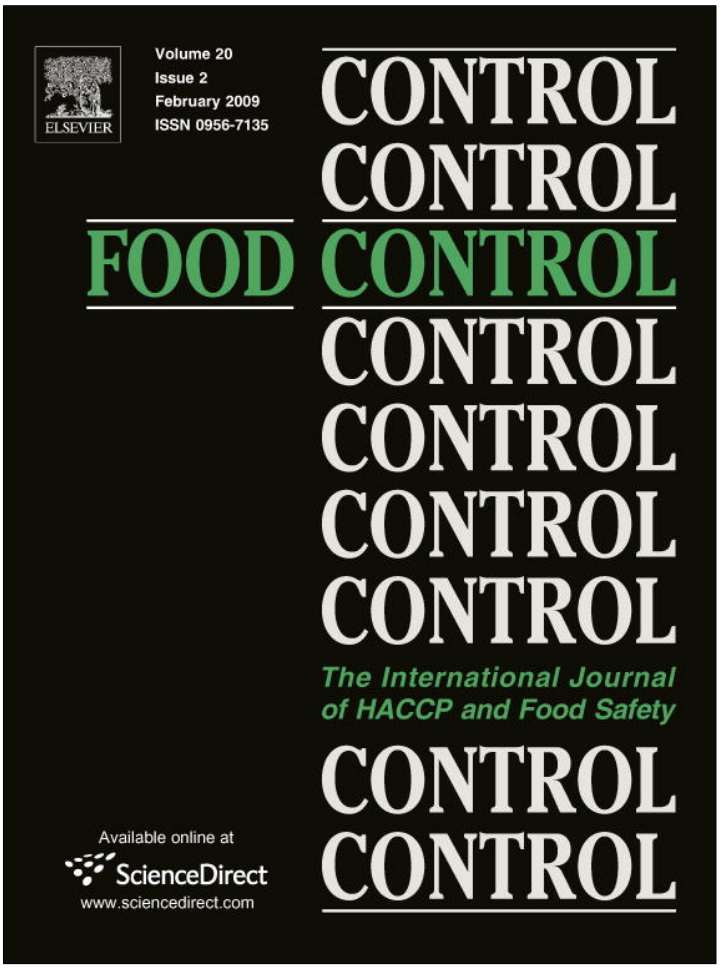

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Prevention of bread mould spoilage by using lactic acid bacteria with antifungal properties

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ABSTRACT

The ability of lactic acid bacteria (LAB) to inhibit *Aspergillus*, *Fusarium*, and *Penicillium*, the main contaminants in bread, was evaluated. Only four strains (*Lactobacillus plantarum* CRL 778, *Lactobacillus reuteri* CRL 1100, and *Lactobacillus brevis* CRL 772 and CRL 796) from 95 strains tested displayed antifungal activity. The major antifungal compounds were acetic and phenyllactic acids. The fermentation quotient (FQ = 2.0) and the leaven volume (80 cm^3) of doughs with LB and yeasts were higher than doughs without LB. The inclusion of antifungal LAB strains in the starter culture allowed a reduction in the concentration of calcium propionate by 50% while still attaining a shelf life similar to that of traditional bread containing 0.4% CP.

1. Introduction

Spoilage of bakery products is mainly due to fungal growth; the major species involved are *Aspergillus*, *Fusarium*, and *Penicillium*. In addition to the great economic losses derived from the presence of mould, another concern is the potential mycotoxin production that may cause public health problems (Legan, 1993). Contamination by moulds can be prevented by irradiating the goods with infrared rays or microwaves, by using modified atmospheres during packaging, or by adding chemical preservatives such as propionic acid (Gould, 1996). The maximal concentration of propionate that is allowed for packaged sliced breads by the Argentine Alimentary Code (AAC) is 0.4% (wt/wt). Most bakeries in the country used this upper limit concentration for the conservation of bread.

In recent years, bio-preservation (the use of microorganisms and/or their metabolites to prevent spoilage and to extend the shelf life of foods) (Stiles, 1996) has gained increasing interest due to consumers' demands. Lactic acid bacteria (LAB) as bio-preservation organisms are of particular interest: they have been used for centuries as starter cultures in the food industry and are able to produce different kind of bioactive molecules, such as organic acids, fatty acids, hydrogen peroxide and bacteriocins. The antifungal activity of LAB is documented (Hassan & Bullerman, 2008; Magnusson, Ström, Roos, Sjögren, & Schnürer, 2003; Sjögren, Magnusson, Broberg, Schnürer, & Kenne, 2003). Authors also reported that sourdough LAB strains are able to inhibit mould growth in bakery (Corsetti, Gobbetti, Rossi, & Damiani, 1998; Lavermicocca et al., 2000). Unfortunately, these studies rely on "in vitro" assays and the applicability of these strains in dough fermentations has not always been considered nor has the quality of the final product been described.

The present study was undertaken to evaluate the potentiality of LAB strains of different origin to inhibit mould growth. The metabolites involved were identified, and the efficacy of antifungal LAB as bio-preservative in bread manufacture was evaluated.

2. Materials and methods

2.1. Microorganisms and culture conditions

The 95 LAB strains (CRL) used in this study are shown in Table 1. The mould strains *Aspergillus* (*A.*) *niger* C_H 101, *Penicillium* (*P.*) sp. C_H 102 and *Fusarium* (*F.*) *graminearum* C_H 103 (previously isolated from contaminated bread) were used as indicators in the bioassays. All strains (CRL and C_H) were obtained from the Culture Collection of the Centro de Referencia para Lactobacilos (CERELA-CONICET) Tucumán—Argentina.

The LAB cultures were grown in wheat flour hydrolysate (WFH) broth (pH 6.0) at 37 °C for 48 h (Gobbetti, 1998). The cell-free supernatants (CFS) were obtained by centrifugation (9000*g* for 10 min at 4 °C), sterilized by filtration (0.2 μm filters) (Sartorius AG, Goettingen, Germany) and used in the antifungal assays. The moulds were grown on Czapek-Dox agar (1.5%) with 0.5% yeast extract (CZ) at 25 °C for 7 days. The conidia were collected in sterile

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Table 1
Lactic acid bacteria evaluated in this study

LAB strains (CRL)		Source
Lactobacillus acidophilus	1063, 1064, 1065 1072, 1071	Dairy products Vegetal
Lactobacillus brevis	**772**, 780, 781, **796** 376	Sourdough Dairy products
Lactobacillus bulgaricus	142, 406	Dairy products
Lactobacillus casei	59, 69, 75, 87, 143, 168, 206, 225, 234, 237, 239, 295, 429, 645, 687	Dairy products
Lactobacillus curvatus	760 689,1000	Sourdough Dairy products
Lactobacillus fermentum	763 220, 236, 250, 251, 345	Sourdough Dairy products
Lactobacillus paracasei	208, 717, 997, 1501	Dairy products
Lactobacillus plantarum	759, 769, 775, **778**, 783, 785, 788, 794, 795 92, 93, 95, 99, 101, 107, 110, 121, 130, 133, 136, 137, 140, 142, 182, 183, 223, 358, 428, 681, 768, 948, 1073 1081, 1090	Sourdough Dairy products Vegetal
Lactobacillus reuteri	1097, 1098, 1099, **1100**, 1101	Sourdough
Lactobacillus rhamnosus	186, 201, 932, 981	Dairy products
Pediococcus acidilactici	767, 770	Sourdough
Pediococcus pentosaceus	761, 771, 773, 779, 791, 792, 793, 797 908 1046	Sourdough Vegetal Dairy products

The evolve numbers corresponding to positive strains.

Tween 80 at 0.05% (vol/vol) and counted at the microscope in a haemocytometer chamber to adjust the concentration at 10^4 conidia/ml in sterile water.

2.2. Antifungal assay

2.2.1. Conidial germination assay

The inhibition of conidial germination was performed using the Microtitre Plate Well Assay (Lavermicocca, Valerio, & Visconti, 2003). Conidial suspensions (10 μl) containing 10^4 conidia/ml were added to 190 μl of the CFS. The assays were performed in sterile multiwell microdilution plates (96 sterile wells) (Corning Incorporated, USA). Conidial germination was determined after 48 h at 30 °C by measuring the optical density (OD_{580}) with a spectrophotometer ($VERSA_{max}$, Molecular Devices, USA). The conidial inoculated WFH broth was used as control of germination. The antifungal activity of the LAB strains was expressed as the germination reduction (%) as measured by OD_{580}, compared to the control after 48 h of incubation at 30 °C. An inhibition of conidial germination more than 20% was considered as positive.

2.2.2. Inhibition of the mycelial growth

Aliquots (70 μl) of CFS were introduced into wells (5-mm diameter) cut in the CZ agar and allowed to diffuse for 2 h at room temperature. The mycelia plugs (cut from the growing edge of a 7-days fungal culture) were placed in the center of a Petri plate, and the plates were further incubated at 30 °C for 48 h for mould growth. The antifungal activity of the CFS was evaluated by measuring the inhibition zone of the mould growth.

2.3. Effects of pH and proteolytic enzymes on antifungal activity

The antifungal activity of the CFS (pH 3.5) after exposure to high temperature (100 °C for 10 min), neutralization to pH 6.0 (with 0.1 M NaOH), or treatment with proteinase K (Sigma, 2000 U/ml CFS) at pH 7.6 (45 °C for 60 min) was determined by using either the agar well diffusion assay or the microtiter plate assay. Before evaluating the antifungal activity, the pH of the CFS treated with proteinase K (pH 7.6) was adjusted to 3.5.

2.4. Determination of the antifungal compounds

The organic acids present in the 48-h CFS were determined by High Performance Liquid Chromatography (HPLC) and enzymatic methods. Lactic and phenyllactic acids were determined by HPLC (ISCO 2350 model) using an ion-exclusion Aminex 87 H column (300 mm × 78 mm, Bio Rad) under the following conditions: mobile phase (2.5 mM) H_2SO_4; flow rate 0.6 ml/min; temperature of column 45 °C. An UV (210 nm) detector (ISCO V 4 model) connected to the software (Peak Simple II) for data analyses was used. Acetic acid was determined by a commercial UV-test (Boehringer Mannheim, Germany).

The fermented dough samples (10 g) were homogenized with distilled water (90 ml) in a homogenizer (The Virtis Company, Gardiner, New York 12525) and centrifuged at 8000*g* for 10 min. The organic acids concentrations were determined in the dough aqueous extracts supernatants.

2.5. Antifungal activity of organic acids

The antifungal activity of the organic acids was determined at pH 6.0 and 3.5. These values corresponded to the pH of the LAB cultures (CFS) at zero time and after 48 h fermentation. Different concentrations of lactic (10–300 mM), acetic (0.5–30 mM), phenyllactic (0.01–10 mM) and propionic acids (0.1–20 mM) (Sigma Chemical Company, St. Louis, MO, USA) were separately added to the WFH broth, and the pH value was adjusted to pH 3.5 and 6.0 with 0.1 M NaOH or 0.1 M H_3PO_4. The inhibitory effect of the organic acids on conidia germination was determined and compared by means of the 50% Minimal Inhibitory Concentration (MIC_{50}) calculated as reported by Cabo, Murado, Gonzalez, and Pastoriza (1999). The MIC_{50} was defined as the lowest concentration (mM) of organic acid that produces 50% inhibition of conidia germination after 48-h incubation compared to the control (conidia germination in WFH without organic acids addition).

2.6. Dough fermentation and bread manufacture

The antifungal LAB strains were inoculated (1%) in MRS broth and incubated for 16 h at 37 °C. Cells were harvested by centrifugation at 8000*g* for 10 min, washed twice, and resuspended in sterile distilled water. This cell suspension (9 log cfu/ml) was used as starter culture together with *Saccharomyces* (*S.*) *cerevisiae* (7 log cfu/g) in bread manufacture (YLB-dough). The dough (Y) without LAB was used as control. The doughs were prepared as follows: 1000 g commercial wheat flour 000 type (71.1% carbohydrates, 2.8% fiber, and 10% protein), 10 g NaCl, 20 g sucrose and 500 ml tap water. Calcium propionate (CP), a chemical additive usually employed in bakery for bread conservation, was used from 0.1% to 0.4%, which is the maximal concentration allowed by the AAC.

Both doughs (Y and YLB) were individually placed in aluminium pans and fermented at 30 °C for 2 h. The LAB and yeast viability was determined by the plate dilution method using MRS agar (Lactobacillus Broth, Oxoid) with cycloheximide (200 μg/ml) and yeast peptone dextrose agar medium (YPD) with chloramphenicol (500 μg/ml), respectively. The plates were incubated at 30 °C (yeast) and at 37 °C (LAB) under conditions of microaerophilia for 48 h. Results were expressed as log cfu/g. The dough pH was measured with a pH meter (Altronix-TPX1 pH/mV-Meter, Argentina). A portion of dough (30 g) was fermented in a graduated tube; the leaving power was determined as V (cm^3) = $V_f - V_i$, where V_f and V_i are the final and the initial volume, respectively. After fermentation, the doughs were baked in a batch oven (180 °C during 20 min) and the bread loaves were cooled at room temperature for 90 min. The fermentation quotient (FQ = lactate/acetate molar ratio) was used as the index of bread quality (optimal FQ = 2–4) (Gobbetti, 1998).

The bread loaves were surface sprayed with a conidial suspension (10^4 conidia/ml, 1 ml per 100-g loaf) of *A. niger* C_H 101, packed into polyethylene bags, and stored at 30 °C. The packaged loaves were observed daily for the manifestation of visible mould growth, and the shelf life was defined as the time (in days) for moulds to become visible in the packaged loaves.

2.7. Statistical analysis

Results of three independent assays are presented as mean values ± standard deviation (SD). Data were compared by the one way Analysis of Variance (ANOVA) and by Dunnett *t*-test. Statistical significance ($p < 0.05$) was assessed with the Minitab-12 software.

3. Results

3.1. Inhibition of mould growth

From the total (95 strains) homo- and hetero-fermentative LAB tested, the majority of them (63 strains) were able to inhibit the conidial germination showing high (⩾70%) antifungal activity on *F. graminearum* C_H103, moderate (40–70%) activity on *Penicillium* sp. C_H 102, and low (<40%) activity on *A. niger* C_H 101. Within these strains, only *Lactobacillus plantarum* CRL 778 and *L. brevis* CRL 796 inhibited the mycelial growth of *A. niger* C_H 101 while *F. graminearum* C_H103 was inhibited by *L. plantarum* CRL 778, *L. reuteri* CRL 1100, and *L. brevis* (strains CRL 772 and CRL 796). No action on the vegetative growth of *Penicillium* sp. C_H 102 by any of the LB strains assayed was observed.

From these results, the four lactobacilli strains were regarded as antifungal (LAB+) strains and selected for further studies. The inhibitory activity of the bacterial CFS on mycelial growth and conidial germination was not changed after either heating or treatment with Proteinase K while it was removed after neutralization with sodium hydroxide indicating the acidic nature of the metabolites involved.

The organic acid production by the LAB+ strains after 48 h fermentation in medium WFH is shown in Table 2. Lactic acid was found in a broad concentration range from 14 mM (*L. reuteri* CRL 1100) to 149 mM (*L. plantarum* CRL 778) while acetic acid was about 2.2–2.9 mM for these strains, and 9.4–11.4 mM for *L. brevis* (strains CRL 792 and 772, respectively). Phenyllactic (PLA) was only detected (0.02–0.03 mM) in the CFS of *L. plantarum* CRL 778 and *L. reuteri* CRL 1100.

To know whether the organic acids profile (and/or the final pH) play a role on the inhibition of mycelial growth, the CFS of LAB+ and LAB− (hetero-fermentative strains without effect on mould growth) was compared. *L. reuteri* CRL 1100 and *L. brevis* (strains CRL 792 and 772) showed a lactic:acetic acid ratio of 4.8, 5.5, and 6.1, respectively, while *L. plantarum* CRL 778 gave a ratio of 66.5. In all cases, the final pH was 3.5. In contrast, the LAB− strains showed a similar range for lactic:acetic acid ratio but higher pH values (>4.5) after fermentation.

To determine the effect of pH on the antifungal efficacy (expressed as MIC_{50}) of the organic acids produced by the LAB+ strains, commercial PLA, acetic and lactic acids were evaluated at pH 6.0 (initial WFH culture pH) and at pH 3.5 (final pH after fermentation). Propionic acid (commonly used as additive in bakery) was also tested. Results are shown in Table 3. The efficacy of the organic acids, which was 2–85-folds enhanced at low pH (3.5) was dependent on the type of acid and the fungi strain evaluated. Thus, the MIC_{50} of PLA on *A. niger* C_H 101 and *F. graminearum* C_H 103 were 30- and 85-folds lower, respectively, at pH 3.5 respect to pH 6.0. PLA was the most effective (MIC_{50} 0.02–6.0 mM) against all fungi strains compared to propionic acid (MIC_{50} 0.1–12 mM), acetic acid (MIC_{50} 0.3–120 mM) and lactic acid (MIC_{50} 2.5–300 mM).

3.2. Dough fermentation and bread manufacture

The LAB+ strains were evaluated both as single and mixed starter culture (together with the commercial yeast) in bread manufacture. The bread obtained with each single culture had a short shelf life (lower than 3 days) and a fermentation quotient (FQ) >4. From these results, a mixed starter culture (LB) was formulated by using the four strains at the ratio 1:1:1:1. Results obtained for doughs and breads elaborated with the commercial yeast alone (Y) and the yeast together with the starter LB (YLB) are presented in Table 4. After 4 h fermentation the YLB-doughs showed higher content in lactic (19.9 mmol/kg) and acetic (10 mmol/kg) acid and reached lower (5.0) pH values compared to the traditional Y doughs (pH 5.7). The YLB-breads displayed values (2.0) of FQ within the standards of high bread quality (optimum 1.5–3.0) while Y-breads and Y-CP-breads showed FQ values of 12.5 and 6.0, respectively. The addition of LB starter culture improved the leavening power (80 cm^3) of YLB doughs without affecting the yeast growth (0.4 log cfu/g dough) (data not shown).

The starter LB allowed extending the shelf life of YLB-breads to 5 days compared to Y-breads without CP (2 days). A similar conservation effect was obtained by acidifying the Y-doughs with

Table 2
Production of lactic, acetic, and phenyllactic acids by antifungal LAB strains after 48 h fermentation in WFH medium

Strains (CRL)	Organic acids (mM)		
	Lactic	Acetic	Phenyllactic
Lactobacillus reuteri 1100	14.2	2.92	0.03
Lactobacillus plantarum 778	148.8	2.24	0.02
Lactobacillus brevis 772	57.7	9.40	0.00
Lactobacillus brevis 796	63.2	11.40	0.00

Table 3
Inhibitory effect (MIC_{50}) of organic acids on conidia germination at pH 3.5 and 6.0

Mould (C_H)	Lactic acid		Acetic acid		Phenyllactic acid		Propionic acid	
	pH 3.5	pH 6.0	pH 3.5	pH 6.0	pH 3.5	pH 6.0	pH 3.5	pH 6.0
Penicillium sp. 102	80[a]	160	0.9	24	0.03	0.06	0.5	12
Fusarium graminearum 103	2.5	50	0.3	4.0	0.02	0.06	0.1	0.8
Aspergillus niger 101	180	>300	18.0	120	0.07	6.0	0.5	6.0

[a] MIC_{50} = defined as the lowest concentration (mM) of organic acid that produces a 50% inhibition of conidia germination after 48 h incubation in WFH broth compared to control (conidia germination in WFH without organic acids addition).

Table 4
Organic acids content, pH value and shelf life of Y-breads and YLB-breads

Dough	pH	Organic acids (mmol/kg)				Shelf life (days)
		Lactic	Acetic	Phenyllactic	Propionic	
Without CP						
Y	5.7	2.5 (0.04)[b]	0.2 (0.02)	0.0	–	2
YLB	5.0	19.2 (1.30)	10.0 (3.65)	0.2 (0.01)	–	5
With CP						
Y-$CP_{0.2}$[a]	5.5	2.4 (0.05)	0.3 (0.11)	0.0	12.9 (2.4)	4
Y-$CP_{0.3}$	5.5	2.4 (0.05)	0.4 (0.16)	0.0	19.0 (3.6)	5
Y-$CP_{0.4}$	5.5	2.3 (0.05)	0.3 (0.11)	0.0	26.4 (5.0)	8
YLB-$CP_{0.2}$	5.0	19.9 (1.34)	9.9 (3.50)	0.2 (0.01)	12.9 (5.0)	8

Y (commercial yeast); LB (LAB starter culture); CP (calcium propionate).
[a] The subscript indicates the concentration (%) of calcium propionate.
[b] Undissociated organic acid fraction (within parenthesis) calculated by the Henderson–Hasselbach equation.

a mixture of commercial PLA, lactic, and acetic acids at the concentrations produced by the LB starter. This preservation effect was lost after dough neutralization (data not shown).

To determine the antifungal efficacy of the starter LB, the shelf life of YLB-breads was compared with that of Y-breads containing 0.2%, 0.3% or 0.4% CP (wt/wt) which corresponds to 12.9, 19.0 and 26.4 mmol/kg, respectively. A 5-days shelf life was obtained by using either the starter LB or 0.3% CP (Y-$CP_{0.3}$) while breads elaborated with lower concentrations of CP were spoiled within 48 h. The maximal shelf life (8-days) was obtained for Y-breads containing 0.4% CP (Y-$CP_{0.4}$) or when combining 0.2% CP and the starter LB (YLB-$CP_{0.2}$ bread).

4. Discussion

Fungal spoilage is the main cause of substantial economic losses in packaged bakery products and might also be regarded as sources of mycotoxins, involving public health problems (Legan, 1993). In this context, LAB may be considered as an alternative for bio-conservation. In this study, 95 LAB strains (isolated from different sources) were screened for antifungal activity against *A. niger* C_H 101, *Penicillium* sp. C_H 102 and *F. graminearum* C_H 103, the most common contaminants in bread. Results obtained evinced that the antifungal ability of LAB was dependent on the LAB strain and the fungus species. From the total (95 strains) homo- and hetero-fermentative LAB tested, the majority of them (63 strains) were able to inhibit the conidial germination, while only four strains inhibited the mycelial growth. The conidia germination is the growth stage that is most sensitive to inhibition. On the other hand, comparison of the potency of a compound as an inhibitor of germination with its activity in a mycelial growth assay can provide preliminary information on its mode of action (Slawecki, Ryan, & Young, 2002).

The antifungal effect of *L. reuteri* CRL 1100, *L. brevis* (CRL 796 and 772) and *L. plantarum* CRL 778 would be related to both the nature of the organic acid produced – lactic, acetic and PLA – and the low pH (3.5) reached after fermentation. The production of PLA would also contribute to the antifungal activity of *L. reuteri* CRL 1100 and *L. plantarum* CRL 778 (Valerio, Lavermicocca, Pascale, & Visconti, 2004; Vermeulen, Gänzle, & Vogel, 2006). PLA is regarded as being active against several fungal species (including some mycotoxigenic isolates such as *Aspergillus ochraceus*, *Penicillium verrucosum* and *Penicillium citrinum*) and certain contaminating bacteria, namely *Listeria* sp., *Staphylococcus aureus* and *Enterococcus faecalis* (Dieuleveux & Gueguen, 1998; Dieuleveux, Van der Pyl, Chataud, & Gueguen, 1998; Gould, 1996).

The higher effectivity of PLA (MIC_{50} 0.02–6.0 mM) compared to lactic and acetic acid (Table 3) would be linked to the lipophility of the undissociated active form. As all organic acids, the rate of dissociation was dependent on the pH. At low pH, the undissociated form can easily pass across the cell membrane and then accumulate within the cytoplasm, thereby causing loss of viability and cell destruction (Torino, Taranto, Sesma, & Font de Valdez, 2001). This fact would explain the differences in activity (expressed as MIC_{50}) observed among the acids and the higher antifungal effect obtained at pH 3.5. Accordingly, a 10-fold greater MIC_{50} was necessary for lactic acid (pK_a = 3.86) to obtain an inhibition similar of *A. niger* C_H 101 to that of acetic acid (pK_a = 4.76, MIC_{50}18 mM) at pH 3.5. From this point of view, it would be expected analogous antifungal activity for organic acids with similar pK_a values, e.g., acetic (pK_a = 4.76) and propionic (pK_a = 4.86) acid, or lactic (pK_a = 3.83) and PLA (pK_a = 3.46). However, propionic acid (MIC_{50}0.5 mM) was 36-fold more effective than acetic acid (MIC_{50} 18 mM), and PLA (MIC_{50} 0.07 mM) about 2500-fold more effective than lactic acid (MIC_{50}180 mM) on *A. niger* C_H 101 (Table 3). These results suggest that different mode of action would be involved in the antifungal effect. In fact, the precise mechanism of antimicrobials can often not be defined because of a complex interaction between the different compounds produced during cell growth and the frequently synergistic effects among them (Legan, 1993). Besides, the differences in sensitivity observed among the mould species may be related to their capacity to change the cell metabolism in response to acid stress conditions (Yang, Bastos, & Chen, 1993).

Numerous studies have described the isolation and characterization of antifungal components from LAB cultures (Corsetti et al., 1998; Lavermicocca et al., 2003) but limited applications of the antifungal strains in baking have been reported (Dal Bello et al., 2007; Lavermicocca et al., 2000). In our work, *L. reuteri* CRL 1100, *L. plantarum* CRL 778, and *L. brevis* CRL 772 and CRL 796 (regarded as antifungal positive strains) were used in the formulation of a mixed starter culture (LB) and used together with *S. cerevisiae* (commercial yeast) in bread elaboration. The FQ value (2.0–3.0), a criterion of bread quality (Gobbetti, 1998) was optimal (FQ = 2.0) for YLB-breads compared to traditional Y-bread (FQ = 12.5). In addition, the fungal growth was delayed for 5-days when using the starter LB compared to Y-breads elaborated with (0.1–0.2%) or without CP, which were spoiled within 2-days of storage.

The bioconservation of YLB-breads was mainly associated to the acetic acid and PLA. At pH 5.0 (pH of YLB-dough) the lactic acid was mainly (93%) dissociated and thus inactive although being at a high concentration (19.2 mM). Likewise, CP was less effective in Y-breads due to the high pH (5.6) of the doughs, which determined a low CP undissociated fraction (2.4 mmol/kg). In contrast, the antifungal effect of CP was 50% improved by using the LB starter culture due to the lower pH (5.0) reached during the dough (YLB-$CP_{0.2}$) fermentation, which determined a higher CP undissociated fraction (5.0 mmol/kg). These factors allowed increasing the shelf life to 8–9 days.

The incidence of moulds in bakery products in the North of Argentina is rather high mainly due to the climate conditions (warm and wet) and manufacturing practices. For these reasons, CP is commonly used at the limit concentration (0.4%) allowed by the AAC despite its potential tumorigenic and neurobiological effects (Griem, 1985; MacFabe et al., 2007). Accordingly, the new EU regulations permit the use of CP in bread manufacture only up to 0.3% (wt/wt) (Anonymous, 1995). Our results supply evidence that CP can be reduced by 50% when using together with selected antifungal LAB strains. This Food Grade bioestrategy would allow fitting in with the EU standard regulations for the use of CP in bakery. Additional studies on the contribution of bioactive molecules to the quality and shelf life of foods will surely widen the use of LAB strains as a novel biocontrol strategy.

References

Anonymous (1995). European Directive 95/2/CE of European Parliament and Council of European Community, 20 February 1995. European Parliament and Council of European Community, Brussels, Belgium.

Cabo, M. L., Murado, M. A., Gonzalez, M. P., & Pastoriza, L. (1999). A method for bacteriocin quantification. *Journal of Applied Microbiology, 87*(6), 907–914.

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

Dal Bello, F., Clarke, C. I., Ryan, L. A. M., Ulmer, H., Schober, T. J., Ström, K., et al. (2007). Improvement of the quality and shelf life of wheat bread by fermentation with the antifungal strain *Lactobacillus plantarum* FST 1.7. *Journal of Cereal Science, 45*(3), 309–318.

Dieuleveux, V., & Gueguen, M. (1998). Antimicrobial effects of D-3-phenyllactic acid on *Listeria monocytogenes* in TSB-YE medium milk and cheese. *Journal of Food Protection, 61*, 1281–1285.

Dieuleveux, V., Van der Pyl, D., Chataud, J., & Gueguen, M. (1998). Purification and characterization of anti-Listeria compounds produced by *Geotrichum candidum*. *Applied and Environmental Microbiology, 64*, 800–803.

Gobbetti, M. (1998). The sourdough microflora: Interaction of lactic acid bacteria and yeasts. *Trends in Food Science and Technology, 9*, 267–274.

Gould, G. W. (1996). Methods for preservation and extension of shelf life. *International Journal of Food Microbiology, 33*, 51–64.

Griem, V. W. (1985). Tumorigenic effects in the mucosa of the forestomach of rats in feeding experiments with propionic acid. *Bundesgesundheitblatt, 23*, 322–327.

Hassan, Y. I., & Bullerman, L. B. (2008). Antifungal activity of *Lactobacillus paracasei* ssp. tolerans isolated from a sourdough bread culture. *International Journal of Food Microbiology, 121*, 112–115.

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

Lavermicocca, P., Valerio, F., & Visconti, A. (2003). Antifungal activity of phenyllactic acid against moulds isolated from bakery products. *Applied and Environmental Microbiology, 69*, 634–640.

Legan, J. D. (1993). Moulds spoilage of bread: The problem and some solutions. *International Biodeterioration and Biodegradation, 32*, 33–53.

MacFabe, D. F., Cain, D. P., Rodriguez-Capote, K., Franklin, A. E., Hoffman, J. E., Boon, F., et al. (2007). Neurobiological effects of intraventricular propionic acid in rats: Possible role of short-chain fatty acids on the pathogenesis and characteristics of autism spectrum disorders. *Behavioral and Brain Research, 176*(1), 149–169.

Magnusson, J., Ström, K., Roos, S., Sjögren, J., & Schnürer, J. (2003). Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. *FEMS Microbiology Letters, 219*, 129–135.

Sjögren, J., Magnusson, J., Broberg, A., Schnürer, J., & Kenne, L. (2003). Antifungal 3-hydroxy fatty acids from *Lactobacillus plantarum* MiLAB 14. *Applied and Environmental Microbiology, 69*, 7554–7557.

Slawecki, R. A., Ryan, E. P., & Young, D. H. (2002). Novel fungitoxicity assays for inhibition of germination-associated adhesion of *Botrytis cinerea* and *Puccinia recondita* spores. *Applied and Environmental Microbiology, 68*(2), 597–601.

Stiles, M. E. (1996). Biopreservation by lactic acid bacteria. *Antonie van Leeuwenhoek, 70*, 331–345.

Torino, M. I., Taranto, M. P., Sesma, F., & Font de Valdez, G. (2001). Heterofermentative pattern and exopolysaccharide production by *Lactobacillus helveticus* ATCC 15807 in response to environmental pH. *Journal of Applied Microbiology, 91*, 846–852.

Valerio, F., Lavermicocca, P., Pascale, M., & 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 Microbiology Letters, 233*, 289–295.

Vermeulen, N., Gänzle, M. G., & Vogel, R. F. (2006). Influence of peptide supply and co-substrates on phenylalanine metabolism of *Lactobacillus sanfranciscensis* DSM20451T and *Lactobacillus plantarum* TMW1.468. *Journal of Agricultural and Food Chemistry, 54*, 3832–3839.

Yang, Y., Bastos, M., & Chen, K. Y. (1993). Effects of osmotic stress and growth stage on cellular pH and polyphosphate metabolism in *Neurospora crassa* as studied by ^{31}P nuclear magnetic resonance spectroscopy. *Biochimica et Biophysica Acta, 1179*, 141–147.