9 (+Glu + α-KG)	0·01	0·01	0·02	0·02	–	0·50
	10 (+Cit)	0·01	0·01	–	–	0·10	0·50
	11 (+Cit + α-KG)	0·01	0·01	–	0·02	0·10	0·50
	12 (+Glu + α-KG + Cit)	0·01	0·01	0·02	0·02	0·10	0·50
3	13 (–Ac)	0·01	0·01	0·02	–	–	–
	14 (+Cit –Ac)	0·01	0·01	0·02	–	0·10	–

α-KG, alpha-ketoglutarate; Phe, phenylalanine; Tyr, tyrosine; Glu, glutamate; Cit, citrate.
*Values within parenthesis are in mmol l^{-1}.

TFA, and solvent B, methanol plus 0·05% TFA. The initial mobile phase (0–15 min) was a linear gradient from 10 to 50% solvent B; this was followed by 15 min with 50% solvent B and 15 min of reequilibration under the initial conditions. The flow rate was 0·6 ml min^{-1}, and the data were monitored using an UV detector (UV/Vis Gilson 152, Middleton, WI, USA) at 210 nm for Phe-PLA and 220 nm for Tyr-OH-PLA.

Organic acids evaluation

The concentrations of lactic and acetic acids were determined by HPLC from filtered (0·45 μm filters, Ministart high flow; Sartorius) cell-free supernatants of fermented samples. Lactic and acetic acids were determined by using an ion-exclusion Aminex HPX-87 H column (300 mm × 7·8 mm; Bio-Rad, CA, USA) under the following conditions: isocratic mobile phase H_2SO_4 (5 mmol l^{-1}); flow rate 0·6 ml min^{-1} and column temperature, 45°C. A refractive index detector (Smartline Knauer, Berlin, Germany) connected to the software (Peak Simple II; Knauer) was used for data analysis. Acetic acid concentrations were expressed as the difference in concentration after 24 h of fermentation in CDM respect to the zero time (Δmmol l^{-1}).

GDH activity

The activity of the GDH enzyme in cells grown in CDM modified with Glu, α-KG and Cit was determined. *Lact. plantarum* CRL 778 was cultured in 100 ml of each medium for 12 h, then washed with 50 mmol l^{-1} sodium phosphate buffer (pH 7·0), and the pellet suspended in 10 ml of the same buffer for cells disruption with a French press (French Pressure Cells; Thermo-Spectronic, Rochester, NY, USA). The cell debris was removed from the cell-free extract by centrifugation (8609 ***g***, 15 min). The enzymatic assay was carried out in multiwell microdilution plates (96 wells, IWAKI; Scitech Div., Asashi Techno Glass, Tokyo, Japan). The reaction mixture contained cell-free extract (1·4 mg protein per ml), glutamate (50 mmol l^{-1}) and NADP (2 mmol l^{-1}) in a final volume of 0·2 ml. The reduced cofactor NADPH (produced through the oxidative deamination of glutamate by GDH) in the reaction mixture was determined by measuring the absorbance in a microplate reader spectrophotometer (VERSAmax, Molecular Devices) at 340 nm during 5 min. Units of GDH enzyme activity (U) were calculated (using a molar extinction coefficient of 6·22 l mol^{-1} cm^{-1}) as nanomoles of NADPH released per min. The enzyme specific activity was calculated as units per milligram of protein (U mg^{-1}). The cell-free extract was inactivated at 90°C for 5 min and incubated in the presence of $NADP^+$ with and without Glu and used as control. The protein concentration in the cell-free extracts was determined according to Bradford (1976) using bovine serum albumin as standard.

Statistical data analysis

The results are mean values of three separated fermentations. Significant differences ($P < 0·05$) in organic acid formation upon the addition of the different compounds were evaluated by using one-way analysis of variance (ANOVA) and by Fisher's test in MINITAB-12 software (Minitab, State College, PA).

Results

Behaviour of *Lactobacillus plantarum* CRL 778 in CDM

The behaviour of *Lact. plantarum* CRL 778 in CDM was evaluated during 72 h of fermentation. The cell viability was maximum (log 9·28 ± 0·09 CFU ml^{-1}) after 12 h of incubation and began to decrease after 24 h (Fig. 2). The increase in the production of PLA (0·10 ± 0·003 mmol l^{-1}) and OH-PLA (0·12 ± 0·01 mmol l^{-1}) was greater for the

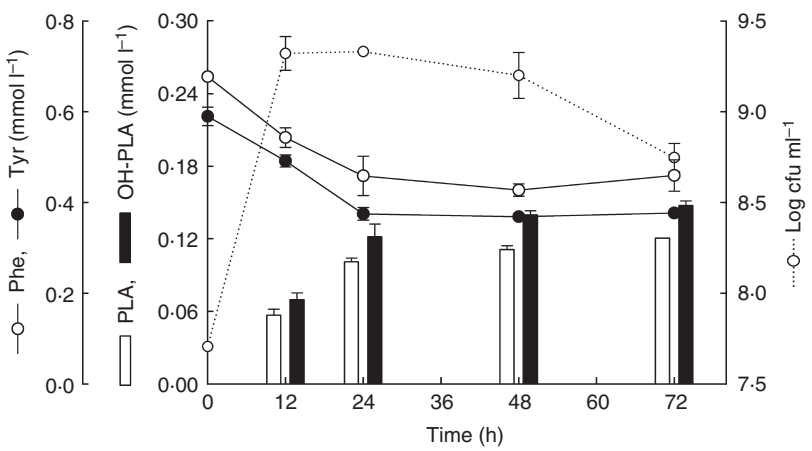


Figure 2 Growth, residual amino acids (Phe and Tyr) and antifungal compounds [phenyllactic acid (PLA) and OH-PLA] production by *Lactobacillus plantarum* CRL 778 in chemically defined medium incubated at 30°C for 72 h.

first 24-h incubation period, a slight (20–25%) increase being observed from then on. Accordingly, Phe and Tyr (0·60/0·55 molar ratio) were consumed within 24 h remaining *c.* 0·22 mmol l^{-1} after 72 h fermentation. From these results, further fermentation assays were performed for 24 h of incubation.

Influence of biosynthetic precursors (Phe and Tyr) on PLA and OH-PLA synthesis

The effect of Phe/Tyr molar ratio on the PLA and OH-PLA formation by *Lact. plantarum* CRL 778 is shown in Fig. 3. The ratio 0·60/0·55 Phe/Tyr was included for comparison (Control). The formation of PLA was strongly increased (0·26 ± 0·02 mmol l^{-1}) in the presence of a high (3 mmol l^{-1}) amount of Phe, which in turn produced a decrease (from 0·12 to 0·06 ± 0·01 mmol l^{-1}) in OH-PLA formation. Similarly, the addition of Tyr in high amounts (2·75 mmol l^{-1}) enhanced the production of OH-PLA (0·28 ± 0·02 mmol l^{-1}) but induced a reduction in PLA (0·05 ± 0·01 mmol l^{-1}). Interestingly, greater concentrations of both amino acids did not induce significant ($P > 0·05$) changes in PLA and OH-PLA formation compared with the control Phe/Tyr molar ratio.

As tryptophan (Trp) might compete with Phe and Tyr by AAT during the transamination step, high concentration of Trp (2·5 mmol l^{-1}) was added to CDM in the presence of low amounts of Phe and Tyr. Results showed that no change in the synthesis of PLA and OH-PLA took place when compared with the control (data not shown).

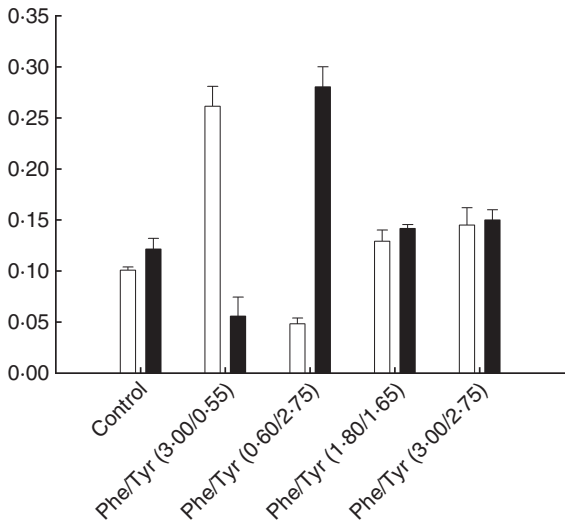
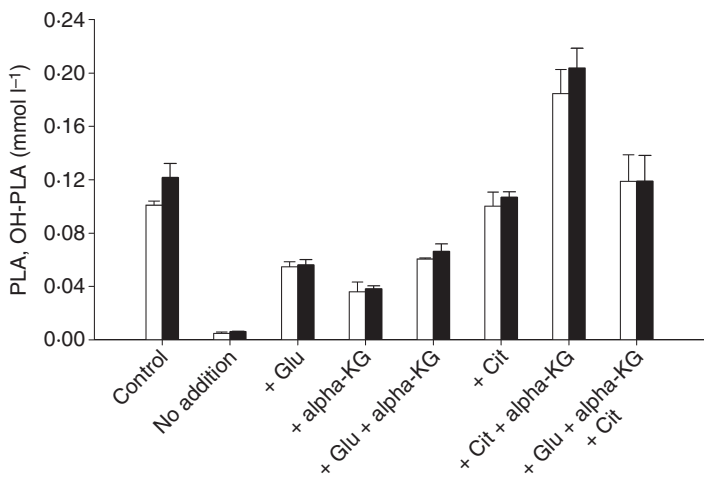


Figure 3 Effect of different concentrations (mmol l^{-1}) of Phe and Tyr on the phenyllactic acid (PLA) and OH-PLA production by *Lactobacillus plantarum* CRL 778 in chemically defined medium incubated at 30°C for 24 h. The ratio 0·60/0·55 (Control) was included for comparison. (□) PLA and (■) OH-PLA (mmol l^{-1}).

Stimulant effect of α-KG and Cit

Glutamate, α-KG and Cit affected in different way the growth of and the antifungal compounds production by *Lact. plantarum* CRL 778 [Fig. 4; CDM without modifications (Control) was included for comparison]. The presence of glutamate was essential for reaching the



Log cfu ml^{-1}	9·28 (± 0·21)	8·20 (± 0·14)	9·20 (± 0·28)	9·03 (± 0·18)	9·13 (± 0·18)	8·68 (± 0·16)	9·22 (± 0·20)	9·18 (± 0·24)
μ (h^{-1})	0·48 (± 0·02)	0·09 (± 0·01)	0·46 (± 0·02)	0·24 (± 0·04)	0·49 (± 0·03)	0·11 (± 0·01)	0·29 (± 0·01)	0·45 (± 0·03)

Figure 4 Effect of Glu, alpha-ketoglutarate and Cit on growth of and phenyllactic acid (PLA) and OH-PLA (mmol l^{-1}) production by *Lactobacillus plantarum* CRL 778 grown in chemically defined medium at 30°C for 24 h. (□) PLA (mmol l^{-1}) and (■) OH-PLA (mmol l^{-1}).

maximum levels of cell growth and μ_{max}. The addition of α-KG instead of Glu produced a decrease in cell viability, μ_{max}, and antifungal compounds formation by 13, 48 and 34%, respectively. Cit was necessary to reach those PLA and OH-PLA concentrations observed in CDM control, in spite of the growth parameter were significantly ($P < 0{\cdot}05$) lower. Surprisingly, when α-KG and Cit were combined in the absence of Glu, a synergic effect on PLA and OH-PLA production was obtained.

The combined effect of Glu, α-KG and Cit on GDH activity of *Lact. plantarum* CRL 778 grown in CDM was also assessed, and the results are shown in Fig. 5. The best enzyme activity results (33·1 nmol mg^{-1} protein per min) was obtained when Cit and α-KG where combined. The presence of Glu decreased the GDH activity levels, which were comprised in a range between 9·2 and 13·2 mmol mg^{-1} protein per min.

The production of the fermentation end products by *Lact. plantarum* CRL 778 cultured in CDM was evaluated as well. The lactic acid production was similar (95–110 mmol l^{-1}) in all growth media except in CDM without α-KG and Glu, where lower concentrations were observed (23 ± 6 and 57 ± 9 mmol l^{-1}) (Fig. 6). Ethanol was not produced in CDM while acetic acid showed an unusual profile. This acid was consumed [(−7·2)–(−8·8) Δmmol l^{-1}] when Cit was omitted and increased (6·2–7·4 Δmmol l^{-1}) or remained unchanged, when Cit was added. These results indicate that acetic acid was only produced when Cit was present. To corroborate this, sodium acetate compound was omitted in CDM. Results allowed us to confirm that Cit was necessary for the production (4·1 ± 0·52 mmol l^{-1}) of acetic acid (data not shown) by *Lact. plantarum* CRL 778.

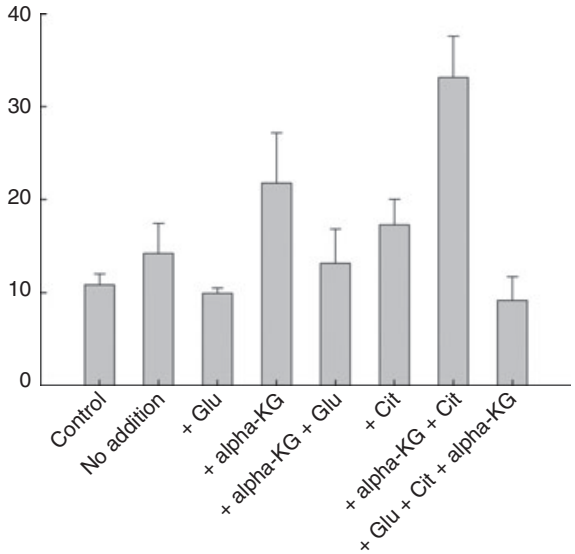


Figure 5 Effect of Glu, alpha-ketoglutarate and Cit on glutamate dehydrogenase activity of *Lactobacillus plantarum* CRL 778 grown (12 h) in chemically defined medium. (▩) GDH specific activity (U mg^{-1}).

Discussion

The sourdough strain *Lact. plantarum* CRL 778 has antifungal properties against the major bread contaminant fungi, such as *Aspergillus*, *Fusarium* and *Penicillum*, mainly because of the production of PLA and acetic acid (Gerez *et al.* 2009). In this work, studies were carried out in a CDM supplemented with various compounds acting as biosynthetic precursors, intermediates and electron acceptors

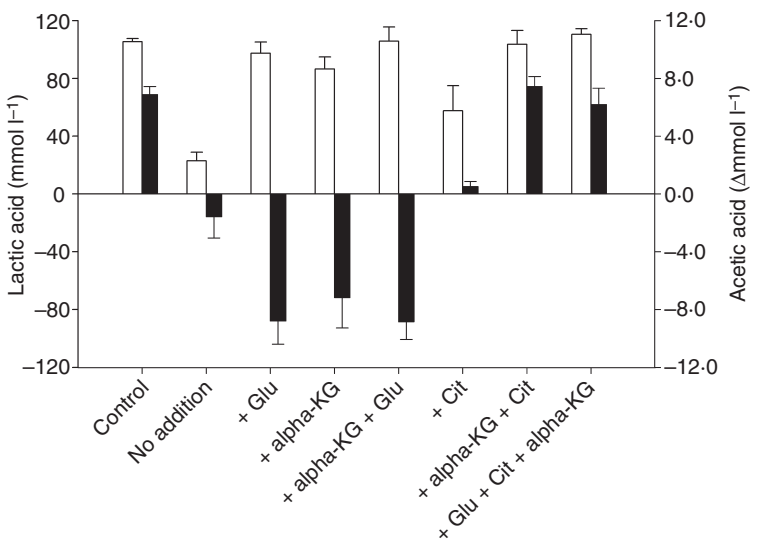


Figure 6 Effect of Glu, alpha-ketoglutarate and Cit on lactic and acetic acids production by *Lactobacillus plantarum* CRL 778 grown at 30°C for 24 h. (□) Lactic acid (mmol l^{-1}) and (■) Acetic acid (△mmol l^{-1}).

to evaluate their effect on the synthesis of PLA and OH-PLA. The CDM medium used allowed to better understand the role of different effectors on the formation of antifungal compounds by *Lact. plantarum* CRL 778 if compared with complex broth cultures where interactions with interfering proteins, peptides and amino acids may occur.

Results indicate that PLA and OH-PLA are primary metabolites because they were mainly accumulated after 12 and 24 h of fermentation. The presence of residual Phe and Tyr at the final of the fermentation (72 h) showed that these amino acids were in excess, and they were not a limiting factor for the production of more antifungal compounds. On the other hand, the final concentrations of PLA and OH-PLA determined in CDM were lower than those observed for Phe and Tyr consumption revealing that these amino acids were converted into antifungal compounds and, about 50% of them were probably diverting to other metabolic reactions.

The use of high concentrations of Phe largely increased PLA synthesis but inhibited OH-PLA formation and *vice versa*, thus indicating the direct relationship existing between Phe and PLA, and Tyr and OH-PLA as previously reported (Valerio *et al.* 2004). In addition, our results evinced that the presence of both amino acids even at quite high concentrations had no effect on the production of PLA or OH-PLA. This fact could be due to a saturation of the aromatic aminotransferase (AAT) by the substrates that would be competing for the active sites of the enzyme, avoiding further synthesis of PLA and OH-PLA. In addition, similar affinity of the AAT for Phe and Tyr occur in *Lact. plantarum* CRL 778 because similar amounts of PLA and OH-PLA were produced. It is well known that AAT have different affinities for substrates; Yvon *et al.* (1997) characterized an AAT form *Lactococcus lactis* NCDO763 where the relative affinity for aromatic amino acids was Phe > Tyr > Trp, while that for AAT characterized by Gao and Steele (1998) from *L. lactis* S3 was Trp > Tyr > Phe. Based on these facts, we also evaluated the effect of Trp on the synthesis of antifungal compounds in *Lact. plantarum* CRL 778, and we observed that large amounts of Trp did not change the PLA and OH-PLA production (data not shown). These findings suggest that the enzyme would have a limited or null activity on Trp.

Glutamate was a very important amino acid for the growth and the production of all organic acids tested by *Lact. plantarum* CRL 778. However, the growth observed in CDM without Glu indicated that the micro-organism was able to obtain this amino acid by an alternative route. The strain could have used glutamine to convert it into Glu through the glutamine synthetase (Lapujade *et al.* 1998) or through the glutaminase enzyme reported by Vermeulen *et al.* (2007). *Lact. plantarurm* CRL 778 also could use α-KG to produce Glu because the cell viability and the lactic acid production were similar to that observed in the medium with Glu. However, α-KG did not largely stimulate the PLA and OH-PLA formation as expected (Kieronczyk *et al.* 2003), indicating that this intermediate was converted in Glu mainly through GDH activity and not by transamination reactions. This result was in concordance with the increase observed on the GDH activity when Glu was replaced by α-KG. Otherwise, α-KG could be also converted into 2-hydroxyglutarate (Zhang and Gänzle 2010). The presence of α-KG combined with Glu did not stimulate the PLA and OH-PLA production, in opposite to reported for *Lact. plantarum* TMW1.468 by Vermeulen *et al.* (2006). However, the presence of citrate had a clear positive effect on the PLA and OH-PLA synthesis in *Lact. plantarum* CRL 778. Probably, Cit stimulated the production of these antifungal metabolites by changing the redox balance of the cells. The catabolism of citrate in LAB leads to the production of large amounts of pyruvate, which may be converted to succinate, lactate, diacetyl, acetoin and 2,3-butanediol (Drinan *et al.* 1976; Verhue and Tjan 1991; Boumerdassi *et al.* 1997). The formation of some of those compounds increase largely the $NAD(P)^+$/NAD(P)H relation (Ardö 2006) which in turn stimulates the deamination of Glu by GDH resulting in an excess of α-KG. Thus, the presence of an electron acceptor would stimulate the Glu synthesis through the AAT activity which increase the Phe and Tyr catabolism and improve the PLA and OH-PLA formation. This fact explains the highest production of PLA and OH-PLA when α-KG was combined with Cit in Glu-free CDM and the down regulation when Glu was added together. Probably, a negative feedback took place when an excess of Glu was available.

The presence of α-KG and Cit had a quite pronounced positive effect on GDH activity, while Glu presence showed an opposite effect, indicating that this enzyme was down regulated by Glu. On the other hand, it remains unclear how Cit could stimulate the GDH activity in *Lact. plantarum* CRL 778.

Cit was an important cosubstrate for the acetic acid production by *Lact. plantarum* CRL 778. Although this micro-organism is a heterofermentative facultative, the acetic acid production was not observed in citrate-free medium, indicating that Cit was necessary for the acetate formation. Torino *et al.* (2005) reported that in LAB, the citric acid once inside the cell is split into acetate and oxaloacetate by the citrate lyase enzyme. The acetate formed can be expelled or part of it can be used in biosynthetic reactions, such as fatty acid synthesis (Arbogast and Henderson 1975; Goldberg and Eschar 1977). These facts could explain the decrease in acetic acid concentrations observed in CDM without Cit.

We concluded that the synthesis of PLA, the main antifungal compound produced by *Lact. plantarum* CRL 778 can be improved with higher amounts of Phe than Tyr. On the other hand, the production of both PLA and OH-PLA can be increased by cometabolism of glucose with Cit. This acid would act as an external electron acceptor increasing the $NAD(P)^+/NAD(P)H$ ratio and stimulating the transaminase reactions (Fig. 1). A synergistic effect was obtained when Cit was combined with α-KG instead of Glu. This later would regulate both transaminase reactions and GDH activity by negative feedback.

Acknowledgements

This work was financed by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) and Consejo de Investigación de la Universidad Nacional de Tucumán (CIUNT) from Argentina. A.D. is recipient of a doctoral fellowship from CONICET. We thank Dr Fernanda Mozzi and Dr Raúl R. Raya (Centro de Referencia para Lactobacilos, Argentina) for helpful suggestions.

References

Arbogast, L.Y. and Henderson, T.O. (1975) Effect of inhibition of protein synthesis on lipid metabolism in *Lactobacillus plantarum*. *J Bacteriol* **123**, 962–971.

Ardö, Y. (2006) Flavour formation by amino acid catabolism. *Biotechnol Adv* **24**, 238–242.

Boumerdassi, H., Monnet, C., Desmazeaud, M. and Corrieu, G. (1997) Effect of citrate on production of diacetyl and acetoin by *Lactococcus lactis* ssp. lactis CNRZ 483 cultivated in the presence of oxygen. *J Dairy Sci* **80**, 634–639.

Bradford, M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.

Corsetti, A., Gobbetti, M., Rossi, J. and Damiani, P. (1998) Antimould activity of sourdough lactic acid bacteria: identification of a mixture of organic acids produced by *Lactobacillus sanfrancisco* CB1. *Appl Microbiol Biotechnol* **50**, 253–256.

De Man, J.C., Rogosa, M. and Sharpe, M.E. (1960) A medium for the cultivation of Lactobacilli. *J Appl Bacteriol* **23**, 130–135.

Drinan, D.F., Tobin, S. and Cogan, T.M. (1976) Citric acid metabolism in hetero- and homofermentative lactic acid bacteria. *Appl Environ Microbiol* **31**, 481–486.

Ferain, T., Schanck, A.N. and Delcour, J. (1996) ^{13}C nuclear magnetic resonance analysis of glucose and citrate end products in an *ldh*L-*ldh*D double-knockout strain of *Lactobacillus plantarum*. *J Bacteriol* **178**, 7311–7315.

Gao, S. and Steele, J.L. (1998) Purification and characterization of oligomeric species of an aromatic amino acid aminotransferase from *Lactococcus lactis* subsp. *lactis* S3. *J Food Biochem* **22**, 197–211.

Gerez, C.L., Torino, M.I., Rollán, G.C. and Font de Valdez, G. (2009) Prevention of bread mould spoilage by using lactic acid bacteria with antifungal properties. *Food Control* **20**, 144–148.

Goldberg, I. and Eschar, L. (1977) Stability of lactic acid bacteria to freezing as related to their fatty acid composition. *Appl Environ Microbiol* **33**, 489–496.

Kieronczyk, S., Skeie, T., Langsrud, D. and Yvon, M. (2003) Cooperation between *Lactococcus lactis* and nonstarter lactobacilli in the formation of cheese aroma from amino acids. *Appl Environ Microbiol* **69**, 734–739.

Lapujade, P., Cocaign-Bousquet, M. and Loubiere, P. (1998) Glutamate biosynthesis in *Lactococcus lactis* subsp. *lactis* NCDO 2118. *Appl Environ Microbiol* **64**, 2485–2489.

Lavermicocca, P., Valerio, F., Evidente, A., Lazzaroni, S., Corsetti, A. and Gobbetti, M. (2000) Purification and characterization of novel antifungal compounds from the sourdough *Lactobacillus plantarum* strain 21B. *Appl Environ Microbiol* **66**, 4084–4090.

Lavermicocca, P., Valerio, F. and Visconti, A. (2003) Antifungal activity of phenyllactic acid against molds isolated from bakery products. *Appl Environ Microbiol* **69**, 634–640.

McSweeney, P.L. and Sousa, M.J. (2000) Biochemical pathways for the production of flavor compounds in cheeses during ripening: a review. *Le Lait* **80**, 293–324.

Pescuma, M., Hébert, E.M., Mozzi, F. and Font de Valdez, G. (2007) Hydrolysis of whey proteins by *Lactobacillus acidophilus*, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* grown in a chemically defined medium. *J Appl Microbiol* **103**, 1738–1746.

Rijnen, L., Delacroix-Buchet, A., Demaizieres, D., Le Quéré, J.L., Gripon, J.C. and Yvon, M. (1999) Inactivation of lactococcal aromatic aminotransferase prevents the formation of floral aroma compounds from aromatic amino acids in semi-hard cheese. *Int Dairy J* **9**, 877–885.

Rijnen, L., Courtin, P., Gripon, J.C. and Yvon, M. (2000) Expression of a heterologous glutamate dehydrogenase gene in *Lactococcus lactis* highly improves the conversion of amino acids to aroma compounds. *Appl Environ Microbiol* **66**, 1354–1359.

Rocken, W. (1996) Applied aspects of sourdough fermentation. *Adv Food Sci* **18**, 212–216.

Ryan, L.A.M., Dal Bello, F., Czerny, M., Koehler, P. and Arendt, E.K. (2009) Quantification of phenyllactic acid in wheat sourdough using high resolution gas chromatography-mass spectrometry. *J Agric Food Chem* **57**, 1060–1064.

Ryan, L.A.M., Zannini, E., Dal Bello, F., Pawlowska, A., Koehler, P. and Arendt, E.K. (2011) *Lactobacillus amylovorus* DSM 19280 as a novel food-grade antifungal

agent for bakery products. *Int J Food Microbiol* **146**, 276–283.

Schnürer, J. and Magnusson, J. (2005) Antifungal lactic acid bacteria as biopreservatives. *Trends Food Sci Technol* **16**, 70–78.

Spicher, G. (1983) Baked goods. In *Biotechnology: Food and Feed Productions with Microorganisms* ed. Reed, G. pp. 1–80. Weinheim: Verlag Chemie.

Ström, K., Sjögren, J., Broberg, A. and Schnürer, J. (2002) *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo (L-Phe–L-Pro) and cyclo (L-Phe–trans-4-OHL-Pro) and 3-phenyllactic acid. *Appl Environ Microbiol* **68**, 4322–4327.

Tanous, C., Kieronczyk, A., Helinck, S., Chambellon, E. and Yvon, M. (2002) Glutamate dehydrogenase activity: a major criterion for the selection of flavour-producing lactic acid bacteria strains. *Antonie Van Leeuwenhoek*, **82**, 271–278.

Torino, M.I., Taranto, M.P. and Font de Valdez, G. (2005) Citrate catabolism and production of acetate and succinate by *Lactobacillus helveticus* ATCC 15807. *Appl Microbiol Biotechnol* **69**, 79–85.

Valerio, F., Lavermicocca, P., Pascale, M. and Visconti, A. (2004) Production of phenyllactic acid by lactic acid bacteria: an approach to the selection of strains contributing to food quality and preservation. *FEMS Microbiol Lett* **233**, 289–295.

Verhue, W.M. and Tjan, F.S.B. (1991) Study of the citrate metabolism of *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* by means of 13C nuclear magnetic resonance. *Appl Environ Microbiol* **57**, 3371–3377.

Vermeulen, N., Gänzle, M.G. and Vogel, R.F. (2006) Influence of peptide supply and cosubstrates on phenylalanine metabolism of *Lactobacillus sanfranciscensis* DSM20451T and *Lactobacillus plantarum* TMW1.468. *J Agric Food Chem* **54**, 3832–3839.

Vermeulen, N., Gänzle, M.G. and Vogel, R.F. (2007) Glutamine deamidation by cereal-associated lactic acid bacteria. *J Appl Microbiol* **103**, 1364–5072.

Yvon, M. and Rijnen, L.A.C.T. (2001) Cheese flavour formation by amino acid catabolism. *Int Dairy J* **11**, 185–201.

Yvon, M., Thirouin, S., Rijnen, L., Fromentier, D. and Gripon, J.C. (1997) An aminotransferase from *Lactococcus lactis* initiates conversion of amino acids to cheese flavor compounds. *Appl Environ Microbiol* **63**, 414–419.

Yvon, M., Berthelot, S. and Gripon, J.C. (1998) Adding α-ketoglutarate to semi-hard cheese curd highly enhances the conversion of amino acids to aroma compounds. *Int Dairy J* **8**, 889–898.

Zhang, C. and Gänzle, M.G. (2010) Metabolic pathway of α-ketoglutarate in *Lactobacillus sanfranciscensis* and *Lactobacillus reuteri* during sourdough fermentation. *J Appl Microbiol* **109**, 1301–1310.

Zhang, C., Brandt, M.J., Schwab, C. and Gänzle, M.G. (2010) Propionic acid production by cofermentation of *Lactobacillus buchneri* and *Lactobacillus diolivorans* in sourdough. *Food Microbiol* **27**, 390–395.