Expression of bifidobacterial phytases in *Lactobacillus casei* and their application in a food model of whole-grain sourdough bread.

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ABSTRACT

Phytases are enzymes capable of sequentially dephosphorylating phytic acid to products of lower chelating capacity and higher solubility, abolishing its inhibitory effect on intestinal mineral absorption. Genetic constructions were made for expressing two phytases from bifidobacteria in *Lactobacillus casei* under the control of a nisin-inducible promoter. *L. casei* was able of producing, exporting and anchoring to the cell wall the phytase of *Bifidobacterium pseudocatenulatum*. The phytase from *Bifidobacterium longum* spp. *infantis* was also produced, although at low levels. *L. casei* expressing any of these phytases completely degraded phytic acid (2 mM) to lower *myo*-inositol phosphates when grown in MRS medium. Owing to the general absence of phytase activity in lactobacilli and to the high phytate content of whole grains, the constructed *L. casei* strains were applied as starter in a bread making process using whole-grain flour. *L. casei* developed in sourdoughs by fermenting the existing carbohydrates giving place to an acidification. We determined that in this food model system the contribution of *L. casei* strains expressing phytases to phytate hydrolysis was low, and the phytate degradation was mainly produced by activation of the cereal endogenous phytase as a consequence of the drop in pH. This work constitutes the first use of lactobacilli engineered to express phytases in food fermentation and it shows their capacity to be modified in order to produce enzymes with relevance in food technology processes. The ability of these strains in reducing the phytate content in fermented food products must be evaluated in further models.

Keywords: *Lactobacillus casei*, *Bifidobacterium*, phytase, phytate, sourdough, whole wheat

Chemical compound studied in this article

Phytic acid or *myo*-inositol hexakisphosphate (PubChem CID: 890)

1. Introduction

The impact of diet on health has led to an increasing demand for functional foods, where high fibre products, as whole grain meal, play an important physiological role in the maintenance of general well-being and health. Epidemiological findings indicate a protective role of whole grain foods against several diseases such as diabetes, certain cancers, cardiovascular disease and obesity, including an improved regulation of blood glucose levels (McIntosh et al., 2003). Refined grains are characterized by a limited nutritional value, whereas whole grains are a better source of fibre, vitamins, minerals and other biologically active compounds as phenolic compounds, lignans, phytosterols and phytic acid. Processing may modify the amount and bioavailability of some of them (Isserliyska et al., 2001; Slavin, 2004). Sourdough fermentation is a traditional process employed since ancient times in baking (Katina et al., 2005). Generally, the microbiota involved in sourdough fermentation is composed of yeasts and lactic acid bacteria (LAB), which represent the majority of the sourdough´s microbiota, with counts ranging from 10^8 to 10^9 CFU per g of sourdough. The strains of LAB most frequently found in sourdough belong to the species *Lactobacillus sanfranciscensis*, *Lactobacillus brevis* and *Lactobacillus plantarum* (Jekle, et al., 2010). During sourdough fermentation LAB produce a number of metabolites which have been shown to have a positive effect on the texture and staling of bread, e.g. organic acids, exopolysaccharides (EPS) and/or enzymes (Arendt et al., 2007). This results in an enhancement of the nutritional and sensory quality of bread (Katina et al., 2005). The sourdough could also increase the bioavailability of minerals. As was mentioned above, whole grain cereals contain significant amounts of phytic acid (*myo*-inositol (1,2,3,4,5,6)-hexakisphosphate, InsP_6) or its salts (phytates). The phytic acid is an organic acid common in plants in which it functions in the storage of phosphorus and cations for growth and it is a well-known inhibitor of mineral, proteins and trace element bioavailability (Sandberg et al., 1999). However, the phytate hydrolysis decreases the negative effects on mineral absorption and generates lower *myo*-inositol phosphates with potential specific biological activity that may positively affect human health (Shi et al., 2006). Phytases are the enzymes capable of sequentially dephosphorylating phytic

acid to products of lower chelating capacity and higher solubility, unlocking the inhibitory effect on mineral absorption (Haros et al., 2009). Cereals have their own endogenous phytase activity that could be enhanced by the low pH resulting from the use of sourdough in the breadmaking process, but this activity is not sufficient to efficiently degrade phytate (Greiner and Konietzny, 2006; Sanz-Penella et al., 2012a). Bacteria of the genus *Lactobacillus* are the main players in sourdough fermentation and LAB:yeast ratio is generally 100:1 (De Vuyst and Neysens, 2005). Yeasts usually show low phytase activity and for high yeast phytase activity to take place, conditions must favour the expression of the phytase genes (Andlid et al., 2004). As far as we know, no real phytases from lactobacilli have been described in the literature. Some reports exist describing the partial degradation of phytate by particular *Lactobacillus* strains (Anastasio et al., 2010; De Angelis et al., 2003; Lopez et al., 2000; Zamudio et al., 2001), but this activity is due to the expression of unspecific phosphatases that act on phytate, although with very low efficiency (Haros et al., 2009a; Sandberg and Andlid, 2002; Zamudio et al., 2001). By the contrary, phytase activity has been described for strains of the genus *Bifidobacterium* and the corresponding genes and enzymes have been characterized, showing that they belong to the Histidine-Acid Phosphatase family (Tamayo-Ramos et al., 2012). The purified bifidobacterial phytases have been applied in several food processes (García-Mantrana et al., 2014; Iglesias-Puig et al., 2014; Sanz-Penella et al., 2012b) and the strain *Bifidobacterium pseudocatenulatum* ATCC27919 has been used in both direct and indirect breadmaking processes (Sanz-Penella et al., 2009, 2012a), showing its potential in the reduction of phytates in breads and in the increase of mineral bioavailability. However, the condition of strict anaerobic and fastidious microorganisms of bifidobacteria limits their use in food fermentations. The purpose of this work was to construct *Lactobacillus casei* strains expressing the phytases from *B. pseudocatenulatum* and *Bifidobacterium longum* spp*. infantis* and their application as starters in the breadmaking process of whole-grain bread.

2. Materials and methods

2.1. Materials

Commercial Spanish whole wheat flour was purchased from the local market. The characteristics of the flour in dry basis were: moisture 14.04±0.08%, protein (N x 5.7) 11.64±0.08%, lipid content 1.67±0.03% and ash 1.36±0.01. Compressed yeast (*Saccharomyces cerevisiae*, Levamax, Spain) was used as starter for the breadmaking process and *Lactobacillus casei* strains genetically modified to produce phytases from bifidobacteria were used as starter in sourdough fermentation. In order to construct these strains we used *L. casei* BL23 (wild type) and *L. casei* BL23[*nisKR*] (Hazebrouck et al., 2007), a BL23 derivative in which the *nisKR* two-component system from *Lactococcus lactis* has been integrated in its genome. *L. lactis* MG1363 was used as a host for cloning.

2.2. Methods

2.2.1. Culture media and growth conditions

L. casei strains were grown in MRS medium (Oxoid) at 37ºC under static conditions and *L. lactis* was grown in M17 medium (Oxoid) supplemented with 0.5% glucose at 30ºC. Antibiotics for plasmid selection (erythromycin and chloramphenicol) were used at 5 μg/ml when added individually and at 2.5 μg/ml when they were used together.

2.2.2. Construction of genetically modified *Lactobacillus casei* expressing phytase genes from bifidobacteria

The phytase genes from *Bifidobacterium pseudocatenulatum* ATCC27919 (BIFPSEUDO_03792) and *Bifidobacterium longum* spp. *infantis* ATCC15697 (BLON_0263) were amplified by PCR with the following primer pairs: (5´-GAACCATGGGGATAATGGCGAAAAAC/5'-CACAAGCTTTCACGTCACGTTTGAACCGGTTTTG) and

(5'AATCCATGGCAACACGAGTGATG/5'GACAAGCTTTCAGACCGAACTTCC GGTACGTGCC), respectively (underlined sequences correspond to the NcoI and HindIII sites introduced for cloning). The PCR reaction was carried out in a mixture containing Expand High Fidelity 1X buffer, 100 ng of genomic DNA of each strain, 200 µM of dNTPs, 10 pmol of each primer, 1 µl of Expand High Fidelity Polymerase (Roche), in a volume of 50 µl. The PCR thermal conditions were as follows: 1 cycle at 94°C for 3 min, 30 cycles of 94 °C for 30 seconds, 50 °C for 30 seconds and 72 °C for 2.5 min and a last cycle of 72 °C for 7 min. The amplified genes were examined by agarose gel electrophoresis, purified with the Illustra GFX PCR and gel band DNA purification kit (GE Healthcare) and digested with NcoI y HindIII for cloning into the pNG8048e vector (Steen et al., 2007) digested with the same endonucleases. Vector and phytase genes were ligated with T4 ligase (Invitrogene) and the ligation mixtures were used to transform *L. lactis* MG1363 electrocompetent cells (Holo and Nes, 1995) using a GenePulser apparatus (Biorad) and 0.2 cm electroporation cuvettes. After electroporation, cells were resuspended in 5 ml of M17 medium containing 0.5 M saccharose, 0.5% glucose, 2 mM $CaCl_2$ and 10 mM $MgCl_2$ and incubated for 1 h at 30ºC. After this period transformants were plated on M17 plates containing 0.5 M saccharose, 0.5% glucose and 5 µg/ml chloramphenicol and incubated overnight at 30ºC. The recombinant plasmids were purified from the lactococcal transformants with the Illustra Plasmid Isolation Kit (GE Healthcare) and examined by sequencing analysis. The obtained plasmids were electrotransformed to competent cells of *L. casei* BL23 [*nisKR*] as previously described (Posno et al., 1991). Transformants were recovered on MRS plates containing erythromycin and chloramphenicol after incubation at 37ºC.

2.2.3. Phytase induction experiments

L. casei BL23 [*nisKR* pNG80548e], *L. casei* BL23 [*nisKR* pNGPHY*pseudo*] and *L. casei* BL23 [*nisKR* pNGPHY*longum*] were grown in 20 ml of MRS medium overnight. These cultures were diluted into 50 ml of fresh MRS medium with antibiotics to an OD_{550} of 0.4, and incubated at 37 °C for 1.5 h. At the end of the incubation each culture was divided in two parts and nisin (Sigma-Aldrich) was

added at a concentration of 20 ng/ml to one of them. Then, cultures were further incubated for 3 h at 37 °C. The cells were centrifuged at 9,000x*g* for 15 min (Hermle Z383K centrifuge), the pellet was washed twice with PBS and frozen at -20°C until use. Phytase expression was analyzed by SDS-PAGE. To this end, to 10 μl of bacterial cells resuspended in PBS, 10 μl of 2X Laemmli buffer were added. After boiling for 5 min, samples were centrifuged at 12,000x*g* for 5 min and loaded onto 10% SDS-PAGE gels that were stained with Coomassie blue.

2.2.4. Preparation of crude extracts for phytase determination

L. casei strains harbouring different plasmids were induced for phytase production and the phytase activity was determined in different cellular fractions. Induced cells were washed twice with Tris-HCl 50 mM pH 7.5 and resuspended in the same buffer. Eight hundred μl of this suspension were mixed with 1 gram of glass beads (0.1 mm diameter) and broken in a BeadBeater apparatus (Biospec Products) for 4 cycles of 40 seconds at maximal speed with 1 min intervals in which the tubes were kept on ice. Unbroken cells were removed after centrifugation for 5 min at 14,000x*g* at 4 ºC. Protein concentration in the crude extracts was determined with the BioRad Dye-binding Protein Assay Kit using BSA as a standard. Cell wall proteins were obtained by enzymatic digestion. The reaction was carried out in 100 μl of Tris-HCl 50 mM pH 7.5 containing bacterial cells to an OD_{550} of 1 and 0.5 M saccharose, 5 mg/ml lysozyme and 5 U/ml mutanolysin. The suspension was incubated at 37 ºC for 30 min and bacteria were removed by centrifugation 5 min at 14,000x*g* and 4 ºC. The supernatant was collected and used as crude cell wall extract. The enzymatic extract of sourdoughs were prepared following the method reported by Haros et al. (2001).

2.2.5. Determination of phytase activity

The phytase activity was determined using 500 μl of 0.1 M sodium acetate pH 5.5, containing 1.2 mM phytic acid dipotassium salt (Sigma-Aldrich) and 100 μl of each fraction (whole cells, crude extracts, cell wall extracts or sourdough

extracts) (Haros et al., 2001, 2005). After 15 minutes of incubation at 50 ºC, the reaction was stopped with 100 µl of trichloracetic acid at 20%, allowed to stand for 10 min at 0 ºC and centrifuged at 14,000x*g*, 5 min and 4ºC (Centrifuge 5415R, Eppendorf). The determination of the enzyme activity was based in a colorimetric quantification at 400nm of free phosphorus released by the hydrolysis of phytate using ammonium molybdovanadate reagent (Fluka Chemika) according to Tanner et al. (Tanner & Barnett, 1986).

2.2.6. Phytate hydrolysis by *L. casei* grown in MRS medium.

In order to estimate the *in vivo* phytate degradation capacity of *L. casei* strains expressing bifidobacterial phytases, bacterial cells were inoculated in MRS broth containing 2 mM phytic acid dipotassium salt, 20 ng/ml of nisin, 2.5 µg/ml of chloramphenicol and 2.5 µg/ml of erythromycin and incubated for 24 hours at 37 ºC. Five ml samples were periodically taken for OD, pH and phytate content determination.

2.2.7. Inoculum and sourdoughs preparation

L. casei strains were grown in 50 ml of MRS medium with appropriated antibiotics at 37 °C for 24 hours. Bacterial cells were centrifuged (9000x*g*, 10 min, 4 °C, Hermle Z383K centrifuge), washed twice in 0.9% NaCl solution, resuspended in 1 ml of 0.9% NaCl and the OD_{550} was determined. The sourdough formulation consisted in a mixture of whole flour and water (1:2, v/v) with an inoculum of 5 x 10^8 CFU per gram of flour of *L. casei* BL23 (wild type strain) or the recombinant strains *L. casei* BL23 [*nisKR* pNG8048e], *L. casei* BL23 [*nisKR* pNGPHY*pseudo*] and *L. casei* BL23 [*nisKR* pNGPHY*longum*]. Twenty ng/ml of nisin were added to the sourdough, which included 2.5 µg/ml of chloramphenicol and 2.5 µg/ml of erythromycin when recombinant strains were used. Incubation was carried out at 37 °C for 18 hours. Two control acid sourdoughs were also prepared consisting in the same formulation and conditions, without inoculated lactobacilli and containing a mixture of antibiotics (penicillin, 50 U/ml; streptomycin, 0.05 mg/ml; neomycin, 0.1 mg/ml and

cycloheximide, 0.5 mg/ml from Sigma-Aldrich) at 1% v/v. The pH of these controls was adjusted to 4 and 5, respectively, by using lactic acid.

2.2.8. Breadmaking procedure

The formula used for making bread dough consisted of (500g): whole wheat flour 100%; tap water 61% (up to optimum absorption corresponding to 500 BU, Brabender Units); compressed yeast 5% and sodium chloride 1.6%. Wheat sourdoughs without yeast and inoculated with the *L. casei* strains were added in a 10% level in flour basis to bread doughs formula for replacement of flour. The ingredients were mixed for 5.5 min, rested for 10 min, divided into 100 gr pieces, kneaded and then rested again for 15 min. Doughs were manually sheeted, rolled and fermented up to the optimum volume increase at 28 ºC and 80% of relative humidity. Finally, the samples were baked at 165 ºC/30min, and then cooled at room temperature for one hour. The formulation samples were done in duplicate.

2.2.9. pH, total titratable acidity

Sourdough, dough and bread pH was determined electrometrically during sampling. Measurements were done in triplicate using a pH meter. For determination of titratable acidity ten grams of sourdough, dough or bread was mixed and blended with 100 ml of acetone:water (5:95, v/v). Later, they were titrated against 0.1 N NaOH up to pH 8.5. The results were expressed as the volume (ml) of NaOH 0.1 N needed for titrating 10 g of sourdough, fermented dough or bread.

2.2.10. Determination of *myo*-inositol phosphates

Phytate (*myo*-inositol hexakisphosphate or InsP_6) present in the supernatant of culture media and in breads and lower *myo*-inositol phosphates generated by phytase action (pentakis-, tetrakis- and triphosphate of *myo*-inositol: InsP_5, InsP_4 and InsP_3, respectively) were extracted by ion-exchange chromatography

and measured by the HPLC method described by Türk and Sandberg (1992), later modified by Sanz-Penella et al. (Sanz Penella et al., 2008). Identification of the *myo*-inositol phosphates was achieved by comparison with standards of phytic acid di-potassium salt (Sigma-Aldrich). Samples were analyzed in quadruplicate.

2.2.11. Statistical analysis

Multiple sample comparison of the means and Fisher's least significant differences (LSD) were applied to establish significant statistical differences between treatments. All statistical analyses were carried out with the Statgraphics Plus 7.1 Software (Statistical Graphics Corporation) and differences were considered significant at $p<0.05$.

3. Results

3.1. Construction of *L. casei* strains expressing bifidobacterial phytases

The phytase genes from *B. pseudocatenulatum* and *B. longum* subsp. *infantis* were cloned in *L. casei* BL23 under the control of a nisin-inducible promoter (plasmids pNGPHY*pseudo* and pNGPHY*longum*, respectively). The genes were cloned preserving their coding regions for the signal peptides and C-terminal motifs of the LPXTG class that promote the secretion and covalent anchoring to the cell-wall peptidoglycan via a sortase-catalized reaction (Tamayo-Ramos*,* et al*.*, 2012). Therefore *L. casei* transformants were expected to express, secrete and display at their surface the bifidobacterial enzymes. The analysis by SDS-PAGE of crude cell extracts from *L. casei* transformed with pNGPHY*pseudo* and induced with 20 ng nisin per ml of culture showed the appearance of an extra protein band of 68 kDa which was not present in extracts of non-induced cells or in cells transformed with the empty vector (pNG8048e) (Fig. 1). This protein size was in agreement with the molecular weight of *B. pseudocatenulatum* phytase. By the contrary no extra bands could be identified in a strain carrying the *B. longum* phytase gene (pNGPHY*longum* plasmid). These results suggested that the *B. pseudocatenulatum* phytase was efficiently expressed in *L. casei*, whereas the *B. longum* enzyme was not expressed or it was expressed at a low level.

3.2. The bifidobacterial phytases expressed in *L. casei* are functional

We determined the phytase activity in several fractions of *L. casei* strains (Table 1). Activity in crude extracts of induced *L. casei* transformed with pNGPHY*pseudo* was 4.5-fold higher compared to non-induced cells. These cells exhibited the highest phytase activity, while the capacity of releasing phosphate from phytate in cells carrying pNGPHY*longum* was comparable to that of a strain carrying the control plasmid. Phytase activity was also detected in whole cells and in proteins extracted from the cell walls of *L. casei* transformed with pNGPHY*pseudo* (Table 1), indicating that part of the enzyme was secreted and displayed at the cell surface.

3.3. Degradation of phytate by recombinant *L. casei* growing cells

We next tested whether the *L. casei* strains with phytase plasmids could degrade phytic acid when grown in MRS medium. Figure 2 shows the growth curves and the medium pH of the recombinant strains *L. casei* BL23 [*nisKR* pNG8048e], *L. casei* BL23 [*nisKR* pNGPHY*pseudo*] and *L. casei* BL23 [*nisKR* pNGPHY*longum*]. Compared to the control, the strains carrying phytase genes grew slower and reached lower final optical densities (4.90±0.08 for *L. casei* BL23 [*nisKR* pNG8048e]; 3.43±0.44 for *L. casei* BL23 [*nisKR* pNGPHY*pseudo*] and 3.34±0.01 for BL23 [*nisKR* pNGPHY*longum*]). Also, differences in the final medium pH were observed (3.83±0.04 for *L. casei* BL23 [*nisKR* pNG8048e] 4.07±0.09 for *L. casei* BL23 [*nisKR* pNGPHY*pseudo*]; and 4.09±0.01 for BL23 [*nisKR* pNGPHY*longum*]). The results clearly showed that the strains harboring the phytase expression plasmids had a drawback in growth. However, phytase-expressing bacteria efficiently degraded the phytic acid (InsP_6), giving rise to different lower *myo*-inositol phosphates (Fig. 3). The degradation rate of InsP_6 was higher for the strain expressing the *B. pseudocatenulatum* phytase and it was completed in around seven hours of growth; whereas transformants expressing the *B. longum* enzyme needed 24 h to almost completely eliminate InsP_6. The lower *myo*-inositol phosphates InsP_5 and InsP_4 showed a transient accumulation and they were finally degraded with accumulation of InsP_3. In the strain carrying pNGPHY*longum* accumulation of InsP_4 was also observed at 24 h. No InsP_6 degradation and generation of lower *myo*-inositol phosphates was seen in the control strain (Fig. 3).

3.4. Characteristics of sourdough prepared with *L. casei* strains

In order to determine the efficacy of the constructed *L. casei* strains in removing InsP_6 in a complex food model, fermentations of sourdough made with whole-wheat flour and the modified lactobacilli were performed. The resulting sourdoughs were afterwards introduced in a whole-wheat breadmaking process. Table 2 shows pH, total titratable acidity (TTA) and LAB counts of sourdoughs. The samples inoculated with wild type *L. casei* BL23 strain showed

an acidification from an initial pH of 5.85 to final pH of 3.68, reaching a TTA value of 13.71 ml. This was attributed to lactic acid production showing that the strain adapted to the dough environment and was metabolically active possibly by fermenting the carbohydrate sources from the flour (mainly maltose and maltodextrins), although BL23 strain is not from a sourdough origin. The cell counts increased one order of magnitude, from 5.4 x 10^8 CFU/g to 6.7 x 10^9 CFU/g per gram of flour after the incubation period. The sourdough prepared with *L. casei* strain BL23 [pNG8048e] showed similar pH as the sourdough inoculated with the wild type (unmodified) strain, but with a total titratable acidity significantly lower (Table 2). The lower acidification could be explained by lower cell counts. In fact this trend was more pronounced in sourdoughs inoculated with *L. casei* BL23 [pNGPHY*pseudo*] and *L. casei* BL23 [pNGPHY*longum*], with significantly lower cell counts, which resulted in significantly lower TTA values and higher pH values (Table 2). Differences in bacterial cell counts were even observed during the first sampling, although all sourdoughs were initially inoculated with the same bacterial numbers per g.

3.5. Characteristics of the dough and bread

During the fermentation period pH, TTA and volume were determined. The control dough without added sourdough showed the highest pH value, 5.76 and the lowest TTA value of 3.51, as was expected (Table 2). However, samples made with sourdough inoculated with wild-type *L. casei* BL23 obtained the highest decrease in pH from 5.76 (control dough) to 5.09. In addition, these values were significantly lower than samples inoculated with lactobacilli strains carrying phytase genes. The inclusion of sourdough in the bread formulation did not cause any significant change in the dough pH evolution, which lasted unchanged during the whole yeast fermentation process for all the formulations. Dough volume of bread showed a progressive increase during the fermentation period, reaching optimum dough volume values of 105-107 ml after 1 hour at 28° C, which did not change by the sourdough inclusion. TTA values in fermented dough ranged from 3.51 to 5.69, showing a significant increase in samples inoculated with lactobacilli, due to the lactic acid production, compared to the values recorded in the control without sourdough. As was mentioned

above for the pH, TTA values for lactobacilli strains carrying phytase genes were significantly lower compared to samples containing the wild type strain.

As a general trend, the inclusion of sourdough to the bread formulation decreased the pH of bread compared to the control sample, whereas TTA values did not show significant differences between samples (Table 2). Nevertheless, TTA values in bread were lower than those obtained in fermented dough. The effect could be explained by weight loss during the breadmaking process. 95% of this loss is due to water evaporation, whereas 5% is due to losses in organic acids content, mainly in crust and outside crumb of the bread during baking (Sanz-Penella et al., 2012a). The observed lower TTA values in bread compared to dough could be attributed to the drop in acetic acid content during baking, due to its volatility compared to lactic acid (Barber et al., 1991). The pH in bread was close to the optimal pH for endogenous phytase and remained constant during mixing and fermentation allowing thus this activity.

3.6. Effect of sourdough on the *myo*-inositol phosphates levels

In order to determine the impact of sourdough inoculated with phytase-expressing lactobacilli in whole-grain breads, phytate and lower *myo*-inositol phosphates were measured (Table 3). As was mentioned above, the endogenous cereal phytase works during mixing, fermentation and first stage of baking as a consequence of a drop in pH. Thus, phytate degradation during cereal dough fermentation has been reported, which was correlated with the endogenous phytase activity (Reale et al., 2007). In our samples this effect was even observed in the control formulation without sourdough when we compared its phytate (InsP_6) contents to those present in flour (1.38±0.04μmoles/g (dry basis) and 1.53±0.01μmoles/g (dry basis), respectively). The addition of sourdough to the bread formula produced a greater and significant decrease in the amount of InsP_6 compared to the control sample (up to 38% InsP_6 degradation). Samples containing sourdough inoculated with the recombinant strains *L. casei* BL23 [*nisKR* pNGPHY*pseudo*] and *L. casei* BL23 [*nisKR* pNGPHY*longum*] showed the lowest InsP_6 contents. Nevertheless, the *myo*-inositol phosphates levels did not present significant differences between samples with *L. casei* BL23 [*nisKR* pNG8048e] and samples with *L. casei* BL23

[*nisKR* pNGPHY*pseudo*] or *L. casei* BL23 [*nisKR* pNGPHY*longum*]. No differences were also observed in the rest of lower *myo*-inositol phosphates (InsP_5 to InsP_3). The acidified control breads, supplemented with lactic acid to reach the pH found in sourdoughs, displayed a lower degree of hydrolysis but, again, at pH 4 there were no statistically significant differences compared with the samples fermented with lactobacilli. The results indicated that bifidobacterial phytases were not being expressed or that they had a minor contribution to InsP_6 degradation in the dough matrix added with sourdough. The phytase activity in extracts prepared from sourdoughs fermented with strains *L. casei* BL23 [*nisKR* pNG8048e], *L. casei* BL23 [*nisKR* pNGPHY*pseudo*] and *L. casei* BL23 [*nisKR* pNGPHY*longum*] did not show significant differences (activities of 0,69; 0,44 and 0,56 µg P released $min^{-1}ml^{-1}$, respectively). In the sourdough samples degradation of InsP_6 was completed for all the strains after 18 hours of fermentation at 37 °C.

4. Discussion

Lactobacillus is a bacterial genus implicated in numerous food fermentation processes. Owing to its relevant role in sourdough fermentation and the need of a greater phytase activity for phytate degradation in breadmaking processes, phytase producing lactobacilli have been isolated and characterized for their use in these processes (Anastasio et al., 2010; Chaoui et al., 2003; De Angelis et al., 2003). However, most *Lactobacillus* strains typically responsible for sourdoughs fermentation lack phytase activity. Their phytase degrading capacity is limited and based on non-specific acid phosphatases that are able to hydrolyse phytates at a low rate (Haros et al., 2009; Palacios et al., 2005; Zamudio et al., 2001). The general lack of phytase in lactobacilli is also supported by the lack of phytase encoding genes in the currently available genomes. This correlated with the fact that lactobacilli displaying phytase-like activity were in some cases not able to degrade phytate when used in fermentation processes (Songré-Ouattara et al., 2010; Tang et al., 2010). Notwithstanding, some reports described that specific strains lowered phytate levels in phytate-containing foods (Anastasio et al., 2010; Fischer et al., 2014; Lopez et al., 2000).

Efforts have been made to construct phytase expressing lactobacilli, but the resulting strains have never been used in food applications The phytase gene of *Aspergillus ficuum* was expressed in *L. casei* (Zuo et al., 2010) but the capacity of the originated strain to degrade phytate was not evaluated. Also, *Lactobacillus gasseri* and *Lactobacillus reuteri* strains have been engineered to express phytase genes from *Bacillus subtilis* (*phyA*) (Askelson et al., 2014) and *Aspergillus fumigatus* (*phyW*) (Wang et al., 2014), respectively. In these examples the strains were administered to broiler chickens to study their effects in growth performance.

L. casei is not a typical species reported in sourdough fermentation. However, in some cases it has been isolated from sourdough samples (Gaggiano et al., 2007; Kitahara et al., 2005). This, together with the genetic amenability of strain BL23 and the fact that no lactobacilli with real phytase activity have been employed in a food process, prompted us to engineer this species in order to explore its capacity for phytate reduction by using a breadmaking model with

sourdough. This model was particularly suited for the biotechnological application of phytases, as phytate concentration in whole-grain products is very high and the endogenosus phytase activity of cereals is clearly not sufficient to reduce phytate to levels which are non-inhibitory for mineral bioavailability (Greiner and Konietzny, 2006; Sanz-Penella et al., 2012a). Here we showed that *L. casei* BL23 was able to express the phytase enzymes from *B. pseudocatenulatum* and *B. longum* spp. *infantis* by using the nisin-inducible system (NICE) for LAB (Mierau and Kleerebezem, 2005). These two bifidobacterial enzymes possess interesting qualities to be used in food products for human consumption (García-Mantrana et al., 2014; Sanz-Penella et al., 2012b). The *B. pseudocatenulatum* enzyme was efficiently expressed and the activity assays confirmed that part of the enzyme was present at the bacterial surface, showing that the *L. casei* cell-wall anchoring machinery was able to recognize its LPXTG signal (Muñoz-Provencio et al., 2012). This fact is crucial, as InsP_6 degradation has to take place extracellularly. We showed that *L. casei* expressing the *B. pseudocatenulatum* phytase efficiently degraded InsP_6 and accumulated InsP_3 in the growth medium. Therefore, the enzymes expressed in lactobacilli showed the same InsP_6 degrading characteristics already reported for the whole cells of bifidobacteria and for the purified enzymes (Sanz-Penella et al., 2009, 2012b; Tamayo-Ramos et al., 2012). Despite of the fact that no clear phytase activity could be measured in *L. casei* extracts carrying the *B. longum* spp. *infantis* phytase gene, this strain was able to slowly degrade InsP_6 in MRS medium, indicating that the phytase gene was being expressed at low level or that this phytase presented lower activity. These results were also in agreement with the fact that the purified enzyme from *B. pseudocatenulatum* has been proved to be superior to the *B. longum* spp. *infantis* phytase in degrading InsP_6 in several food matrices (García-Mantrana et al., 2014; Sanz-Penella et al., 2012b). The presence of plasmids expressing phytase resulted in detrimental effects on *L. casei* growth in laboratory medium (MRS) and this effect was more patent for the strain harboring pNGPHY*pseudo* plasmid, which gave the higher expression level of phytase.

The *L. casei* strains were used in sourdough fermentations for breadmaking. *L. casei* BL23 was able to develop in the sourdough to levels that were in the range of LAB counts found in other works (Hammes et al., 2005; Sanz-Penella

et al., 2012a). The efficient acidification of *L. casei* BL23 was in agreement with the presence of genes for the uptake and metabolism of maltose and maltotriose in its genome (Monedero et al., 2008). However, as also observed for growth in laboratory medium, phytase expression had an adverse effect on *L. casei* growth during sourdough development, resulting in lowered bacterial counts that were evident even at the beginning of the fermentation, indicating a toxic effect that probably reduced the *L. casei* viability.

Despite of the high capacity of the strains to degrade phytate in liquid medium, phytate removal in the final bread products was not enhanced by the recombinant strains and it was mainly related to acidification. Lactic acid production does not only participate in flavor, storability and nutritional and functional value of sourdough breads (Jekle et al., 2010; Liljeberg et al., 1995) but it also contributes to activate the endogenous phytase in cereals (Leenhardt et al., 2005; Reale et al., 2004, 2007). Thus, it has been reported that an acidification of the sourdough is sufficient to explain the partial phytate degradation (Leenhardt et al., 2005). This degradation is independent of the LAB employed in the sourdough fermentation and only relates to the endogenous phytase activity of each flour (Reale et al., 2007) which, again, questions the presence of phytase in wild lactobacilli.

The inability of *L. casei* strain harboring phytase genes to further reduce phytate levels in dough may respond to a deficient induction capacity of nisin in this kind of food matrix, as the measured phytate activity in sourdoughs did not differ between samples. Although many proteins and enzymes have been expressed in LAB by means of the NICE system (Mierau and Kleerebezem, 2005), induction was usually carried out in liquid laboratory medium and very few reports on the induction in complex food matrices (de Ruyter et al., 1997), as sourdough, are reported. In addition, to be effective phytases must have access to the phytate in the dough. The flour used in this study was a whole-wheat flour. Therefore, the sourdough mixture (flour plus water) was a suspension containing a high amount of bran particles. The enzymatic accessibility of phytates could be limited by these particles in the flour. Laurikainen et al. (1998) reported that part of the substrate may be inaccessible to the enzymes by steric hindrance. Other researchers have explored the ability of baker yeast with high phytase activity to reduce phytate levels during simulated digestion of wheat

gruel with similar negative results (Haraldsson et al., 2005). Therefore, this matrix seems especially refractory to this kind of approach and additional factors such as InsP_6 solubility and cell structure of the meal have to be considered. However, the purified bifidobacterial phytases have been employed in other whole-grain products with excellent results (García-Mantrana et al., 2014; Sanz-Penella et al., 2012b). This also points to low diffusion of the microbial produced enzyme in the whole-flour matrix as one likely cause of the poor efficacy. Finally, although the pH reached in doughs was optimal for the bifidobacterial phytases (Tamayo-Ramos et al., 2012), the presence of inhibitors of the activity of these enzymes cannot be excluded.

This investigation showed the ability of modified *L. casei* to produce enzymes with technological relevance in functional foods application. Nevertheless, the contribution of phytases from modified *L. casei* to phytate hydrolysis in bread was not satisfactory, resulting in products with a substantial amount of residual InsP_6. This was due to low expression/activity of the bifidobacterial phytases in the sourdough model or a lack of accessibility of the phytate present in the whole grain by the enzymes. Consequently, the phytate hydrolysis was mainly produced by activation of the cereal endogenous phytase as a consequence of the drop in pH. Current research is under way to test the efficacy of the constructed strains in reducing InsP_6 levels during the fermentation of alternative food matrices.

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563

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FIGURE LEGENDS

Figure 1.

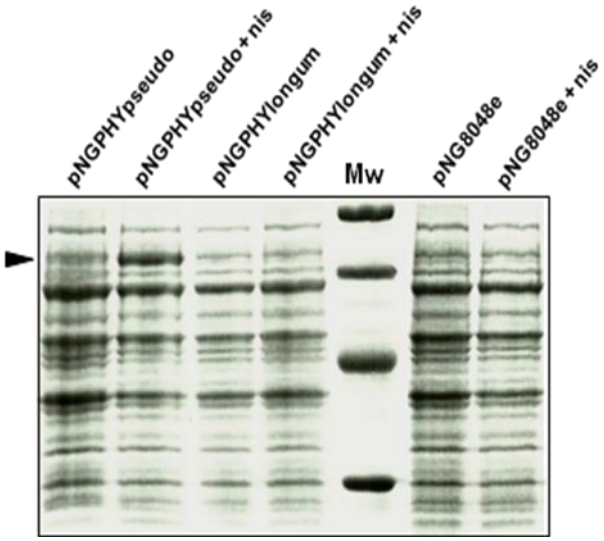
SDS-PAGE analysis of crude extracts from *L. casei* strains carrying different phytase expression plasmids. The clones were uninduced or induced by the addition of 20 ng/ml of nisin (nis). The black arrowhead points to the position of the phytase enzyme from *B. pseudocatenulatum* (pNGPHY*pseudo* plasmid). Mw is a protein molecular weight standard (97, 66, 45 and 30 kDa, respectively).

Figure 2.

Growth and pH evolution of *L. casei* strains in MRS medium under inducing conditions. The inoculated medium contained 2 mM phytate and 20 ng/ml nisin. The different plasmids carried by *L. casei* BL23 [*nisKR*] are indicated.

Figure 3.

Evolution of *myo*-inositol phosphates in *L. casei* strains grown in MRS medium containing 2 mM phytate. InsP_6, InsP_5, InsP_4 and InsP_3 are *myo*-inositol hexakis-, pentakis-, tetrakis- and tri-phosphate, respectively. The inoculated medium contained 20 ng/ml nisin as inducer of phytase expression. The different plasmids carried by *L. casei* BL23 [*nisKR*] are indicated.



770

771 Fig. 1

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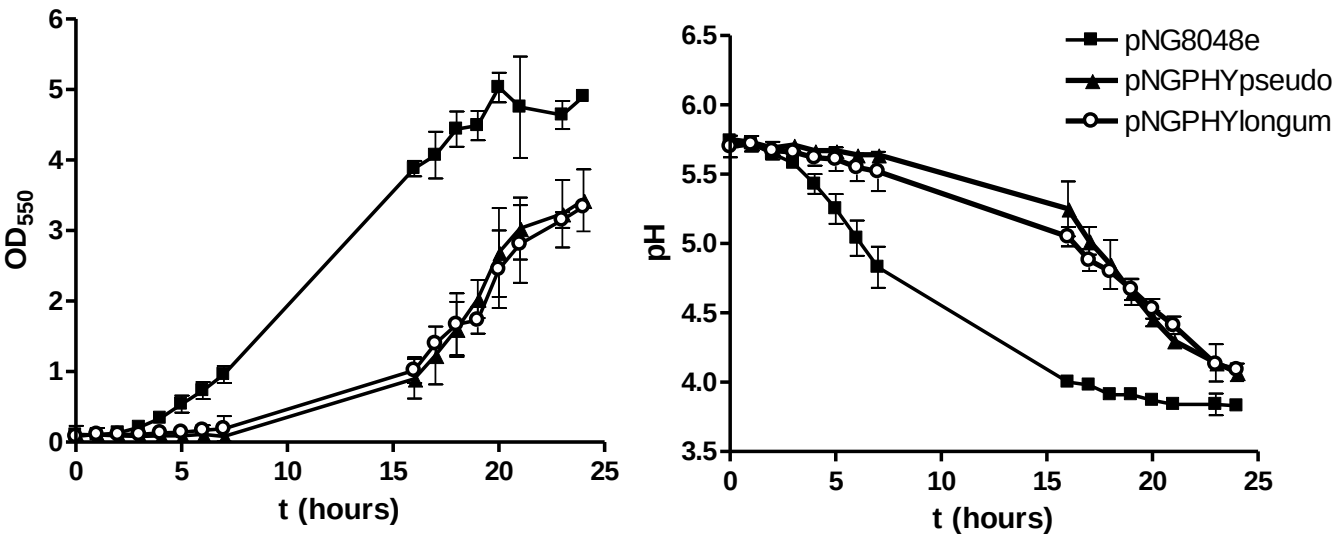
OD550
t (hours)
pH
t (hours)
pNG8048e
pNGPHYpseudo
pNGPHYlongum

Fig. 2

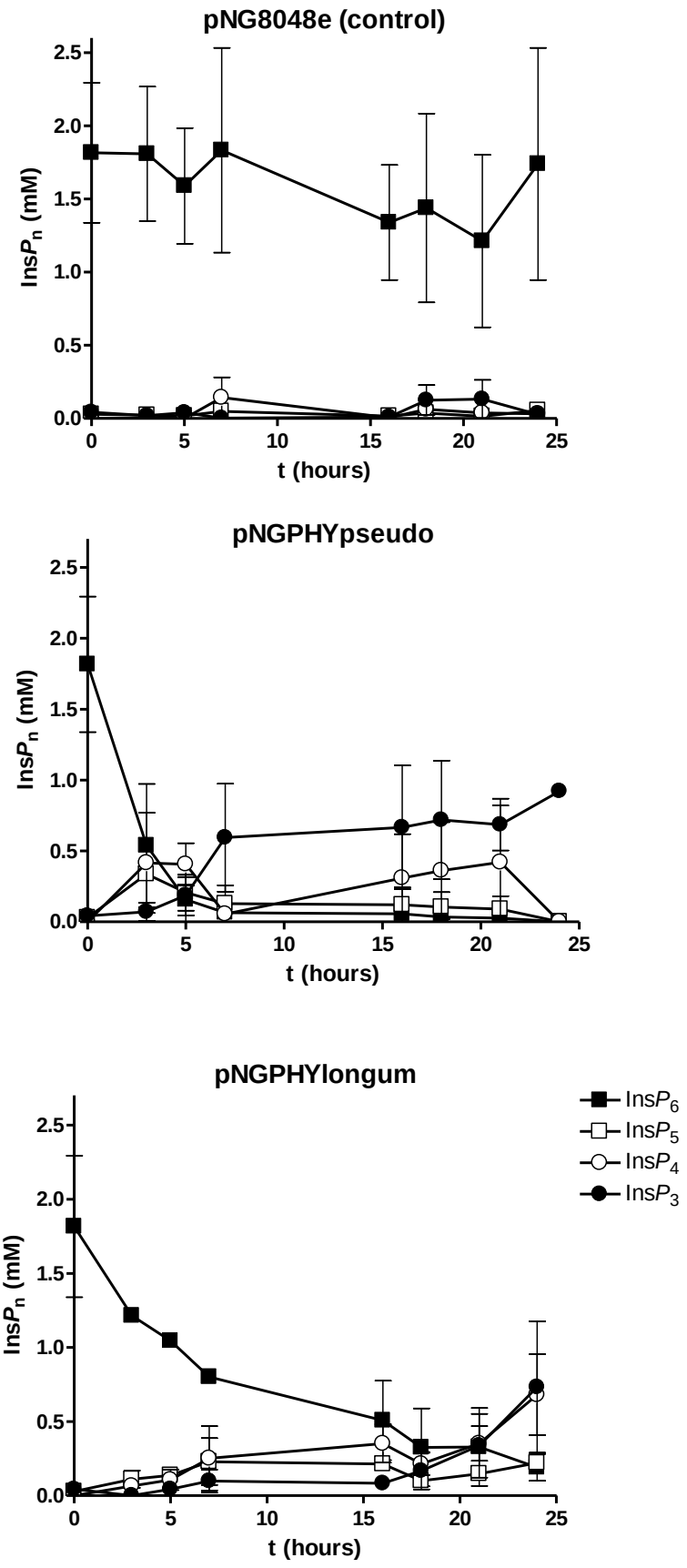
pNG8048e (control)
pNGPHYpseudo
pNGPHYlongum
InsP$_n$ (mM)
t (hours)
InsP$_6$
InsP$_5$
InsP$_4$
InsP$_3$

Fig. 3