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ORIGINAL ARTICLE

Gluten breakdown by lactobacilli and pediococci strains isolated from sourdough

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Abstract

Aims: To evaluate the growth and metabolic activity of lactobacilli and pediococci strains in a gluten base medium (GBM), formulated for a proper selection of proteolytic strains to be used in sourdough fermentation.
Methods and Results: Proteolytic activity by lactic acid bacteria (LAB) was evaluated by SDS-PAGE and by the amino acids released determined by reversed-phase high-performance liquid chromatography. Only 13 LAB (nine lactobacilli and four pediococci), among the 42 evaluated were able to utilize gluten as nitrogen source and to grow in GBM. *Pediococcus pentosaceus* CRL 797 showed a similar proteolytic activity to lactobacilli strains. In the majority of the cultures, basic amino acid group increased (*c.* 80% after 12 h incubation) mainly due to the release of ornithine, a flavour precursor of bread. Lysine, a limiting essential amino acid in wheat flour, increased by 150% in cultures of *P. pentosaceus* CRL 797.
Conclusions: This study allows selecting *P. pentosaceus* CRL 797 and *L. plantarum* CRL 759 as potential starter culture for type III sourdough fermentation. It is shown for the first time that pediococci strains isolated from sourdough are proteolytically active on gluten.
Significance and Impact of the Study: The physiological studies on gluten breakdown by LAB will contribute to the better selection of strains to produce breads with enhanced organoleptic characteristics.

Introduction

Sourdough fermentation is a traditional process for improving the bread quality and producing different wheat and rye breads (Thiele *et al.* 2002; Gänzle and Vogel 2003). Numerous genera and species of lactic acid bacteria (LAB) have been identified in sourdoughs: *Lactobacillus sanfranciscensis*, *Lactobacillus pontis*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus reuteri*, *Pediococcus pentosaceus*, *Leuconostoc mesenteroides*. The use of sourdough offers different advantages in baked food technology such as the pH decrease during fermentation, gas retention and resistance of the gluten network, inhibition of flour amylases, better achievement of aromatic bread flavour (Gobbetti 1998), and lengthening the mould-free period of bread (Lavermicocca *et al.* 2000; Gänzle and Vogel 2003).

Sourdoughs have been classified into three types based on the kind of technology applied for their production and the LAB microflora present (Böcker *et al.* 1995; Hammes and Gänzle 1998).

Four major classes of proteins are traditionally distinguished in wheat: albumins, globulins, gliadins and glutenins (Osborne 1924). Gluten proteins (gliadins and glutenins) are responsible for the unique ability of wheat to be made into bread, and they are determinative for the rheological properties of doughs and the texture properties of breads, contributing to the gas retention during dough fermentation (Gan *et al.* 1995). LAB play a key role during fermentation. Besides acidification, the proteolytic systems of LAB release amino acids and small peptides, which can promote growth and metabolic activities of other micro-organisms and also enhance flavour

development (Schieberle 1996; Gobbetti 1998) and rheological parameters (Martinez-Anaya 1996). Moreover, the study of proteolytic system of LAB contributes to the selection of starter culture strains to produce bread with low contents of allergenic peptides (Di Cagno *et al.* 2002).

In previous studies, homemade sourdoughs obtained in the mountain area of north-west Argentina were used as a source for the isolation of wild LAB strains. Besides heterofermentative lactobacilli, the prevalent lactic microbiota, pediococci strains were isolated as well.

The aim of this study was to evaluate the growth and metabolic activities of sourdough pediococci and heterofermentative lactobacilli strains in a gluten base medium (GBM) formulated for the proper selection of proteolytic strains.

Materials and methods

Micro-organisms and culture conditions

Forty-two strains of LAB isolated from homemade sourdough and belonging to the Culture Collection of Centro de Referencia para Lactobacilos (CERELA, Tucumán, Argentina) were studied: *L. brevis* (six), *Lactobacillus curvatus* (one), *Lactobacillus fermentum* (one), *L. plantarum* (nine), *L. reuteri* (five), *Pediococcus acidilacticci* (two), and *P. pentosaceus* (18). Cultures were grown in MRS broth (Britania, Buenos Aires, Argentina) at 37°C for 18 h; the cells were harvested by centrifugation (B-22M; International Equipment, Needham Heights, MA, USA) (9000 ***g***, 15 min at 4°C) washed twice with 10 mmol l^{-1} potassium phosphate buffer (pH 7·0), suspended in sterile distilled water and inoculated (1%, v/v) in GBM broth at pH 6·0. This medium was prepared with distilled water (w/v) as follows: gluten from wheat flour (Sigma, St Louis, MO, USA) (9%), glucose (2%), KH_2PO_4 (1%), K_2HPO_4 (1%) and Tween-80 (0·1%, v/v). This mixture was homogenized (The Virtis Company, Gardiner, NY, USA) and sterilized at 116°C for 10 min. Cell suspensions of each LAB strains (10^8 CFU ml^{-1}) were inoculated in GBM and incubated at 30°C for different time intervals. Cell counts of the cultures were performed by the pour plate dilution method in MRS agar (MRS plus 1·5% w/v agar) and plates were incubated at 37°C for 48 h. Results were expressed as log CFU ml^{-1}. The pH of the cultures was measured as well (Altronix-TPX1, Saen, Argentina).

Glucose and organic acids determinations

Glucose consumption and acetic acid formation by LAB strains were determined in the supernatants cultures (100 000 ***g***, 10 min, 4°C). Sugar and organic acid analysis were performed using commercial available kits (Boehringer-Mannheim, Germany). L-(+) and D-(−) lactic acid were estimated according to Gutmann and Wahlefeld (1974) and Gawehn and Bergmeryer (1974), respectively. The lactic acid yield ($Y_{p/s}$) was calculated by diving the mmol lactic acid produced by the mmol of glucose consumed.

Proteolytic activity

Proteolysis was evaluated in 24-h-old culture supernatant by SDS-PAGE (Laemmli 1970). The gels contained 12% (w/v) acrylamide and were stained with Coomassie Brilliant Blue R 250 (Sigma). The gels were scanned and analysed with QuantiScan 1·5 software (Biosoft, Ferguson, MO, USA). The percentages of hydrolysis were calculated on the basis of the average of the bands intensities of three gels, and the standard deviations were calculated. The protein concentration was determined according to Bradford (1976) using bovine serum albumin as standard.

Amino acids determination

The culture supernatants (0, 6, 12 and 24 h) were filtered through a 0·22-μm pore size filter (Sartorius AG-W-3400, Goettingen, Germany), derivatized in *o*-phthaldialdehyde derivates, and the amino acid concentrations was determined by reversed-phase high-performance liquid chromatography analysis. The chromatographic separation was carried out using an ISCO system (ISCO, Lincoln, NE, USA) on a Spherisorb ODS2 C18 column (4·6 × 250 mm); with an elution flow rate of 1·0 ml min^{-1}, and a fluorometer detector (excitation 340-nm emission 425). Identification and quantification of amino acids were carried out by comparison using a standard mixture of amino acids (Sigma).

Statistical analysis

All measurements were performed in three independent assays and mean values with standard deviation (SD) are presented. Data were compared by a one-way analysis of variance (ANOVA) and by Dunnett's *t*-test. Statistical significance ($P < 0·05$) was determined with Minitab-12 software (State College, PA, USA).

Results

Only 13 LAB strains of the 42 evaluated in this study were able to grow in GBM broth and utilize gluten as a sole nitrogen source: *L. brevis* CRL 781; *L. curvatus* CRL 760; *L. plantarum* CRL 759, CRL 769, CRL 775 and CRL 778; *L. reuteri* CRL 1098, CRL 1099 and CRL 1100, and

Table 1 Cell viability and glucose consumption by lactobacilli and pediococci strains during growth in GBM

Strains	Viable cell number (Δ log CFU ml^{-1})		Glucose consumed (mmol l^{-1})	
	12 h	24 h	12 h	24 h
Lactobacillus plantarum				
CRL 759	2·9 ± 0·04*	3·9 ± 0·10	6·1 ± 1·2	12·6 ± 1·7
CRL 769	2·2 ± 0·07	2·8 ± 0·03	8·9 ± 0·5	14·9 ± 0·8
CRL 775	2·7 ± 0·14	3·1 ± 0·01	8·2 ± 1·0	13·9 ± 1·8
CRL 778	3·3 ± 0·19	4·3 ± 0·08	10·3 ± 0·9	13·4 ± 0·9
Lactobacillus reuteri				
CRL 1098	1·9 ± 0·01	2·7 ± 0·05	8·5 ± 0·6	13·5 ± 0·7
CRL 1099	2·0 ± 0·91	3·5 ± 0·12	7·0 ± 0·4	11·5 ± 1·8
CRL 1100	2·5 ± 0·21	3·4 ± 0·20	7·3 ± 0·8	10·2 ± 1·0
Lactobacillus curvatus (CRL 760)	3·4 ± 0·23	3·7 ± 0·01	8·7 ± 0·6	13·9 ± 1·6
Lactobacillus brevis (CRL 781)	2·6 ± 0·33	3·3 ± 0·04	12·2 ± 1·5	14·9 ± 1·0
Pediococcus pentosaceus				
CRL 761	3·1 ± 0·11	3·5 ± 0.84	8·9 ± 0·4	13·3 ± 0·9
CRL 792	3·2 ± 0·11	3·7 ± 0·19	9·4 ± 1·3	13·3 ± 0·8
CRL 793	3·1 ± 0·15	3·6 ± 0·90	9·9 ± 0·5	13·8 ± 0·9
CRL 797	2·9 ± 0·29	3·4 ± 0·03	9·8 ± 0·8	13·9 ± 1·4

*Mean ± standard deviation of three independent measurements.

P. pentosaceus CRL 761, CRL 792, CRL 793 and CRL 797. In general, cell count of the strains increased by 3–4 log units while glucose consumption was between a range of 10–15 mmol l^{-1} after 24 h of incubation (Table 1). *Lactobacillus plantarum* CRL 778 (4·3 Δ log CFU ml^{-1}) and *L. reuteri* CRL 1098 (2·7 Δ log CFU ml^{-1}) showed the highest and lowest cell growth in GBM after 24 h, respectively. Regarding the organic acids production the pediococci strains (facultative heterofermentative LAB) produced only lactic acid (8–17 mmol l^{-1}) while the obligated heterofermentative lactobacilli produced lactic (11–22 mmol l^{-1}) and acetic (0·59–0·99 mmol l^{-1}) acids. $Y_{p/s} = 1{\cdot}7$ for *L. plantarum* CRL 775 and *P. pentosaceus* CRL 792 and CRL 761, while $Y_{p/s} = 1{\cdot}2$ for *L. reuteri* CRL 1098 and CRL 1100 after 12 h fermentation. Production of acetic acid was observed in 24-h cultures of *L. reuteri* CRL 1100 (0·99 mmol l^{-1}) and CRL 1099 (0·94 mmol l^{-1}). On the whole, the LAB strains mainly produced L(+) lactic acid (about 80%) with the exception of *L. plantarum* CRL 759 and *P. pentosaceus* CRL 792, which produced the D(−) isomer by 91% and 76%, respectively (Table 2). The culture pH decreased from 6·0 to 3·7 in most of the cultures after 24 h fermentation. These 13 strains were selected for further proteolysis assays.

With SDS-PAGE analysis the presence of nine gluten peptides (135, 83, 60, 49, 42, 35, 32, 27 and 9 kDa) were

Table 2 Organic acid production by lactobacilli and pediococci strains during the growth in GBM

Strains	Acetic acid (mmol l^{-1})		L-Lactic acid (mmol l^{-1})		D-Lactic acid (mmol l^{-1})	
	12 h	24 h	12 h	24 h	12 h	24 h
CRL 759	0·0 ± 0·0*	0·0 ± 0·0	0·89 ± 0·6	1·24 ± 0·7	5·0 ± 0·5	10·2 ± 1·2
CRL 769	0·33 ± 0·01	0·50 ± 0·02	5·7 ± 1·0	11·3 ± 1·0	4·2 ± 0·7	10·4 ± 1·8
CRL 775	0·16 ± 0·02	0·50 ± 0·04	7·6 ± 0·5	11·2 ± 0·5	6·3 ± 0·6	10·3 ± 1·5
CRL 778	0·58 ± 0·05	0·59 ± 0·06	5·4 ± 0·7	7·0 ± 0·9	4·9 ± 0·8	9·0 ± 1·6
CRL 1098	0·67 ± 0·08	0·83 ± 0·08	5·0 ± 0·9	10·2 ± 1·6	4·8 ± 0·5	9·4 ± 1·0
CRL 1099	0·41 ± 0·04	0·94 ± 0·05	4·5 ± 0·5	8·0 ± 0·5	3·6 ± 0·8	6·0 ± 0·9
CRL 1100	0·67 ± 0·05	0·99 ± 0·04	4·4 ± 0·9	5·1 ± 0·8	5·2 ± 0·9	6·6 ± 0·4
CRL 760	0·0 ± 0·0	0·0 ± 0·0	10·2 ± 0·8	16·5 ± 0·4	0·3 ± 0·3	0·3 ± 0·2
CRL 781	0·33 ± 0·03	0·67 ± 0·02	8·2 ± 0·9	10·2 ± 0·8	0·0 ± 0·0	1·3 ± 0·6
CRL 761	0·0 ± 0·0	0·0 ± 0·0	10·2 ± 0·7	10·6 ± 0·5	5·0 ± 0·4	7·3 ± 0·6
CRL 792	0·0 ± 0·0	0·0 ± 0·0	4·1 ± 0·5	5·0 ± 0·8	10·9 ± 0·6	11·2 ± 0·5
CRL 793	0·0 ± 0·0	0·0 ± 0·0	9·0 ± 0·9	5·4 ± 0·6	1·9 ± 0·5	2·5 ± 0·5
CRL 797	0·0 ± 0·0	0·0 ± 0·0	10·4 ± 0·2	11·6 ± 0·3	0·0 ± 0·4	5·1 ± 0·1

*Mean ± standard deviation of three independent measurements.

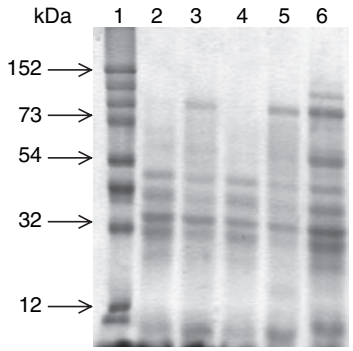

Figure 1 SDS-PAGE of peptides present in lactic acid bacteria cultures in MBG. Lane 1: molecular weight markers; lane 2: *Pediococcus pentosaceus* CRL 797; lane 3: *Lactobacillus plantarum* CRL 759; lane 4: *Lactobacillus brevis* CRL 781; lane 5: *Lactobacillus plantarum* CRL 778; lane 6: GBM control.

observed in GBM (control) (Fig. 1). All strains completely hydrolysed the peptides of 135 and 83 kDa except for *L. plantarum* CRL 778, which displayed only a slight degradation (3%) (Table 3). Lactobacilli strains showed higher proteolytic activity on peptides between 42 and 27 kDa with respect to pediococci, except *P. pentosaceus* CRL 797, which showed a similar activity than lactobacilli. New peptide fractions of 16 kDa were detected in cultures of *L. brevis* CRL 781, *P. pentosaceus* CRL 797 and other 7-kDa peptide in cultures of *P. pentosaceus* CRL 761, *L. plantarum* CRL 759 and CRL 769 after 24 h fermentation which were not observed at zero time (data not shown).

The modifications in the amino acid content in GMB cultures due to the growth of LAB strains is shown in Fig. 2. For a better analysis of the results obtained, individual amino acids were grouped according to their chemical structure into: basic (ornithine, arginine, lysine, histidine), aliphatic (leucine, alanine, valine, glycine, isoleucine), aromatic (trytophan, phenylalanine, tyrosine), dicarboxylic (aspartic acid, glutamic acid, asparagine), and hydroxyl sulfur (serine, methionine, treonine). The concentration of each group was calculated from the sum of the individual amino acid concentrations. The uninoculated GBM broth (control) showed no significant ($P < 0{\cdot}05$) differences after 24 h incubation.

In general, a decrease in all amino acid groups was observed after 12 h incubation except for the basic amino acid group, which increased (80%) respect to the control (Fig. 2). This increase was mainly observed in cultures of *L. reuteri* CRL 1100, *L. plantarum* (CRL 759, CRL 775) and *L. brevis* (CRL 781) at 12 h, and in *P. pentosaceus* strains and *L. reuteri* CRL 1099 cultures at 6 h. Lengthening the fermentation period up to 24 h resulted in the reduction of the basic amino acid group. A decrease in certain amino acid concentrations was observed during the growth of pediococci strains in GBM. The variations of the other amino acid group were not significant.

During fermentation some amino acids were consumed while others were increased due to the LAB metabolism. The aroma precursors glycine, treonine, ornithine and serine showed the main increases (up to 90-folds) after 12 h fermentation (data not shown).

Discussion

Thirteen LAB strains (nine lactobacilli and four pediococci) of 42 screened were selected for their ability to grow in

Table 3 Degradation of gluten peptides by lactobacilli and pediococci strains in GBM cultures (24 h)

	Gluten peptides (kDa)								
Strains	135	83	60	49	42	35	32	27	9
CRL 759	100 ± 0·0*†	78·8 ± 7·3	61·8 ± 9·7	73·8 ± 9·2	67·1 ± 0·3	66·4 ± 8·8	64·1 ± 8·0	63·2 ± 8·2	96·3 ± 5·1
CRL 769	100 ± 0·0	100 ± 0·0	21·7 ± 5·4	56·9 ± 9·7	54·7 ± 9·9	66·9 ± 6·9	57·6 ± 4·0	65·3 ± 9·9	100 ± 0·0
CRL 775	100 ± 0·0	95·9 ± 4·9	0·9 ± 0·6	40·6 ± 8·7	40·1 ± 9·8	34·5 ± 6·2	44·0 ± 4·5	41·3 ± 9·9	69·2 ± 9·8
CRL 778	100 ± 0·0	3·0 ± 1·3	30·1 ± 6·6	63·6 ± 4·6	66·1 ± 5·4	62·7 ± 4·7	61·4 ± 6·2	71·8 ± 2·8	89·7 ± 0·5
CRL 1098	100 ± 0·0	100 ± 0·0	20·7 ± 7·2	62·5 ± 3·1	67·7 ± 4·3	49·0 ± 2·4	57·5 ± 2·3	67·2 ± 5·2	86·0 ± 4·2
CRL 1099	100 ± 0·0	100 ± 0·0	56·7 ± 2·8	68·9 ± 0·4	68·2 ± 1·9	57·4 ± 1·1	60·5 ± 1·9	67·9 ± 2·1	24·6 ± 0·2
CRL 1100	100 ± 0·0	100 ± 0·0	21·2 ± 5·4	62·8 ± 0·9	58·3 ± 2·7	56·3 ± 6·7	54·6 ± 4·9	71·9 ± 2·3	79·6 ± 9·9
CRL 760	100 ± 0·0	93·8 ± 0·5	31·3 ± 6·9	65·8 ± 2·2	66·9 ± 4·1	56·9 ± 2·9	64·3 ± 2·0	74·9 ± 5·9	86·7 ± 1·5
CRL 781	100 ± 0·0	100 ± 0·0	78·9 ± 9·9	74·7 ± 9·5	70·6 ± 5·1	66·9 ± 4·5	66·0 ± 9·8	74·5 ± 5·3	49·9 ± 9·6
CRL 761	100 ± 0·0	100 ± 0·0	74·5 ± 5·1	68·9 ± 1·9	59·5 ± 1·0	39·9 ± 3·6	38·3 ± 2·7	39·0 ± 9·7	100 ± 0·0
CRL 792	100 ± 0·0	100 ± 0·0	57·4 ± 6·6	67·7 ± 4·1	44·7 ± 3·5	37·3 ± 2·7	29·8 ± 0·1	35·9 ± 7·2	9·0 ± 8·3
CRL 793	100 ± 0·0	100 ± 0·0	67·9 ± 3·9	61·9 ± 0·5	47·9 ± 5·1	44·2 ± 3·2	29·5 ± 5·3	41·6 ± 9·8	9·2 ± 7·8
CRL 797	100 ± 0·0	100 ± 0·0	30·3 ± 1·9	67·9 ± 5·6	73·5 ± 4·4	74·8 ± 8·9	67·3 ± 5·1	63·2 ± 6·9	81·8 ± 9·9

*Degradation (%) of peptides by lactic acid bacteria with respect to control (un-inoculated GBM broth).
†Mean ± standard deviation of three independent measurements.

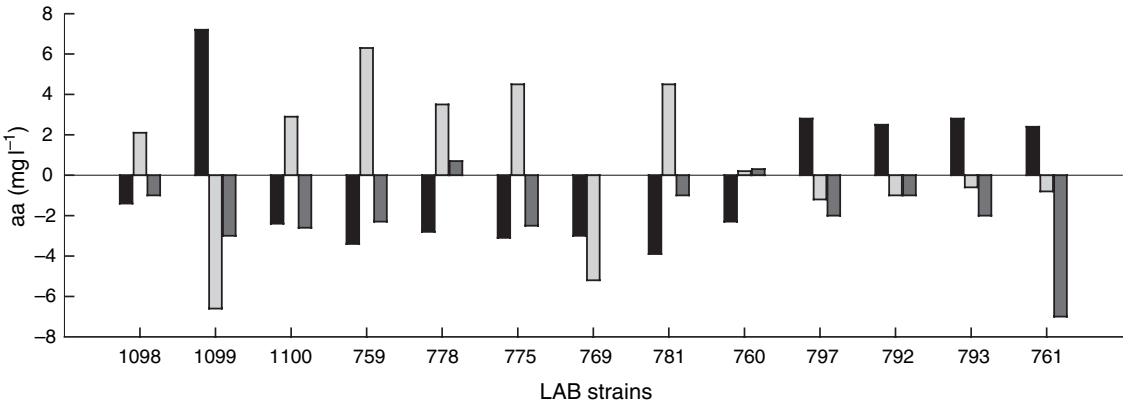

Figure 2 Release and consumption of basic amino acids by lactobacilli and pediococci strains during growth in gluten-based medium. ■, 0–6 h; □, 6–12 h; ■, 12–24 h.

GBM broth with gluten as the sole nitrogen source. The cell growth in this medium was similar for both microbial groups. *Lactobacillus plantarum* CRL 759 showed a low consumption of glucose, although its growth was similar to that observed in *L. plantarum* CRL 778. The organic acid production of both strains was different. During sourdough fermentation, lactic and acetic acids combine with ethanol and other products of dough fermentation and enhance the perception of aroma (Gobbetti *et al.* 1995; Schieberle 1996). The production of L(+) or D(−) lactic acid or the D–L racemic mixture was both, time- and strain-dependent. However, most of the cultures produced the L(+) isomer, which is a desirable characteristic for starter strains to be used in food fermentation (Caplice and Fitzgerald 1999).

Changes in the peptide profiles were observed in GBM cultures at different incubation periods. *Pediococcus pentosaceus* CRL 797 was the most proteolytic strain among pediococci, and displayed a similar activity on gluten to that of *L. plantarum* CRL 759. Wehrle *et al.* (1999) reported a similar rate of gluten hydrolysis for two pediococci strains of meat origin compared with *Lactobacillus sanfrancisco* 1 strain. Our results show the first evidence of pediococci strains isolated from sourdough, which are proteolytically active on gluten.

The ability of *L. plantarum* CRL 759 to hydrolyse (73%) a synthetic peptide homologous to the α-gliadin 31–43 amino sequences was seen previously (Rollán *et al.* 2005) indicating that selected sourdough LAB strains might reduce gluten-allergen compounds during sourdough fermentation. Proteolysis by LAB in sourdough is also important for increasing the amino acid concentration in dough. *Lactobacillus reuteri* CRL 1099 and pediococci strains increased the basic amino acid concentration in GBM cultures after 6 h incubation mainly due to an increase in the amount of ornithine. The slow removal of basic amino acid is an important selection criteria for starter cultures as they are the most reactive compounds during baking (El-Dash 1971). Ornithine is considered one of the key flavour precursors in wheat bread (Gassenmeier and Schieberle 1995). The pattern increase of the aroma precursors amino acid during LAB growth in GBM broth was similar to those reported by other authors (Schieberle 1990, 1996; Gobbetti 1998) during sourdough fermentation. The net decrease suggests that the amino acids are utilized as metabolites to a greater extent than the amount release by proteolysis.

Flours obtained from cereals and used as human or animal food are deficient in amino acids (Kamel and Stauffer 1993), which are essential for humankind. We observed an increase in essential amino acids (treonine, valine, lysine and phenylalanine) in LAB cultures in GBM medium. Lysine, an essential amino acid that is limiting in wheat flour, increased by 150% in cultures of *P. pentosaceus* CRL 797 after 6 h of fermentation. In this sense, the addition of lysine-producing pediococci to wheat dough might be considered as an alternative to increase this amino acid concentration in wheat bread.

Type III sourdough mostly contains LAB that are resistant to drying and are able to survive in that form, e.g. facultative heterofermentative *P. pentosaceus* and *L. plantarum* strains (Stolz and Böcker 1996). The results of this work show that the *P. pentosaceus* CRL 797 and *L. plantarum* CRL 759 strains display interesting properties to be used as starter cultures for type III sourdough. Research work is being currently carried out to evaluate mixed cultures in bread dough fermentations.

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