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Assessment of Iron Bioavailability in Whole Wheat Bread by Addition of Phytase-Producing Bifidobacteria

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ABSTRACT: In this study, the influence of phytase-producing *Bifidobacterium* strains during the breadmaking process (direct or indirect) on final bread Fe dialyzability and ferritin formation in Caco-2 cell as a measure of cell Fe uptake was assessed. The addition of bifidobacteria significantly reduced the InsP_6 + InsP_5 concentrations compared to control samples. Fe-dialyzable contents for samples with bifidobacteria were increased 2.3−5.6-fold, and dialyzability was improved by 2.6−8.6% compared to controls. However, this was not reflected in an increase of Fe uptake by Caco-2 cells as was predicted by the phytate/Fe molar ratios. The results demonstrated the usefulness of phytase-producing bifidobacteria to reduce phytate during the breadmaking process and to increase Fe accessibility, although the effects appeared to be still insufficient to improve Fe bioavailability in Caco-2 cells. Further refinement of the use of phytase-producing bifidobacterial strains and/or breadmaking technological processes is deserved for improving Fe uptake.

KEYWORDS: *whole wheat bread, sourdough, Bifidobacterium, phytate-degrading enzyme, Fe dialyzability, Fe uptake, Caco-2 cells*

INTRODUCTION

For many years, public health concerns have focused on Fe deficiency because this is a health problem in most countries of the world. Fe deficiency results in the depletion of body Fe stores and is believed to affect 20−50% of the world's population. This problem is even more pronounced in populations consuming monotonous plant-based diets with little meat, where most of the dietary Fe is in nonheme form. Nonheme Fe is found mainly in plant foods such as cereals, legumes, fruits, and vegetables, and its absorption is often <10%.[1] In addition, the absorption of this kind of Fe can be affected by many dietary components that are able to act as enhancers or inhibitors.[1]

Bread is a staple food in many countries and is therefore of global importance in international nutrition. Nevertheless, the presence of phytic acid or phytate (*myo*-inositol hexaphosphate, InsP_6) in whole grains, which have strong chelating properties, interferes with mineral absorption by forming insoluble complexes with nutritionally important minerals such as Fe, Zn, and Ca.[2] Food fortification, such as fortified cereal flours, is the most practical and best long-term strategy to prevent Fe deficiency. However, Fe addition often causes unacceptable sensory changes in food vehicles and has low bioavailability.[3] Many studies have indicated that hydrolysis of phytate is a way to overcome its negative effect on mineral absorption,[4,5] so that substantial decreases of phytic acid in cereal products could improve Fe availability to the consumers. Cereal grains contain endogenous phytase, an enzyme capable of hydrolyzing phytate to free inorganic phosphate and lower inositol phosphate esters, thus decreasing or eliminating the antinutritional effect of phytates.[4] However, in cereal grain products phytates remain at high concentrations due to inefficient enzymatic degradation.[6] To overcome this limitation, the addition of exogenous enzymes, mainly from fungal origin, to increase phytase activity has been the best strategy to reduce the phytate content of cereals. The effect of phytase from *Aspergillus niger* in InsP_6 degradation during the breadmaking process was studied by Türk and Sandberg[7] and later by Haros et al.[6] Its use to reduce phytate content in bread was an effective method for increasing Fe absorption in humans.[5] The addition of sourdough for breadmaking has also been used for InsP_6 degradation by activation of cereal endogenous phytase due to the decrease of pH.[8] Some studies have improved Fe absorption in humans by reducing phytate via the addition of fungal phytase or promoting the endogenous phytase activity into food processing.[9−11] Nevertheless, human studies are often impractical because they are costly, lengthy, and complex.

The Caco-2 cell line has been used extensively as an in vitro method to assess Fe bioavailability. A strong correlation has been found between the published human absorption data and Fe uptake by the Caco-2 cells, indicating the usefulness of this method in assessing human Fe absorption.[12] The study of the phytate effects on Fe bioavailability in whole grain products by the Caco-2 cell line has been well documented.[13−15] Some years ago it was reported that strains of the *Bifidobacterium* genus have phytase activity,[16,17] suggesting their possible utility in the production of bakery products with low InsP_6 levels.[18,19] The use of fungal phytases is currently approved in animal feed, but so far has not been certified for use in foods intended for human consumption. Thus, the use of bifidobacteria, which are GRAS/QPS (generally regarded as safe/qualified presumption of safety) microorganisms, could be a strategy particularly suitable to reduce the phytate content in whole grain products for human consumption.

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The objective of the present investigation was to study the utility of phytase-producing *Bifidobacterium* strains as starter in the breadmaking process (direct or indirect) and to assess their influence on Fe dialyzability and ferritin formation in Caco-2 cell as a measure of cell Fe uptake. The results were compared with a positive control (sample with commercial fungal phytase) and a negative control (sample without either exogenous phytase or bifidobacteria).

MATERIALS AND METHODS

Materials. *Samples.* Whole wheat breads and whole wheat sourbreads were the materials of the current investigation made according to the procedures described by Sanz-Penella et al.[18,19] The formulation of whole wheat breads (Ctrl and Bif) on flour basis was 100% of whole wheat flour, 2.5% compressed yeast, 1.8% sodium salt, 65% tap water, 0.01% ascorbic acid, and $\sim 10^8$ CFU of phytase-producing bifidobacteria (*Bifidobacterium infantis* ATCC 15697 and *Bifidobacterium pseudocatenulatum* ATCC 27919) per gram of flour.[18] The formulation of whole wheat sourbreads (0-SD, 10-SD, and 20-SD) was the same as above-described with the exception of the inclusion of bifidobacteria, which were included as prefermented dough (sourdough) at different levels: 0, 10, and 20% on a flour basis. The sourdough formulation consisted of a mixture of whole wheat flour and water (1:2, v/v) with an inoculum $\sim 10^8$ CFU of *B. pseudocatenulatum* ATCC 27919 per gram of flour and was incubated for 18 h at 37 °C in anaerobic conditions.[19] The control acid sourdough for preparing the 20-SD-AcC bread sample consisted of the same formulation and conditions as described above without the addition of a *Bifidobacterium* strain, including a mixture of antibiotics; the pH was adjusted with a mixture of lactic and acetic acids to reach the same pH of sourdough biologically acidified with bifidobacteria.[19] Commercial phytase was added as positive control to dough formulations (Phy and 0-SD-Phy bread samples) prepared in parallel at a concentration equivalent to 10 times the flour activity.

Reagents. Digestive enzymes and bile salts were purchased from Sigma Chemical (St. Louis, MO, USA): porcine pepsin (EC 232-629-3), porcine pancreatin (EC 8049-47-6), and porcine bile extract (EC 8049-47-6). Working solutions of these enzymes were prepared immediately before use. All glassware used in the sample preparation and analyses was soaked in 10% (v/v) of HCl concentrated (37%) for 24 h and then rinsed with deionized water (18 MΩ cm) (QRG, quality reagent grade) before being used to avoid mineral contamination.

Methods. *Fe Content Determination.* Total Fe content in breads was measured by atomic absorption spectrophotometry (AAS) with a model 2380 instrument (Perkin-Elmer, Norwalk, CT, USA). Prior to the Fe AAS determination, all samples were mineralized by adding 3 mL of HNO_3; samples were then heated to dryness and placed in a muffle furnace model Controller B170 (Nabertherm GmbH, Germany) at 450 °C for 24 h. The process was repeated as many times as necessary to obtain a white residue. After cooling, the residue was dissolved with 1 mL of HCl concentrated (37%) and 10 mL of distilled deionized water.[20]

Determination of myo-Inositol Phosphates. Initial InsP_6 and InsP_5 concentration in flour and its remaining concentration in bread were measured according to the high-pressure liquid chromatographic method described by Türk and Sandberg[7] and later modified by Sanz-Penella et al.[21]

In Vitro Digestion. Bread samples were subjected to a simulated gastrointestinal digestion procedure as described elsewhere[12] with slight modifications (Figure 1). Pepsin (800−2500 units/mg protein), pancreatin (activity, 4× USP specifications), and bile extract were demineralized with Chelex-100 before use. Briefly, 6 mL of an isotonic saline solution (140 mM NaCl, 5 mM KCl) was added to sample breads (1.000 ± 0.001 g), and the mixtures were acidified to pH 3.0 with 0.1 mol/L HCl. Then, 0.96 mL of a pepsin solution (0.01 g/mL)

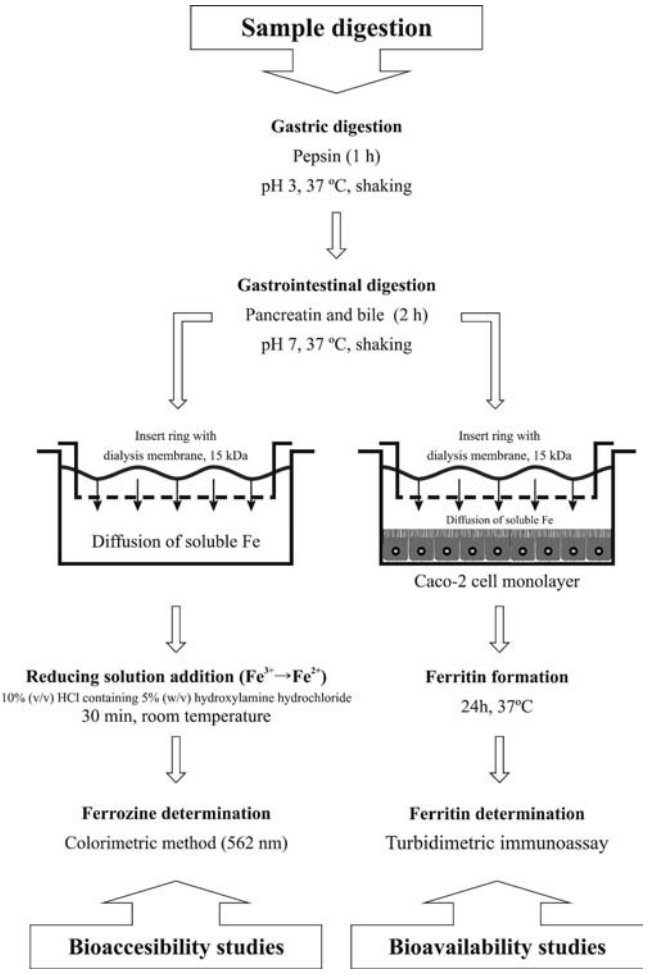

Figure 1. Schematic representation of the in vitro digestion of samples.

was added, and the mixture was incubated for 1 h at 37 °C (gastric digestion). Afterward, the digest was adjusted to pH 5.5 with 1 mol/L $NaHCO_3$. The intestinal phase of digestion was then initiated with the addition of 1.19 mL of a pancreatin−bile extract solution (0.004 g/mL pancreatin and 0.025 g/mL bile) and adjusted to pH 7.0 with 0.5 mol/L NaOH. The volume was brought to 10 mL, and the gastrointestinal digestion was carried out in the upper chamber of a bicameral system created with a 15000 Da molecular weight cutoff dialysis membrane attached to a plastic insert ring to separate the "gastrointestinal digest" from the Caco-2 cell monolayer. Next, an additional 1 mL of minimum essential medium (MEM, Gibco) was added to the lower chamber, and the plates were returned to the incubator for an additional 22 h. The next day, the cells from each well were washed twice with the isotonic saline solution and harvested in 2 mL of QRG water. Control solutions containing digestive enzymes but no sample were used throughout the experiments in parallel to digestions of breads. Cell ferritin formation was used as a measure of cell Fe uptake.

Cell Culture and Ferritin Analysis in Cell Monolayer. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) at passage 17 and used in experiments at passage 33−38. Cells were maintained with Dulbecco's modified Eagle's medium (DMEM, Gibco) under conditions previously described.[22] For the assays, Caco-2 cells were seeded at 50000 cells cm^{-2} in collagen-treated 6-well culture plates (Costar, Cambridge, MA, USA) and were grown with DMEM. On the day prior to the experiments, the DMEM was replaced by 2 mL of MEM (Gibco), and then the cells were returned to the incubator. A latex-enhanced turbidimetric immunoassay (Ferritin-turbilatex, Spinreact, Girona, Spain) was used to measure Caco-2 cell ferritin content. The concentrations of ferritin were normalized by determination of total protein content in cell

cultures. Control cells, exposed to in vitro digestions of control solutions containing digestive enzymes but no bread sample, were used throughout. Baseline cell ferritin in cultures grown in MEM averaged 3.84 ng/mg cell protein. Samples were analyzed in triplicate.

Quantification of Soluble Fe by the Ferrozine Assay. For the experiments the in vitro digestion was carried out as described above, but 1 mL of isotonic saline solution was added on the bottom chamber of the bicameral system, instead of Caco-2 cells growing. The ferrozine assay[23] with slight modifications was used to determine the total amount of soluble Fe present in the dialysates.[15] Aliquots (0.1 mL) of the stock reducing solution (10% (v/v) HCl containing 5% (w/v) hydroxylamine hydrochloride) were added to each dialysate (1 mL), and the mixture was allowed to react at room temperature for 30 min. Then, 0.1 mL of a ferrozine solution (5 mg/mL) and the 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (final concentration of 1 M) were added to each dialysate. After 1 h of incubation at room temperature, the absorbance (at 562 nm) (spectrophotometer model 8453, Hewlett-Packard, Waldbronn, Germany) was measured to quantify the total Fe content. For the quantification a standard curve was prepared with an Fe atomic absorption standard solution (1000 μg Fe/mL in 1% HCl) (Titrisol; Merck, Barcelona, Spain) treated as described above.

Statistical Analysis. Multiple sample comparison of the means (ANOVA) and Fisher's least significant differences (LSD) were applied to establish statistical significant differences between treatments. All statistical analyses were carried out with the software Statgraphics Plus 7.1 (Bitstream, Cambridge, MN, USA), and the significance level was established at $P < 0.05$.

RESULTS AND DISCUSSION

Fe and Phytate Contents. The use of phytase-producing bifidobacteria strains in the direct or fermented sourdough breadmaking processes evaluated had an additional positive effect, reducing the InsP_5 and InsP_6 levels in comparison to control samples (Figures 2A and 3A, respectively). The direct breadmaking process with the addition of bifidobacteria significantly reduced the InsP_6 + InsP_5 concentrations by 32.0%, but the use of fermented sourdough had a more notable effect, reducing the amount of InsP_6 and InsP_5 by 44.6% (10-SD) and 64.2% (20-SD), respectively. Bread samples made with chemically acidified sourdough (20-SD-AcC) exhibited significantly lower InsP_6 and InsP_5 hydrolysis than 20-SD, which indicates the higher InsP_6- or InsP_5-degrading capacity in fermented sourdough inoculated with phytase-producing bifidobacteria. Breads formulated with fungal phytase (0-SD-Phy) showed a significant reduction of InsP_6 + InsP_5 compared to control (0-SD), similar to that found in 20-SD samples. In the direct process the addition of fungal phytase (Phy) decreased to almost negligible levels the InsP_6 and InsP_5 concentrations. Phytase-producing bifidobacterial strains emerged as promising ingredients in bran-enriched wheat breads without affecting either sensory or technological qualities of the final product.[18] Their use was motivated to reduce the InsP_6 and InsP_5 concentrations in breads because of the marked inhibitory effect of these *myo*-inositol phosphates of Fe bioavailability. However, the use of bifidobacteria in an indirect breadmaking process was demonstrated to effectively reduce the InsP_6 and InsP_5 concentrations and increase those of InsP_4 and InsP_3.[19] The data obtained in the current study indicate the preferential hydrolysis of InsP_6 and InsP_5 when using the direct or indirect breadmaking process, respectively (Figures 2A and 3A). This effect can have important consequences in Fe bioavailability because not only InsP_6 but also InsP_5 exerts negative effects in Fe bioavailability.[24] The important influence of these *myo*-inositol phosphates in the

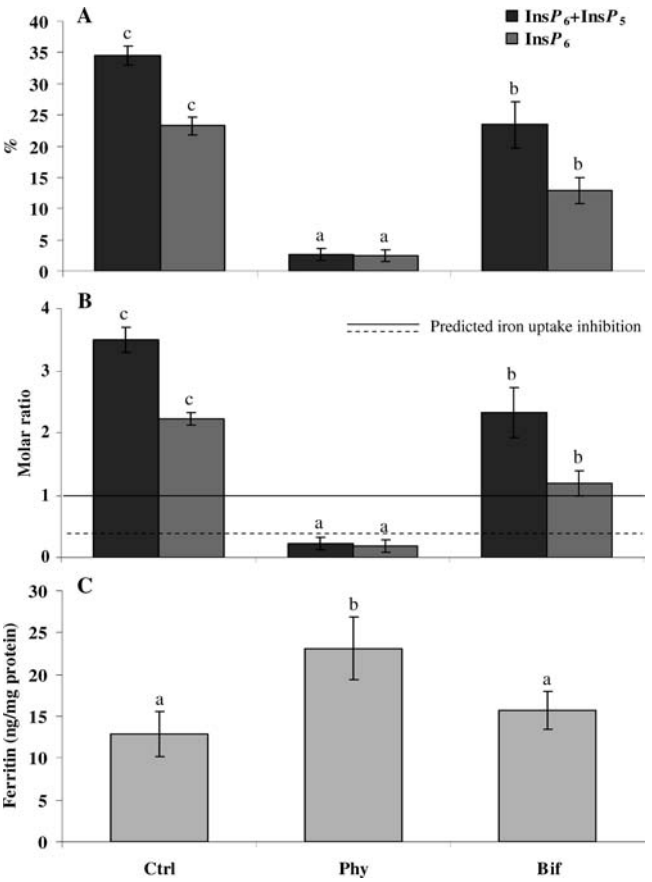

Figure 2. Effect of exogenous phytase addition during a direct process of whole wheat bread on (A) InsP_6 and InsP_5 + InsP_6 residual percentages in breads compared to values found in flour, (B) InsP_6/Fe and InsP_6 + InsP_5/Fe molar ratios, and (C) ferritin formation in Caco-2 cells exposed to gastrointestinal digestion of whole wheat breads. Ctrl, control sample; Phy, sample with fungal phytase; Bif, sample with phytase-producing bifidobacteria; InsP_6, *myo*-inositol hexakisphosphate; InsP_5, *myo*-inositol pentakisphospahte. Mean ± SD, $n = 3$, bar values with the same color and different letters are significantly different ($P < 0.05$).

nutritional quality of breads, concerning micronutrients, has been recognized and especially the significant impact on health status, growth, and development of populations that rely on bread as a staple food, especially in developing countries or populations at risk.[10,24] The bread samples made either through a direct breadmaking process using bifidobacterial strains or an indirect process with different proportions of wheat sourdough inoculated with bifidobacteria had similar Fe concentrations ranging between 31.7 and 35.8 μg/g (dry matter, dm). Taking into account the Fe levels in bread and its intake recommendation of 250 g per day by the World Health Organization, whole wheat bread could provide >60% of the dietary reference intakes (DRIs) for this micronutrient to males.[25] On the other hand, in the case of females, the contribution could range between 28 and 38% because of higher recommendations established according to their physiological requirements.[25] However, the Fe content in food does not show a linear correlation with its availability due to inhibiting factors, such as the presence of phytates, which could be predicted by the phytates/Fe molar ratio (Figures 2B and 3B).

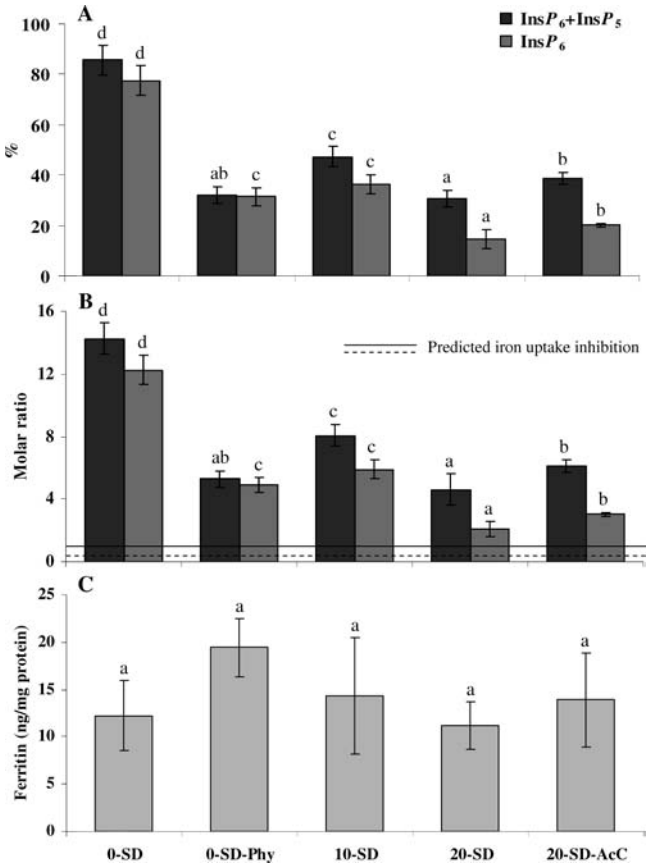

Figure 3. Effect of exogenous phytase addition during an indirect process of whole wheat bread on (A) InsP_6 and InsP_5 + InsP_6 residual percentages in breads compared to values found in flour, (B) InsP_6/Fe and InsP_6 + InsP_5/Fe molar ratios, and (C) ferritin formation in Caco-2 cells exposed to gastrointestinal digestion of whole wheat breads. 0-SD, control sample; 0-SD-Phy, sample with fungal phytase; 10-SD and 20-SD, breads made with 10 and 20% of sourdough inoculated with phytase-producing bifidobacteria, respectively; 20-SD-AcC, bread made with 20% of sourdough chemically acidified; InsP_6, *myo*-inositol hexakisphosphate; InsP_5, *myo*-inositol pentakisphosphate. Mean ± SD, n = 3, bar values with the same color and different letters are significantly different ($P < 0.05$).

Fe Dialyzability. Fe dialyzability has been used as an estimator of Fe availability from dephytinized breads.[26] The influence of bifidobacterial strains in the direct breadmaking process or the inoculation of fermented sourdough with bifidobacteria in Fe-dialyzable contents is shown in Table 1. Fe-dialyzable contents were increased in both the direct and indirect breadmaking processes, although there were slightly significant ($P < 0.05$) differences in the Fe contents loaded. The addition of phytase-producing bifidobacterial strains in the direct breadmaking process increased 2.3-fold the Fe-dialyzable contents, compared to controls. Of note, the inoculation of fermented sourdough with bifidobacteria produced a most marked increase of Fe-dialyzable contents (5.6-fold) relative to controls. However, this effect was less marked (3.8-fold) in bread samples formulated with 20-SD-AcC. The addition of fungal phytase presented significantly increased Fe dialyzable contents only in the positive control made for the indirect process respecting control sample. When these results were expressed as a percentage of the Fe content loaded in the upper chamber (dialyzability, %), the direct breadmaking process had less influence on Fe dialyzability, increasing this value by 2.6%.

Table 1. Dialyzable Fe and Dialyzability Percentages in Whole Wheat Breads[a]

whole wheat bread[b]	Fe in the upper chamber (μg)	dialyzable Fe (μg/mL)	dialyzability (%)
Ctrl	4.875 ± 0.002 c	0.107 ± 0.036 a	2.2 ± 0.7 a
Phy	6.314 ± 0.003 i	0.125 ± 0.016 a	2.0 ± 0.2 a
Bif	5.084 ± 0.003 d	0.243 ± 0.051 c	4.8 ± 1.0 b
0-SD	4.800 ± 0.003 b	0.104 ± 0.009 a	2.2 ± 0.2 a
0-SD-Phy	5.260 ± 0.002 f	0.151 ± 0.012 b	4.0 ± 0.3 b
10-SD	4.754 ± 0.001 a	0.294 ± 0.047 c	6.2 ± 0.9 c
20-SD	5.369 ± 0.002 g	0.578 ± 0.033 e	10.8 ± 0.6 d
20-SD-AcC	5.234 ± 0.002 e	0.398 ± 0.064 d	7.4 ± 1.2 c

[a]Mean ± standard deviation, n = 3. Values followed by the same letter in the same column are not significantly different at the 95% confidence level. [b]Ctrl, control sample; Phy, sample with fungal phytase; Bif, sample with phytase-producing bifidobacteria; 0-SD, control sample; 0-SD-Phy, sample with fungal phytase; 10-SD and 20-SD, breads made with 10 and 20% of sourdough inoculated with phytase-producing bifidobacteria, respectively; 20-SD-AcC, bread made with 20% of sourdough chemically acidified.

Nevertheless, increasing proportions of fermented sourdough could be associated with higher dialyzability, showing an increases of 4.0, 8.6, and 5.2 percentage points for SD-10, SD-20, and 20-SD-AcC, respectively. The use of fungal phytase showed no influence on Fe dialyzability of breads or slight increases by 1.8% (Table 1).

Fermentation processes improve Fe solubility,[27] but the reduction of pH values in bread samples by sourdough addition can favor the activity of endogenous phytase,[28] reducing the inhibitory effect of InsP_6 and/or InsP_5 on Fe availability. According to the obtained results, the lesser influence of the use of phytase-producing bifidobacterial strains than fermented sourdough in breadmaking processes seems likely caused by the lower reduction of pH values in bread samples.[18,19] This effect has important influence favoring the activity of the endogenous phytase activity, which results markedly affected by moderate decrease of pH.[29] In this study, the increased endogenous phytase activity is supported by the preferential hydrolysis of InsP_6 and InsP_5 that could explain the increased Fe dialyzability (Table 1). This behavior is concordant with previous studies in which an improved phytase activity in whole grain cereal products was associated with increased Fe dialyzability values.[13,14] In addition, the fermentation of sourdough with bifidobacteria led to the production of lactic and acetic acids in the fermented dough[19] that help to solubilize Fe, as previously indicated.[27] Porres et al.[26] demonstrated the positive effect of citric acid addition, alone or together with phytase, on Fe dialyzability.

Fe Uptake by Caco-2 Cells. Fe-deficient cell cultures produce ferritin as a response to the micronutrient that has been internalized into cells.[12] Ferritin concentrations in Caco-2 cell cultures exposed to the digests of the different bread samples are shown in Figures 2C and 3C. The basal level quantified in untreated cell cultures grown in MEM was 3.84 ± 2.47 ng/mg cell protein. Additionally, a positive control using Caco-2 cells grown in MEM with the addition of ascorbic acid + Fe was run, showing ferritin concentrations above 400 ng/mg cell protein. Cell cultures did not show significant increases in ferritin formation compared to controls despite their being exposed to breads produced by a direct or an indirect breadmaking process. Taking together these results and the

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different Fe dialyzability (%) values from the products tested, it can be assumed that only a minor fraction of the micronutrient released from breads remains bioavailable to Caco-2 cells. In contrast, breads made by the direct process and added fungal phytase exhibited higher ($P < 0.05$) ferritin concentrations, indicating an improved Fe bioavailability. These data can be explained considering the marked extent of InsP_6 hydrolysis below the critical values established as inhibitory of Fe uptake, InsP_6 concentrations of 0.135 μmol/g of bread[5] or phytate/Fe molar ratios lower than 1 or preferably lower than 0.4.[11] The amount of InsP_6 on Phy sample was 0.142 μmol/g of bread, close to this value; however, the rest of the samples analyzed in this study registered values at least 5-fold above this limit. Figures 2B and 3B show the InsP_6/Fe and InsP_6 + InsP_5/Fe molar ratios from breads. In all breads analyzed, these molar ratios observed were >0.4, except for the Phy sample, from which could be expected the only sample to have improved Fe availability. The samples Bif and 20-SD, made with phytase-producing *Bifidobacterium*, showed InsP_6/Fe ratios between 1 and 2. On the other hand, although organic acids could help to enhance Fe uptake,[30] no positive influence on the products made with sourdough fermented with bifidobacteria was detected.

The inhibitory effect of InsP_6 and InsP_5 on Fe bioavailability is well-known.[5,11] Nowadays, most current research attempts using dephytinization processes to abolish or minimize the negative effect of these compounds on Fe bioavailability. One of the major drawbacks or inconveniences is that complete dephytinization can be achieved only with the addition of fungal phytases to bread formulation;[5] however, these enzymes are not considered for human consumption, and the food industry requires the development of alternative processes to reduce the concentration of InsP_6 and InsP_5 in cereal byproducts. In the current study, the strategy applied was the use of phytase-producing bifidobacterial strains in two different technological approaches, direct and indirect, to the breadmaking process. The data obtained demonstrated the usefulness of these bifidobacterial strains to reduce the concentration of InsP_6 and InsP_5 in whole wheat breads, although the extent of reduction seems to be insufficient to improve Fe bioavailability to Caco-2 cells. Interestingly, the inclusion of phytase-producing bifidobacterial strains had a positive effect on Fe availability, increasing the soluble fraction of the micronutrient released from breads, the use of fermented sourdough being more effective than the direct breadmaking process. This observation is important because the static nature of the in vitro model used should not be ruled out and the increased soluble fraction of the micronutrient could be more effectively absorbed in vivo because of the larger absorption area. Another aspect of interest is the fact that phytase-producing bifidobacterial strains can behave differently if they participate in different breadmaking technological processes where further refinement of these processes is encouraged.

In summary, the use of phytase-producing bifidobacterial strains significantly reduced the InsP_6 and InsP_5 concentrations in both the direct and indirect breadmaking processes, demonstrating their usefulness to reduce phytates in breads. Fe availability was increased in both processes; however, this was not reflected in an increase of Fe uptake by Caco-2 cells, probably due to insufficient phytate reduction below inhibitory limits to improve Fe bioavailability. To optimize phytate degradation by phytase-producing bifidobacterial strains during food processing, the use of a long fermentation process or purified phytase from bifidobacteria should be evaluated to reduce phytates more effectively and improve mineral bioavailability.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS AND NOMENCLATURE

0-SD, control bread; 0-SD-Phy, bread with fungal phytase; 10-SD, bread made with 10% of sourdough inoculated with phytase-producing bifidobacteria; 20-SD, bread made with 20% of sourdough inoculated with phytase-producing bifidobacteria; 20-SD-AcC, breads made with 20% of sourdough chemically acidified; AAS, atomic absorption spectroscopy; Bif, bread with phytase-producing bifidobacteria direct process; Ctrl, control bread direct process; dm, dry matter; DMEM, Dulbecco's modified Eagle's medium; EC, Enzyme Commission; GRAS/QPS, generally regarded as safe/qualified presumption of safety; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-pressure liquid chromatography; InsP_6, phytic acid, *myo*-inositol hexakisphosphate or phytate; InsP_5, *myo*-inositol pentakisphosphate; InsP_4, *myo*-inositol tetrakisphosphate; InsP_3, *myo*-inositol triphosphate; MEM, minimum essential medium; NAS, National Academy of Sciences; Phy, bread with fungal phytase direct process; QRG, quality reagent grade; SD, standard deviation; USP, U.S. Pharmacopeia.

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