# Strains of Lactic Acid Bacteria Isolated from Sour Doughs Degrade Phytic Acid and Improve Calcium and Magnesium Solubility from Whole Wheat Flour

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Five strains of lactic bacteria have been isolated from sour doughs and examined for their ability to degrade phytic acid. In white flour medium in which phytic acid was the only source of phosphorus, the disappearance of phytate and an elevation of inorganic phosphate were observed after only 2 h of incubation in all strains tested (−30 and +60%, respectively). Both phenomena correspond to phytate breakdown. No difference was observed in the levels of phytic acid hydrolysis among strains, suggesting that phytase enzymes are similar among these bacteria. Using whole wheat flour medium naturally rich in phytic acid in the presence of *Leuconostoc mesenteroides* strain 38, a 9 h fermentation established that the degradation of PA and the production of lactic acid lead to greater Ca and Mg solubility than in control medium.

**Keywords:** *Calcium; fermentation; magnesium; phosphorus; phytate*

## INTRODUCTION

Phosphorus in unrefined cereals, legumes, nuts, seeds, and tubers is mostly present as phytic acid (PA). Because PA is negatively charged, it complexes with positively charged ions or proteins and subsequently inhibits the absorption of these compounds. The interactions of PA with protein and several minerals is considered to be one of the primary factors limiting the nutritive value of cereal grains and legume seeds. PA levels may be decreased by phytase, an enzyme that catalyzes the stepwise hydrolysis of phytate to phosphate and inositol via penta- to monophosphates. The enzyme is widely distributed in plants (Peers, 1953), animal tissues (Cooper and Gowing, 1983; Iqbal et al., 1994), and microorganisms. Microbial phytase activity has been found in fungi such as *Aspergillus ficuum* (Irving and Cosgrove, 1971) or *Aspergillus niger* (Nagashima et al., 1999), in yeast (Lambrechts et al., 1992), and in bacteria (Greiner et al., 1993, 1997; Lopez et al., 1983; Shirai et al., 1994). Wholemeal bread is a staple fermented food in many countries and is an important source of minerals. However, the presence of high PA contents compromises the bioavailability of the mineral fraction. Although traditional bread-making (made with baker's yeast) leads to phytate hydrolysis (Türk et al., 1996), bakery sour doughs contain a large proportion of bacteria that may also degrade PA. However, the role of these bacteria in PA hydrolysis remains to be established. In this study, we have isolated the predominant bacteria strains from the sour doughs and compared their abilities to degrade PA in order to select the most active strain for sour dough bread-making. The isolated strains were studied in a whole wheat flour medium to appreciate the real impact of the selected bacteria on Ca and Mg solubility during the fermentation process.

## MATERIALS AND METHODS

**Starter Culture.** Doughs were purchased from regional bakers known for their sour dough bread taste. For each sour dough, a 10% solution was prepared in trypton salt. Different lactic acid strains were then isolated in MRS medium plus maltose (20 g/L) and cycloheximide (200 mg/L) and in Mayeux medium (only for dextrane-producing *Leuconostoc mesenteroides*). These media were inoculated with the 10% solution for 48 h at 30 °C. After several purifications on MRS agar plus maltose, heterofermentation was tested using a modified hot loop method (Bervas, 1991). Gram coloration, catalase test, and 45 °C growth were performed for each strain. Pure strains were identified by API 50CH kits (BioMérieux, Charbonnières-les-Bains, France), and the profiles obtained were analyzed by BioMérieux for identification. Pure strains were maintained in MRS agar: *Lactobacillus plantarum* S18 (from rye sour dough), *Lactobacillus plantarum* S29 (from wheat sour dough), *Lactobacillus acidophilus* S37 (from organic wheat sour dough), *Leuconostoc mesenteroides* subsp. *mesenteroides* S38 (from rye sour dough), and *Leuconostoc mesenteroides* subsp. *mesenteroides* S50 (from rye sour dough).

**Incubation of Sodium Phytate with Lactic Acid Bacteria for 6 h.** To estimate in vitro phytate degradation in different isolated strains and to select the most active phytasic activity strain for sour dough bread-making, a pasteurized white flour medium was made: in the presence of water, white wheat flour (Uli3 variety) was microwaved at maximum power for 1 min at 1000 W and then diluted in sterile water (25:75). Phosphate was provided as sodium phytate (0.6 g/L) (Sigma Chemical, St. Louis, MO). The medium was inoculated with different bacteria [$10^7$ colony-forming units (cfu) $g^{-1}$ of solution], and a control (not inoculated) was performed in parallel.

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Fermentation was performed at pH 4.3 and 30 °C, and each fermentation was replicated three times.

**Kinetics of the Changes in Whole Wheat Flour Medium Inoculated by a Selected Bacterium Strain for 9 h.** The chosen strain was inoculated in whole wheat flour medium naturally rich in PA (0.8 g of PA/100 g of flour). The pasteurized whole flour medium was made as follows: in the presence of water, whole wheat flour (Uli3 variety) was microwaved at maximum power for 1 min at 1000 W and then diluted in sterile water (35:65). The medium was inoculated with *Ln. mesenteroides* S38 ($10^7$ cfu $g^{-1}$ of solution), and a control (not inoculated) was performed in parallel. Fermentation was performed at 30 °C. The fermentations were replicated three times. For the determination of soluble Ca and Mg, the samples were placed in 2 mL microfuge tubes and immediately centrifugated at 20000*g* for 5 min at 4 °C. Supernatant fractions were stored at −20 °C. Medium water was determined as the difference between wet weight and dry weight on aliquots of medium that were dried to a constant weight.

**Determination of pH and Total Amount of Titratable Acids.** The pH and acid equivalent were determined every 2 h with a Delta 320 pH meter (Mettler, France). A sample (10 g) was mixed with distilled water (90 mL). The mixture was then titrated with NaOH (0.1 M) during agitation to pH 8.5. The total titratable acidity (TTA) was expressed as the amount of NaOH consumed, in milliliters.

**Determination of Viable Counts.** The viable counts, expressed as cfu, were determined in the fermented media after 6 h using the pour plate method. The counts of lactic acid bacteria were made on MRS agar, and the plates were incubated at 30 °C for 2 days.

**Chemical Assays.** PA was determined using HPLC (Dionex, Sunnyvale, CA) as described previously (Lopez et al., 2000). The HPLC system consisted of a gradient pump (Dionex series 4500) equipped with a 25 $\mu$L injector loop and an anion-exchange Dionex HPIC AS-11 analytical column (0.5 cm i.d. × 25 cm). An anion-exchange Dionex HPIC AG-11 guard column was used. An anion micromembrane suppressor (AMMS) in which circulated $H_2SO_4$ (25 mM, 1 mL/min) was used to mimize basal conductivity. Samples in the range 1500−2500 mg (routinely 2000 mg) were extracted with 40 mL of HCl (0.65 mol/L) under vigorous mechanical agitation (Ika-Werk HS 500, Germany) for 4 h at room temperature. The extracts were centrifuged at 5000*g*, and 2 mL of the supernatant was diluted to 10 mL with deionized water (Millipore water system). The diluted supernatant was passed through a 200−400 mesh AG 1-X8 chloride anion exchange column (Bio-Rad, Richmond, CA). The columns were washed with 15 mL of HCl (0.025 mol/L), and PA was eluted from the resin with 15 mL of HCl (2 mol/L). The eluates were evaporated to dryness in an evaporator concentrator (Jouan SA, St Herblain, France) and resuspended in deionized water. Potassium phytate (Sigma, St. Louis, MO) was used as the external calibration standard. Inorganic phosphorus was determined by Biotrol kit (Merck, Nogent sur Marne, France). The increase in formation of the unreduced phosphomolybdate complex measured at 340 nm is directly proportional to the amount of inorganic phosphorus in the sample. Soluble Ca and Mg from whole flour medium were determined on the supernatant fractions after dry-ashing (10 h at 500 °C) and extraction at 130 °C in $HNO_3/H_2O_2$ (2:1) (Merck, Suprapur, Darmstadt, Germany) until decoloration. Mineral concentrations were determined by atomic absorption spectrophotometry (Perkin-Elmer 420, Norwalk, CT) in an acetylene−air flame at the following wavelengths: 422 nm (Ca) and 285 nm (Mg). Appropriate quality controls were analyzed with each set of measurements. Lactic acid was determined spectrophotometrically on neutralized perchloric acid extracts by enzymatic method (Bergmeyer, 1974).

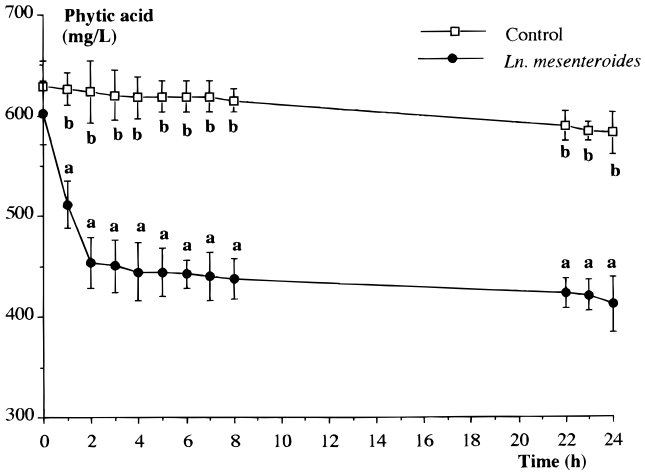


**Figure 1.** Changes in PA in the absence (control) or in the presence of lactic acid bacterium (*Ln. mesenteroides* S38) in white wheat flour medium supplemented with PA. Values are means ± SE for triplicate assays at each experimental point. Different subscript letters indicate significant differences ($P < 0.05$).

**Statistical Analysis.** Where appropriate, significance of the differences among means was determined by one-way ANOVA coupled with the Student−Newman−Keuls test (StatView, Abacus, Berkeley, CA). Differences with $P < 0.05$ were considered significant.

## RESULTS AND DISCUSSION

Testing of four different incubation media (data not shown) revealed that only the white flour medium allowed phytate degradation. Figure 1 shows the phytate variations in the control (not inoculated) and in the medium incubated in the presence of *Ln. mesenteroides* strain 38. After fermentation by strain 38 for 24 h, there was a PA loss only in the inoculated medium. Although lactic acid bacteria have complex growth requirements, microorganisms survived and phytate appeared to be degraded in the white flour medium. Thus, to estimate phytate degradation by lactic acid bacteria, the white

**Table 1. Changes in pH and TTA in the White Flour Medium[a,b]**

| | | 0 h | 2 h | 4 h | 6 h |
|---|---|---|---|---|---|
| control, no inoculation | pH | 4.25 ± 0.32 | 4.25 ± 0.21 | 4.29 ± 0.24 | 4.26 ± 0.14[a] |
| | TTA | 20 ± 1 | 18 ± 2 | 20 ± 1 | 19 ± 2 |
| *La. plantarum* strain 18 | pH | 4.31 ± 0.29 | 4.19 ± 0.19 | 4.10 ± 0.15 | 3.67 ± 0.21[b] |
| | TTA | 18 ± 2 | 26 ± 3 | 24 ± 1 | 29 ± 2 |
| *La. plantarum* strain 29 | pH | 4.24 ± 0.27 | 4.23 ± 0.28 | 4.08 ± 0.34 | 3.69 ± 0.19[b] |
| | TTA | 18 ± 1 | 26 ± 2 | 32 ± 2 | 31 ± 3 |
| *La. acidophilus* strain 37 | pH | 4.21 ± 0.31 | 4.24 ± 0.29 | 3.96 ± 0.37 | 3.68 ± 0.15[b] |
| | TTA | 19 ± 1 | 20 ± 3 | 33 ± 2 | 30 ± 3 |
| *Ln. mesenteroides* strain 38 | pH | 4.25 ± 0.28 | 4.26 ± 0.34 | 4.11 ± 0.27 | 3.69 ± 0.18[b] |
| | TTA | 18 ± 3 | 20 ± 2 | 20 ± 3 | 29 ± 1 |
| *Ln. mesenteroides* strain 50 | pH | 4.27 ± 0.32 | 3.92 ± 0.36 | 3.91 ± 0.39 | 3.82 ± 0.20[b] |
| | TTA | 20 ± 2 | 35 ± 3 | 32 ± 2 | 32 ± 3 |

[a] Values are means ± SE for triplicate assays. [b] Means within columns containing different superscript letters are different ($P < 0.05$).

**Table 2. Viable Counts of Lactic Acid Bacteria (cfu per Gram) in the White Flour Medium after 6 h of Fermentation at 30 °C**

| | 6 h |
|---|---|
| control, no inoculation | 0 |
| *La. plantarum* strain 18 | $4.9 \times 10^7$ |
| *La. plantarum* strain 29 | $5.8 \times 10^7$ |
| *La. acidophilus* strain 37 | $4.7 \times 10^7$ |
| *Ln. mesenteroides* strain 38 | $6.1 \times 10^7$ |
| *Ln. mesenteroides* strain 50 | $5.9 \times 10^7$ |

flour medium was used. The pH and TTA allowed the assessment of the evolution of strain fermentation (Table 1). The rate of pH decrease and the final pH varied in the solutions. Inoculation of $10^7$ cfu of lactic acid bacteria per gram of medium caused a pH decrease, whereas the control pH remained constant (between 4.25 and 4.29) for 6 h of incubation. The acidity of the solutions at 0 h was 20 mL of NaOH consumed to pH 8.5. If the acidity in the control was still near 20 mL of NaOH consumed after 6 h of incubation, the lactic acid bacteria inoculation led to an acidification of white flour medium: the TTA varied between 24 and 32, indicating that normal lactic acid fermentation occurred. After 6 h of incubation at 30 °C, the viable counts of different lactic acid bacteria varied between $4.7 \times 10^7$ and $6.1 \times 10^7$ cfu $g^{-1}$ (Table 2). The highest viable counts were noted with strain 38 (*Ln. mesenteroides*), whereas the lowest were obtained with strain 37 (*Lactobacillus plantarum*). As these lactic acid bacteria strains were isolated from cereal sour doughs, it appears that white flour medium is suitable for their viability.

In a short time (2 h), all strains of the lactic acid bacteria tested caused a significant release of phosphorus (Table 3) and a significant degradation of PA (Table 4) in the white flour medium compared with the control. The disappearance of PA and an accumulation of inorganic phosphate were observed, and both phenomena reflect phytate breakdown. No difference was observed in the levels of PA hydrolysis between strains, suggesting that phytase enzymes are similar among these bacteria. There are some microorganisms that are established in their capacity to degrade phytate, for example, the yeasts *Saccharomyces cerevisae* (Türk et al., 1996) and *Schwanniomyces occidentalis* (Segueilha et al., 1993). Also, 12 strains of lactic acid bacteria have been shown to decrease phytate in a medium in which PA was the only source of phosphates (Shirai et al., 1994). Marklinder et al. (1995) confirmed these results using *La. plantarum*, which can also degrade PA. For all strains considered in our study, most phytate hydrolysis occurred before the first 2 h of fermentation. After 2 h of fermentation, the degradation rate was strongly reduced. A possible explanation of this phenomenon is that the enzyme is inhibited by the phosphate released (Frölich et al. 1986).

To test phytase potentialities in food products, *Ln. mesenteroides* S38 was chosen among tested lactic acid bacteria. This strain did not express the highest phytasic activity in the white flour medium. However, it exhibited the fastest growth over 6 h and was able to rapidly colonize a medium. The kinetics of the changes in whole wheat flour medium incubated in the presence or absence of *Ln. mesenteroides* (strain 38) were observed. *Ln. mesenteroides* S38 produced high levels of lactic acid, leading to an acidification of the medium compared to control medium (Figure 2A). Moreover, after 9 h of incubation, almost all of the PA present in the medium was degraded by strain 38, whereas there was no change in phytate content in the control (Figure 2B). The destruction of PA as well as the acidification led to increased Ca and Mg solubility (Figure 3).

The microorganisms involved in the natural fermentation of cereals such as sour doughs are essentially the surface flora of the seeds. They are known to play a dual

**Table 3. Release of Inorganic Phosphorus ($P_i$) from PA Lactic Bacteria and Incubation in White Flour Medium[a,b]**

| | | 0 h | 2 h | 4 h | 6 h |
|---|---|---|---|---|---|
| control, no inoculation | $P_i$ (mg/L) | 120 ± 16 | 110 ± 15[a] | 127 ± 11[a] | 123 ± 14[a] |
| | % $P_i$ release | | −8 | +5 | +3 |
| *La. plantarum* strain 18 | $P_i$ (mg/L) | 141 ± 11 | 228 ± 18[b] | 233 ± 21[b] | 240 ± 10[b] |
| | % $P_i$ release | | +62 | +65 | +70 |
| *La. plantarum* strain 29 | $P_i$ (mg/L) | 147 ± 9 | 225 ± 16[b] | 230 ± 25[b] | 236 ± 19[b] |
| | % $P_i$ release | | +53 | +56 | +61 |
| *La. acidophilus* strain 37 | $P_i$ (mg/L) | 145 ± 14 | 233 ± 21[b] | 239 ± 16[b] | 247 ± 22[b] |
| | % $P_i$ release | | +61 | +65 | +70 |
| *Ln. mesenteroides* strain 38 | $P_i$ (mg/L) | 149 ± 11 | 222 ± 24[b] | 236 ± 25[b] | 244 ± 24[b] |
| | % $P_i$ release | | +49 | +58 | +64 |
| *Ln. mesenteroides* strain 50 | $P_i$ (mg/L) | 141 ± 13 | 234 ± 31[b] | 234 ± 28[b] | 250 ± 27[b] |
| | % $P_i$ release | | +66 | +66 | +77 |

[a] Values are means ± SE for triplicate assays. [b] Means within columns containing different superscript letters are different ($P < 0.05$).

**Table 4. Reduction of PA by Lactic Bacteria and Incubation in White Flour Medium[a,b]**

| | | 0 h | 2 h | 4 h | 6 h |
|---|---|---|---|---|---|
| control, no inoculation | PA (mg/L) | 629 ± 65 | 624 ± 49[a] | 618 ± 52[a] | 619 ± 43[a] |
| | % reduction | | 0 | −2 | −2 |
| *La. plantarum* strain 18 | PA (mg/L) | 626 ± 52 | 446 ± 29[b] | 429 ± 37[b] | 414 ± 31[b] |
| | % reduction | | −29 | −31 | −34 |
| *La. plantarum* strain 29 | PA (mg/L) | 611 ± 43 | 446 ± 41[b] | 447 ± 42[b] | 435 ± 51[b] |
| | % reduction | | −27 | −27 | −29 |
| *La. acidophilus* strain 37 | PA (mg/L) | 618 ± 39 | 448 ± 39[b] | 439 ± 37[b] | 437 ± 42[b] |
| | % reduction | | −28 | −29 | −29 |
| *Ln. mesenteroides* strain 38 | PA (mg/L) | 603 ± 42 | 454 ± 42[b] | 445 ± 52[b] | 443 ± 34[b] |
| | % reduction | | −25 | −26 | −27 |
| *Ln. mesenteroides* strain 50 | PA (mg/L) | 631 ± 52 | 461 ± 56[b] | 435 ± 53[b] | 427 ± 49[b] |
| | % reduction | | −27 | −31 | −32 |

[a] Values are means ± SE for triplicate assays. [b] Means within columns containing different superscript letters are different ($P < 0.05$).

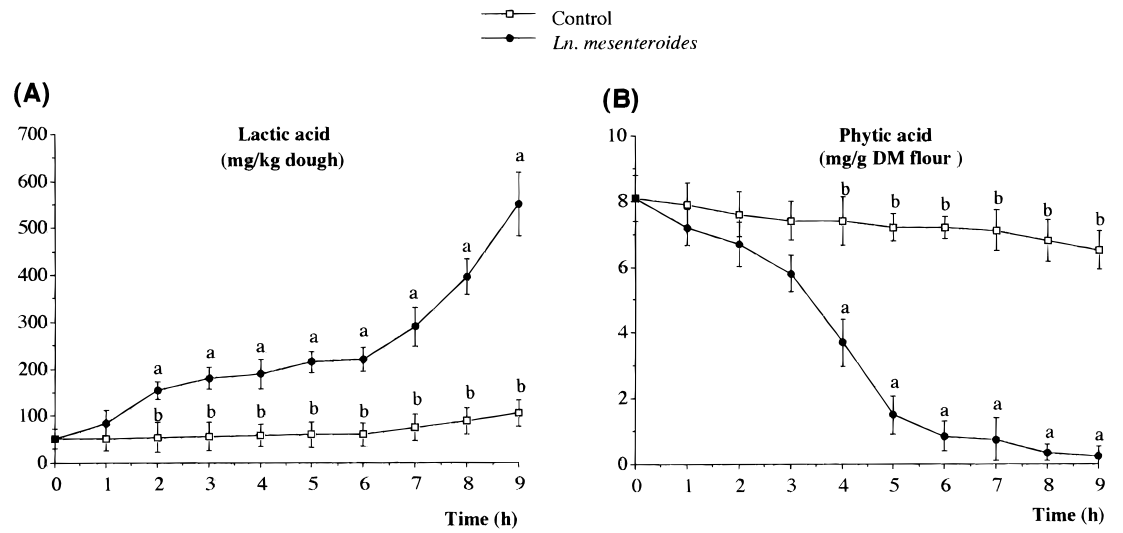


**Figure 2.** Changes in (A) lactic acid production and (B) PA degradation in whole wheat flour medium incubated in the absence (control) or presence of lactic acid bacterium (*Ln. mesenteroides* S38). Values are means ± SE for triplicate assays at each experimental point. Different subscript letters indicate significant differences ($P < 0.05$).

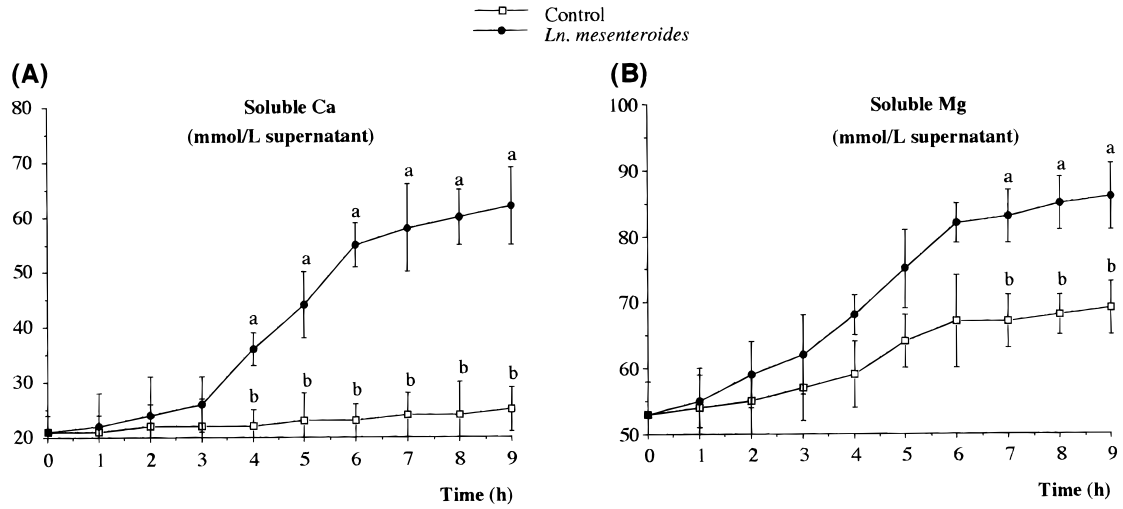


**Figure 3.** Changes in (A) Ca solubility and (B) Mg solubility in whole wheat flour medium incubated in the absence (control) or presence of lactic acid bacterium (*Ln. mesenteroides* S38). Values are means ± SE for triplicate assays at each experimental point. Different subscript letters indicate significant differences ($P < 0.05$).

role as rising and acidifying agents and promote both the aroma and the volume of bread. During the course of the fermentation process, there are four genera of lactic acid producing bacteria that prevail: *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Lactococcus*, all of which require carbohydrates for energy. Lactic acid (and other organic acids) produced from hexoses lowers the pH of the product from 6.5−7 to ∼5. Thus, the fermentation process results in suitable pH conditions for the degradation of phytate. Svanberg et al. (1993) found that lactic fermentation of maize or sorghum can shift a "low iron bioavailability" diet into an "intermediate to high bioavailability" diet. The fermentation process is considered to have a promotive effect on iron absorption, probably through the formation of organic acids. In the present study, the whole wheat flour fermentation with *Ln. mesenteroides* led to lactic acid production. This acidification is accompanied by the improvement of the solubility of minerals. Because lactic acid bacteria from bakery leavened products degrade PA and improve Ca and Mg solubility, mineral bioavailability in fermented cereal foods (bread) should be higher than that of unleavened products (extruded cereals). In this case, the consumption of fermented cereal products such as bread should be recommended to provide bioavailable minerals.

In summary, all strains of lactic acid bacteria isolated from sour dough express phytase activity. In this study, microbial phytase activity did not differ in the strains tested. All strains were able to degrade ≈30% of PA in only 2 h. In whole wheat flour medium, in which PA is naturally present, fermentation leads to better mineral solubility by improving PA hydrolysis and through acidification. To obtain whole cereal products with a higher mineral bioavailability, it may be possible to decrease phytate levels to stimulate mineral absorption. Although the observation that lactic acid bacteria are able to degrade PA is an interesting result, this bacterial phytate hydrolysis should be compared with that observed with yeast fermentation to assess the phytasic potentialities of the two microorganism classes. In addition, the relative importance of vegetal and microbial phytases in a technological processes (for instance, bred-making) should be assessed.

## ABBREVIATIONS USED

cfu, colony-forming units; PA, phytic acid; TTA, total titratable acidity.

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