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ORIGINAL PAPER

# Application of phytases from bifidobacteria in the development of cereal-based products with amaranth

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**Abstract** The effects of the inclusion of purified phytases from *Bifidobacterium longum* spp. *infantis* ATCC15697 and *Bifidobacterium pseudocatenulatum* ATCC27919 on phytate (Ins$P_6$) levels were analyzed during breadmaking process. Two different levels of whole amaranth (*Amaranthus cruentus*) flour (25 and 50 %) were used in bread dough preparation, and they were compared to control doughs made with 100 % wheat flour and 100 % whole wheat flour. Bread samples made with 50 % of amaranth flour showed a significant decrease in technological quality parameters in comparison with control white breads. However, a 25 % of amaranth flour improved the nutritional value of the bread, with only a slight depreciation in the quality. Addition of bifidobacterial phytases resulted in higher Ins$P_6$ degradation compared with a commercial fungal phytase, without affecting the bread quality. Ins$P_6$ reduction was especially efficient in breads with 25 % amaranth, leading to Ins$P_6$ levels below the threshold of mineral bioavailability inhibition for Fe and Zn in human nutrition.

**Keywords** Whole amaranth flour · Bread · Phytates · Bifidobacterial phytase · Mineral availability

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## Introduction

Cereals and their derivatives constitute a considerable part of a balanced diet and are at the first level in the food pyramid, according to the international dietary guidelines. Cereal grain products are divided into two categories: refined and whole grain products. Refined grains possess a limited nutritional value, whereas whole grains are a better source of fiber, vitamins and minerals and contain also a variety of other phytochemicals and antioxidants [1–3]. Due to this fact, an increasing interest exists in the development of dietary fiber-rich cereal products with this nutritional added value. In addition, epidemiological studies confirmed that consumption of whole grain cereals and pseudocereals is associated with reduced risk of type-2 diabetes, constipation, obesity, cardiovascular diseases and some types of cancer [4].

One possibility for increasing the nutritional value of bakery products could be the inclusion of whole amaranth grain in their formulations. *Amaranthus* is a common flowering plant genus that yields the nutritious staple amaranth grain, a pseudocereal [5]. This genus comprises more than 60 species, but only three of them are usually used for human consumption: *Amaranthus caudatus, Amaranthus cruentus* and *Amaranthus hypocondriacus* [5]. The nutritional quality of amaranth grain is significantly higher when compared with most cereal seeds such us wheat, barley or rice. Compared to wheat flour, *A. cruentus* flour possesses a significantly higher protein (14–18 %), lipid (6–8 %) and dietary fiber (11–23 %) contents, and it also contains vitamins, minerals and other biologically active compounds [6, 7]. The balanced essential amino acid composition is also a characteristic of amaranth seeds [8]. Furthermore, amaranth grain protein is rich in lysine content, a deficient amino acid in cereals. The lipid content (6–8 %) is also higher than that

of most cereal grains, with an enhanced presence of unsaturated oil (45 % linoleic acid) which plays an important role in cardiovascular health [9]. In addition to these nutritional components, amaranth seed also contains other organic compounds such as tocotrienols, squalene and phytosterols, involved in the metabolism of cholesterol, and able to lower LDL-cholesterol levels and plasma triglycerides concentration [10]. Aside from these substances, amaranth seeds count with bioactive peptides with antihypertensive effect and with the anticarcinogenic properties of the peptide lunasin [11] and their lack of gluten makes them ideal for the manufacture of products for celiacs. However, whole grains contain significant amounts of phytic acid [*myo*-inositol (1,2,3,4,5,6)-hexakisphosphate or Ins$P_6$] or its salts (phytates), a well-known inhibitor of mineral, proteins and trace elements bioavailability [12]. The negative effects of phytates in human nutrition are more relevant in developing countries, in risk populations such as pregnant women or those who follow an unbalanced diet and also in animal feed [13, 14]. The phytic acid is an organic acid common in plants in which it functions in the storage of phosphorus and cations for growth [15]. Phytic acid has a strong ability to form complexes with bi/multivalent metal ions, especially iron, calcium and zinc [16, 17]. A partial dephosphorylation of phytates not only decreases this negative effect, but also generates lower *myo*-inositol phosphates with potential benefits to human health [18, 19]. Phytases are a class of phosphatases that catalyze the sequential hydrolysis of Ins$P_6$ to lower *myo*-inositol phosphates and inorganic phosphate [19]. There are several strategies to increase the phytase activity present in raw materials. Thus, cereals have their own endogenous phytase and the addition of sour dough into breadmaking process improves the degradation of phytates, due to the decrease in pH [20]. Aside from the own cereal phytase, the addition of an exogenous phytase (generally from microbial sources) is other alternative. This strategy is broadly used in feed production for monogastric animals, and it has also been explored in the production of cereal and legume foods for human consumption [21]. Phytase activity has been described for food-grade strains of the genus *Bifidobacterium*, and these bacteria have been applied in both direct and indirect breadmaking processes [20, 22]. *Bifidobacterium longum* spp. *infantis* ATCC15697 and *Bifidobacterium pseudocatenulatum* ATCC27919 bacterial cells demonstrated their efficacy in the reduction of phytates in breads and increased iron availability in both processes [20, 22]. The genes encoding these phytases have been recently cloned and the enzymes purified and characterized, showing that they belong to the histidine acid phosphatase family and possess distinctive biochemical characteristics, such as a remarkable thermal stability and the ability to degrade Ins$P_6$ to Ins$P_3$ [23]. The inclusion of these purified phytases in the process of production of infant cereals resulted in a significant decrease in Ins$P_6$ contents and an enhanced solubility of zinc [22].

The objective of the present investigation was to develop new bakery cereal-based products with improved nutritional quality. To this end, breads were made with addition of whole amaranth flour. In these breads, the undesirable effects of the high level of phytates found in amaranth seeds were avoided by including purified phytases from intestinal bifidobacteria during the process. The likely contribution of the product to mineral intake, according to the daily reference intake (DRI) and its possible overestimation by the presence of phytates, was also evaluated.

## Materials and methods

### Materials

Commercials flours were purchased from the local market. The characteristics of wheat, whole wheat and whole amaranth flours (*A. cruentus*) were: moisture, 14.53 ± 0.03, 14.04 ± 0.08 and 11.89 ± 0.04 %; protein, 13.6 ± 0.1, 11.6 ± 0.1 and 14.9 ± 0.1 % in dry basis; fat, 1.30 ± 0.09, 1.67 ± 0.03 and 5.60 ± 0.04 % in dry basis; ash, 0.62 ± 0.03, 1.36 ± 0.01 and 2.94 ± 0.08 % in dry basis; and phytate contents were: not detected, 7.5 ± 0.1 and 13.3 ± 0.3 μmol $g^{-1}$ in dry basis, respectively. The flour alveograph parameters were: tenacity, *P*: 81 mm; extensibility, *L*: 110 mm; *P*/*L* ratio, 0.74; and deformation work, *W*: 308 × $10^{-4}$ J. Compressed yeast (*Saccharomyces cerevisiae*, Levamax, Spain) was used as starter. Commercial fungal phytase (E.C 3.1.3.8) from *Aspergillus niger* produced by submerged fermentation of a genetically modified *Aspergillus oryzae* (73 U $ml^{-1}$, Ronozyme Phytase from Novozymes, Bioindustrial, Madrid, Spain) was used as a positive control in dough formulations. Recombinant phytase enzymes expressed in *Escherichia coli* clones carrying the phytase genes from *B. longum* spp. *infantis* ATCC 15697 and *B. pseudocatenulatum* ATCC 27919, originally isolated from infant feces, were also used (see below).

### Expression of bifidobacterial phytases

The phytases from bifidobacteria were overexpressed in *E. coli* as 6xHis-tagged proteins lacking their N-terminal signal peptides and C-terminal cell-wall anchor endogenous sequences [23]. *E. coli* M15 clones carrying the recombinant plasmids (pQE80 derivatives) were grown in 500 ml of LB medium with ampicillin at 100 μg $ml^{-1}$ at 37 °C under shaking, until an optical density of 0.6 at 600 nm was reached. The expression of the phytases was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to 0.1 mM and incubating at 30 °C for 4 h. After

centrifugation, the obtained bacterial cell pellets were washed with 0.9 % NaCl, resuspended in 100 mM Tris–HCl pH 7.4 buffer containing 1 mg $ml^{-1}$ lysozyme and incubated at 37 °C for 30 min. Bacterial cells in this suspension were broken by sonication and after removing the cellular debris by centrifugation at 15,000×*g* 15 min at 4 °C; the supernatants were filtered through 0.45 μm-pore-size nitrocellulose filters. Filtered supernatants were applied to Ni–NTA agarose chromatography columns (1 ml bed volume), and recombinant proteins were purified according to the supplier instructions (Qiagen). After several washes, proteins were eluted from the columns in 1-ml fractions that were analyzed by SDS-PAGE. The fractions containing the expressed proteins were dialyzed against 100 mM Tris–HCl pH 7.4, 1 mM EDTA, 10 % glycerol and 50 mM NaCl at 4 °C for 24 h and stored at −80 °C until use.

### Determination of phytase activity

The enzymatic flour extracts were prepared following the method reported by Haros et al. [24]. The phytase activity was determined using 500 μl of 0.1 M sodium acetate pH 5.5, containing 1.2 mM potassium phytate and 100 μl fractions of each purified enzyme or flour extracts [24, 25]. After 15 min of incubation at 50 °C, the reaction was stopped by adding 100 μl of 20 % trichloracetic acid, allowed to stand for 10 min at 0 °C and centrifuged at 14,000×*g*, 5 min and 4 °C (Centrifuge 5415R, Eppendorf AG, Hamburg, Germany). The determination of the enzyme activity was based in a colorimetric quantification at 400 nm of free phosphorus released by the hydrolysis of phytate using ammonium molybdovanadate reagent (Fluka Chemika) according to Tanner et al. [26]. One unit of phytase activity (U) was defined as the amount of enzyme releasing 1 mg of phosphorous from phytate per minute at pH 5.5 and 50 °C. Determinations were carried out in duplicate.

### Determination of *myo*-inositol phosphates

Ins$P_6$ present in flours and the remaining Ins$P_6$ and lower *myo*-inositol phosphates generated during the breadmaking process were extracted by ion-exchange chromatography and measured by high-pressure liquid chromatographic methods described by Türk and Sandberg [27], later modified by Sanz-Penella et al. [28]. Identification of the *myo*-inositol phosphates was achieved by comparison with standards of phytic acid di-potassium salt (Sigma-Aldrich, St. Louis, MO). Samples were analyzed in triplicate.

### Determination of minerals in bread samples

The total Fe, Ca and Zn concentrations in bread samples were determined using a flame atomic absorption spectrometer at the *Servei Central de Suport a la Investigació Experimental* from the University of Valencia. Previously, samples were placed in a Teflon perfluoroalkoxy (PFA) vessel and treated with 1 mL $HNO_3$ (14 M, Merck) and 1 mL of $H_2O_2$ (30 % v/v, Panreac Química, Spain). The Teflon PFA vessel was irradiated at 800 W (15 min at 180 °C) in a microwave accelerated reaction system (MARS) from CEM (Vertex, Spain). At the end of the digestion program, the digest was placed in a tube and made up to volume with 0.6 M HCl (Merck). Samples were analyzed in triplicate.

### Breadmaking process

Four flour formulations were used for bread doughs: 100 % wheat flour (WF), 100 % whole wheat flour (WWF), 25 % whole amaranth flour and 75 % wheat flour (WAF25), and 50 % whole amaranth flour and 50 % wheat flour (WAF50). The bread dough formula expressed in flour basis consisted of different flour formulations (300 g), compressed yeast (5 %), sodium chloride (1.6 %) and water up to optimum absorption corresponding to 500 BU (Brabender Units) (between 60 % and 66.8 % depending on bread dough formulation). A sponge method mixing dough in a two stage was used (Fig. 1). The first stage involved mixing half water and flour amount together with the total yeast amount and fermenting for 24 h at 4 °C. The sponge is then mixed in with the rest of ingredients in a second stage. The ingredients were mixed for 5.5–6.0 min, rested for 10 min, divided into 100-g 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 180 °C/20 min, 165 °C/30 min, 160 °C/35 min, and 160 °C/30 min for WF, WWF, WAF25 and WAF50, respectively, and cooled at room temperature for 75 min. The formulation samples were done in duplicate. The different phytases were added during mixing at a concentration equivalent to the phytase endogenous activity in the flours (Fig. 1).

### Bread quality

The technological parameters analyzed were moisture (%), loaf specific volume (ml $g^{-1}$), width/height ratio of the central slice (cm $cm^{-1}$) and crumb firmness (*N*) using texture analyzer TA-XT plus. The color parameters $L^*$ (lightness), $a^*$ (redness to greenness) and $b^*$ (yellowness to blueness) of crumb and crust were determinate using a digital colorimeter (Chromameter CR-400, Konika Minolta Sensing, Japan) [22]. From the color parameters, the total color difference ($\Delta E^*$) was calculated with the formula: $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$. Samples were analyzed at least in triplicate.

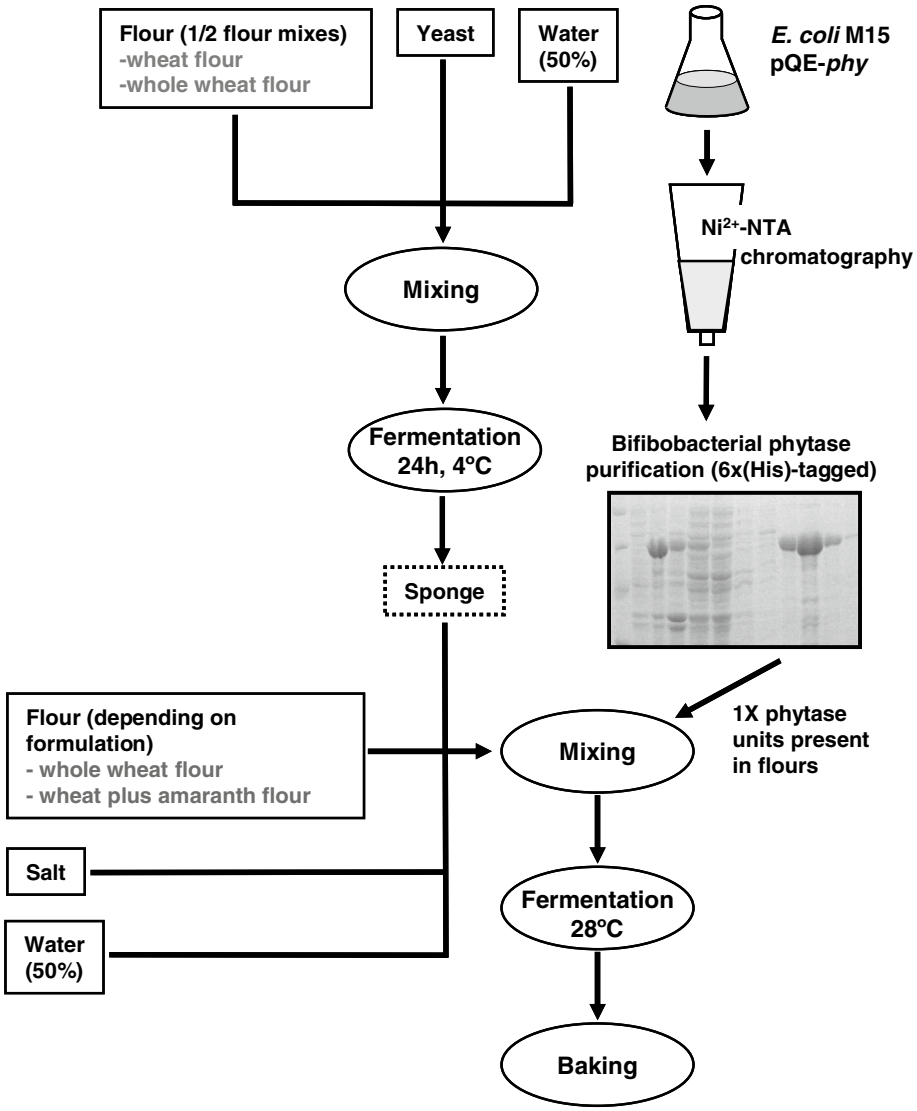


**Fig. 1** Flow diagram of the breadmaking process with different whole grain flours (wheat or amaranth) and bifidobacterial phytases

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 (Bitstream, Cambridge, MN) and differences were considered significant at $p < 0.05$.

## Results and discussion

Technological quality of breads containing amaranth flours and microbial phytases

The characteristics of whole amaranth-wheat mixed bread and the effect of the inclusion of bifidobacterial phytases on bread quality was investigated. The quality of the final products was analyzed by measuring technological parameters such as moisture, loaf specific volume, width/height ratio of the central slice, crust and crumb color and crumb firmness and compared to control breads made with wheat or whole wheat flours.

The moisture parameters for whole wheat breads (WWF samples) were between 30.8 and 33.8 %. Moisture of wheat flour breads (WF samples) was 33.1 %, whereas in breads containing 25 and 50 % of whole amaranth flour (WAF25 and WAF50 samples) moisture ranged between 30.4–33.6 % and 32.5–34.3 %, respectively (Table 1). In general, when the levels of whole grain flours or bran are increased, the water retention capacity of doughs is higher compared with refined flours [28]. However, in the current investigation, strong flour with high protein content (13.6 %) was used, which accounts for a higher water absorption in the wheat flour breads.

**Table 1** Effect of treatment and formulation on the technological parameters of breads

| Sample | Treatment | Moisture (%) | Specific volume (ml $g^{-1}$) | Width/height (cm $cm^{-1}$) | Firmness (*N*) |
|---|---|---|---|---|---|
| WF | | $33.1 \pm 0.1^{e}$ | $4.1 \pm 0.0^{g}$ | $1.6 \pm 0.0^{a,b,c}$ | $0.5 \pm 0.1^{a}$ |
| WWF | C | $33.8 \pm 0.2^{f,g,h}$ | $3.7 \pm 0.1^{d}$ | $1.5 \pm 0.0^{a}$ | $1.0 \pm 0.1^{c,d}$ |
| WAF25 | | $31.5 \pm 0.1^{c}$ | $3.9 \pm 0.1^{f,g}$ | $1.9 \pm 0.0^{f}$ | $0.7 \pm 0.1^{b}$ |
| WAF50 | | $34.3 \pm 0.5^{i}$ | $2.6 \pm 0.2^{a}$ | $2.0 \pm 0.0^{g}$ | $2.0 \pm 0.3^{g}$ |
| WWF | | $33.4 \pm 0.5^{e,f}$ | $3.8 \pm 0.1^{d,e,f}$ | $1.7 \pm 0.0^{b,c,d}$ | $2.5 \pm 0.1^{h}$ |
| WAF25 | FP | $33.6 \pm 0.1^{e,f,g}$ | $3.9 \pm 0.1^{e,f,g}$ | $1.7 \pm 0.0^{b,c,d}$ | $1.1 \pm 0.2^{c,d}$ |
| WAF50 | | $32.5 \pm 0.1^{d}$ | $3.1 \pm 0.1^{c}$ | $1.8 \pm 0.1^{e,f}$ | $1.9 \pm 0.2^{f,g}$ |
| WWF | | $30.8 \pm 0.1^{a,b}$ | $3.8 \pm 0.1^{d,e}$ | $1.8 \pm 0.0^{e,f}$ | $2.5 \pm 0.3^{h}$ |
| WAF25 | PS | $30.4 \pm 0.1^{a}$ | $3.8 \pm 0.1^{d,e,f}$ | $1.7 \pm 0.0^{c,d,e}$ | $0.8 \pm 0.1^{b,c}$ |
| WAF50 | | $34.2 \pm 0.3^{h,i}$ | $2.8 \pm 0.1^{b}$ | $1.7 \pm 0.0^{c,d,e,f}$ | $1.5 \pm 0.1^{e}$ |
| WWF | | $31.0 \pm 0.1^{b,c}$ | $3.8 \pm 0.1^{d,e}$ | $1.7 \pm 0.0^{a,b,c,d}$ | $1.8 \pm 0.2^{f}$ |
| WAF25 | IN | $33.5 \pm 0.1^{e,f}$ | $4.0 \pm 0.1^{g}$ | $1.6 \pm 0.0^{a,b}$ | $0.9 \pm 0.1^{c,d}$ |
| WAF50 | | $34.1 \pm 0.2^{g,h,i}$ | $2.8 \pm 0.1^{b}$ | $1.8 \pm 0.0^{d,e,f}$ | $1.4 \pm 0.1^{e}$ |

*WF* 100 % wheat flour, *WWF* 100 % whole wheat flour, *WAF25* 25 % whole amaranth flour/75 % wheat flour, *WAF50* 50 % whole amaranth flour/50 % wheat flour, *C* control without phytase, *FP* fungal phytase, *PS B. pseudocatenulatum* phytase, *IN B. longum* spp. *infantis* phytase

[a–i] Mean ± SD. Values followed by the same letter in the same column are not significantly different at 95 % confidence level

A decrease in the bread quality was observed by the use of whole wheat flour and whole amaranth flour (Table 1). The presence of these whole grain flours produced significant changes in specific volume and in crumb firmness, especially in WWF and WAF50 samples (Table 1). These changes were related to the gluten content decrease in these formulations. Crumb firmness is a quality parameter in bakery products that is closely related to the tenderness perception by the consumer [29]. Samples with 50 % of amaranth (WAF50) were the most affected, with a 2.8- to 3.8-fold increase in firmness and 1.3- to 1.6-fold decrease in specific volume compared with bread made with wheat flour (WF, Table 1). However, the inclusion of 25 % of amaranth flour (WAF25) resulted in technological parameters closer to the control white bread. Thus, WAF25 samples displayed significantly lower firmness and higher specific volume compared with WWF samples (Table 1).

The color of both whole grain flours showed a lower lightness than the wheat flour without significant differences between them. The values were 84 ± 2; 78 ± 4 and 79 ± 3 for wheat, whole wheat and whole amaranth flour, respectively. The amaranth flour color parameters showed a slight increase in the yellowness (data not shown). This was related to the presence of natural pigments such as flavonoids and other polyphenols, especially present in the whole amaranth flour, which were responsible for the changes in the crust and crumb color observed in breads [30]. In general, the tristimulus color values in crust and crumb were affected by the inclusion of amaranth. The crust redness was significantly higher when the whole grain flour concentration was increased, especially with the increase in amaranth content (values of $a^*$ ranging from 4.9 ± 0.2 to 14.1 ± 0.7 for WF and WAF50 breads, respectively). On the other hand, the crust lightness decreased significantly with the inclusion of whole amaranth (from 65 ± 2 to 51 ± 1), whereas the yellowness remained around 32.

The crumb tristimulus color parameters were more affected than the crust parameters by the inclusion of amaranth flour. The values of $a^*$ varied from −1.5 ± 0.3 to 1.8 ± 0.3 for WF and WAF50 breads, respectively, while the breads made with 100 % whole wheat flour (WWF) had the higher values of redness (3.6 ± 0.5). The inclusion of 25 or 50 % of whole amaranth flour in bread formulations did not affect significantly the crumb lightness. However, crumb lightness in whole wheat bread (55 ± 2) was significantly lower than in control sample (63 ± 3). The crumb yellowness was also correlated to a high concentration of amaranth flour, probably due to a higher content of flavonoids ($b^*$ value was 24.7 ± 0.7 for WAF50 breads, whereas they were 12.9 ± 0.6 and 17.7 ± 0.8 for WF and WWF breads, respectively). The total color difference between samples (crust and crumb color), $\Delta E$, were higher than five units, indicating that significant differences are perceptible to consumers by visual observation [20]. The phytase treatment did not show significant differences in color parameters compared with control (results not shown).

With the exception of some specific conditions, the technological parameters did not change with the addition of the different microbial phytases (control fungal phytase and bifidobacterial phytases). The differences comprised an increased firmness in WWF breads treated with both types of phytases, compared with the same untreated samples (Table 1). This behavior was not observed in breads

**Table 2** Effect of formulation on the *myo*-inositol phosphates levels in bread

| Samples | | $\mu$mol $g^{-1}$ of bread (dry base) | | | | |
|---|---|---|---|---|---|---|
| | Phytase | Ins$P_6$ | Ins$P_5$ | Ins$P_4$ | Ins$P_3$ | Ins$P_6$ + Ins$P_5$ |
| WF | – | n.d. | n.d. | n.d. | n.d. | n.d. |
| WWF | C | $2.3 \pm 0.5^{e}$ | $0.6 \pm 0.1^{e}$ | $0.3 \pm 0.1^{d}$ | $0.5 \pm 0.1^{f}$ | $2.9 \pm 0.7^{c}$ |
| | FP | $0.6 \pm 0.4^{a,b}$ | $0.1 \pm 0.1^{a,b}$ | $0.1 \pm 0.1^{a}$ | $0.1 \pm 0.1^{a}$ | $0.7 \pm 0.7^{a}$ |
| | PS | $0.3 \pm 0.1^{a}$ | $0.1 \pm 0.1^{a,b}$ | $0.1 \pm 0.1^{b}$ | $0.3 \pm 0.1^{e}$ | $0.5 \pm 0.1^{a}$ |
| | IN | $0.3 \pm 0.2^{a}$ | $0.1 \pm 0.1^{a}$ | $0.1 \pm 0.1^{a,b}$ | $0.1 \pm 0.1^{a}$ | $0.4 \pm 0.3^{a}$ |
| WAF25 | C | $1.8 \pm 0.3^{c,d}$ | $0.3 \pm 0.1^{b,c,d}$ | $0.1 \pm 0.1^{b}$ | $0.2 \pm 0.1^{d,e}$ | $2.1 \pm 0.4^{b}$ |
| | FP | $1.3 \pm 0.1^{b,c}$ | $0.2 \pm 0.1^{a,b,c}$ | $0.1 \pm 0.1^{a,b}$ | $0.1 \pm 0.1^{a,b,c}$ | $1.5 \pm 0.2^{b}$ |
| | PS | $0.2 \pm 0.1^{a}$ | $0.1 \pm 0.1^{a}$ | $0.1 \pm 0.0^{a}$ | $0.1 \pm 0.0^{a,b}$ | $0.2 \pm 0.1^{a}$ |
| | IN | $0.2 \pm 0.1^{a}$ | $0.1 \pm 0.0^{a}$ | $0.1 \pm 0.0^{a}$ | $0.1 \pm 0.1^{a,b,c}$ | $0.3 \pm 0.1^{a}$ |
| WAF50 | C | $6.8 \pm 0.2^{g}$ | $1.0 \pm 0.2^{f}$ | $0.2 \pm 0.1^{c}$ | $0.2 \pm 0.1^{b,c,d,e}$ | $7.8 \pm 0.5^{e}$ |
| | FP | $3.4 \pm 0.6^{f}$ | $0.4 \pm 0.1^{c,d,e}$ | $0.1 \pm 0.0^{a,b}$ | $0.1 \pm 0.1^{a,b,c}$ | $3.7 \pm 0.6^{d}$ |
| | PS | $2.0 \pm 0.1^{d,e}$ | $0.3 \pm 0.1^{b,c,d}$ | $0.1 \pm 0.1^{a,b}$ | $0.2 \pm 0.1^{a,b,c,d}$ | $2.4 \pm 0.0^{b,c}$ |
| | IN | $3.5 \pm 0.3^{f}$ | $0.5 \pm 0.1^{d,e}$ | $0.1 \pm 0.0^{a,b}$ | $0.2 \pm 0.1^{c,d,e}$ | $3.9 \pm 0.4^{d}$ |

*WF* 100 % wheat flour, *WWF* 100 % whole wheat flour, *WAF25* 25 % whole amaranth flour/75 % wheat flour, *WAF50* 50 % whole amaranth flour/50 % wheat flour, *C* control without phytase, *FP* fungal phytase, *PS B. pseudocatenulatum* phytase, *IN B. longum* spp. *infantis* phytase

[a–g] Mean ± SD, n = 3. Values followed by the same letter in the same column are not significantly different at 95 % confidence level; [d]n.d., not detected; Ins$P_{6\text{-}3}$, hexakis, pentakis, tetrakis and tri phosphate of *myo*-inositol, respectively

with amaranth flour, although no clear explanation for this could be hypothesized. Another change was represented by a slight increment in loaf volume in formulations with amaranth and treated with phytases compared with untreated samples (Table 1). This effect was significantly higher in the WAF50 formulation. Haros et al. [31] hypothesized that an advanced hydrolysis of phytate could activate the wheat endogenous alpha amylase through calcium liberation, which acts as cofactor of this enzyme. This leads to an increase in the volume of the bakery pieces during the fermentation process. Finally, the width/height ratio of the central slice remained constant in all the formulations, except in the WAF50 breads, where higher values were obtained in samples not treated with phytases (Table 1).

## Effect of bifidobacterial phytases on the *myo*-inositol phosphates levels in bread

The whole grain flours used in this study contained high levels of phytate (whole wheat, 7.5 ± 0.2 μmol $g^{-1}$; whole amaranth, 13.3 ± 0.4 μmol $g^{-1}$ of Ins$P_6$). However, they also showed a high endogenous phytase activity (whole wheat, 18.8 ± 0.5 U $g^{-1}$; whole amaranth, 22.0 ± 0.8 U $g^{-1}$). Usually, the phytate degradation during cereal dough fermentation is positively correlated with the endogenous phytase activity [32]. This fact was observed in the control formulations without added phytase, which displayed a 70 % of phytate hydrolysis for whole wheat bread (WWF) and 46 % for the 25 % whole amaranth bread (WAF25) compared to raw materials (Table 2). However, endogenous Ins$P_6$ hydrolysis in samples with the highest concentration of amaranth flour (WAF50) was not observed. The addition of exogenous phytases resulted in a further decrease in Ins$P_6$ contents which reached up to an 89 % reduction relative to control samples. Ins$P_5$ contents, which also have a strong chelating potential on minerals [33] were also reduced to similar levels. Here, a better performance was found for bifidobacterial phytases compared with the control fungal phytase, especially in WWF and WAF25 breads: hydrolysis of 87–89 % of Ins$P_6$ for bifidobacterial phytases compared to control breads, whereas hydrolysis was only between 28 and 74 % for fungal phytase. A better Ins$P_6$ hydrolysis by bifidobacterial phytases compared with a commercial fungal phytase has been previously reported in the treatment of cereals for infants [34]. In these studies, more than 90 % Ins$P_6$ hydrolysis was reported for the *B. pseudocatenulatum* enzyme, whereas hydrolysis of 68 % were obtained with a *A. niger* phytase. Factors like pH and temperature drastically affect phytase activity [23] and may account for these differences. In this regard, it is worth mentioning that the bifidobacterial enzymes showed a high thermal stability [23] that allows their activity during the first stage of baking. Also, it cannot be excluded that inhibitory substances present in the flours were differentially affecting the activity of fungal and bifidobacterial phytases. In WAF50 breads, phytate hydrolysis by the added phytases reached values from 47 to 70 %, although these products still contained a substantial amount of phytate (up to 3.5 μmol $g^{-1}$, Table 2). This can be attributed to the elevated Ins$P_6$ concentration present in

this formulation, to the presence of likely phytase inhibitory substances in the amaranth flour or to a feedback inhibition on phytase activity by phosphate released to the medium [24]. In these samples, a better Ins$P_6$ degrading activity for the *B. pseudocatenulatum* enzyme, compared with *B. longum* spp. *infantis*, was determined. This was also observed during the phytase treatment of infant cereals, which contained more than 2 μmol $g^{-1}$ of Ins$P_6$ [34].

The biochemical characterization of *B. longum* spp. *infantis* and *B. pseudocatenulatum* phytases showed that these enzymes, although displaying a high specificity for phytate, are not able to degrade Ins$P_3$. Therefore, this *myo*-inositol phosphate is accumulated during the reaction [23]. Ins$P_3$ accumulation was also observed when Ins$P_6$ hydrolysis products were analyzed in bacterial cultures of these bifidobacteria [19, 35] or when the purified enzymes were employed during the dextrinization step of infant cereals [34]. In addition, other whole grain breads made with doughs fermented by bifidobacteria also showed accumulation of Ins$P_3$ [22]. In contrast to this, Ins$P_3$ accumulation was not observed in the present study. This suggests that the high activity of the cereal/pseudocereal endogenous phytases and/or unspecific phosphatases present in our flours is responsible for a further degradation of Ins$P_3$ to lower *myo*-inositol phosphates (Ins$P_2$ and Ins$P_1$) or *myo*-inositol. This indicates that the hydrolysis profile depends not only on the utilized phytases but also on the flour (raw material).

### Phytase treatments and phytate/mineral ratios

The substitution of 50 % of wheat flour by whole amaranth flour (WAF50) increased the amount of Ca from 43 ± 3 to 125 ± 13 mg/100 g. The amount of Fe and Zn was also increased from 1.1 ± 0.1 to 3.2 ± 0.6 mg/100 g and from 2.3 ± 0.2 to 3.3 ± 0.7 mg $g^{-1}$, respectively (Table 3). In general, white bread has a low mineral content and should be supplemented to meet the daily requirements for different elements. In this context, whole grain breads are known to be richer sources of macro- and micro-elements than breads made of refined flours. The amount of Fe in whole wheat bread (WWF) was close to the amount in breads with 25 % of whole amaranth flour (WAF25), while the amount of Zn was similar to the content in bread with 50 % of amaranth (WAF50). Table 3 shows the contributions of mineral intake from bread to the dietary reference intakes (DRIs) given by the Food and Nutrition Board of the Institute of Medicine, National Academy of Science [36], taking into account the World Health Organization's recommendation of a daily intake of 250 g of bread [7]. When expressed in terms of DRIs, the control breads (WF and WWF) contribute between 8.2 and 9.0 % of the Ca recommended for adults, whereas the breads incorporating amaranth contribute to increased intakes of this mineral, ranging from 17.6 to 23.8 % of DRIs (WAF25 and WAF50, respectively). Regarding Zn, consumption of the WF bread would provide 38.8 % for men and 53.4 % for women of the daily requirement in adults, while WAF50 breads could provide 57 and 78 % of the daily requirements in men and women, respectively. The same trend was observed with Fe, where 25 % amaranth flour substitution could supply near 50 % of the daily requirement of this mineral in men and 22 % in women. Notwithstanding, it is known that the bioavailability of minerals depends on the presence of phytates, which act as inhibitors of mineral uptake and have adverse effects on their bioavailability, owing to the formation of insoluble complexes [37, 38]. Solubility of nutrients in the gastrointestinal environment (or bioaccessibility) is a prerequisite for absorption by enterocytes in the intestine. In this sense, the predicted intakes that are derived from DRIs for the minerals analyzed in this study are certainly overestimated due to the presence of phytate [7]. Small amounts of phytate can seriously compromise minerals bioavailability. As an example, more than 0.135 μmol $g^{-1}$ of Ins$P_6$ in fortified bread (dry basis) can affect iron absorption in humans [39]. The phytate/minerals molar ratios are used to predict this inhibitory effect [12, 40], and in this regard, a series of values have been established representing critical thresholds (Table 3). Mineral bioavailability was predicted for calcium, iron and zinc in our samples based on these ratios. Due to the high calcium content, their Ins$P_6$/mineral ratios were below the critical threshold in all samples. These ratios were further decreased by the use phytases. For iron and zinc, bifidobacterial phytases generally proved to be more effective than the control fungal phytase in reducing the ratios below the critical values (<1 and <5, respectively). Thus, molar ratios for iron were still above the critical value in WAF25 samples treated with the fungal phytase, whereas these ratios were below this value in samples with bifidobacterial phytases. Owing to the high phytate contents, breads with 50 % amaranth flour still displayed values indicative of an inhibition in Fe and Zn availability. In these samples, inhibition was only relieved for zinc by treatment with the *B. pseudocatenulatum* phytase, further confirming the better performance of this enzyme in cereal mixes [34].

## Conclusions

We showed that a 25 % of amaranth flour can be used as a replacement for wheat flour in bread formulations. This produces an improvement in the nutritional value of bread with only a slight depreciation in quality. Bifidobacterial phytases are able to lower Ins$P_6$ levels during the breadmaking process. The reduction in breads with 25 %

**Table 3** Effect of bread formulation on mineral dietary reference intake contribution and mineral availability prediction

| Parameter | Units | DRI (mg per day) or Ins$P_6$/mineral (mol mol$^{-1}$) | Bread formulation | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | WF | WWF | | | | WAF25 | | | | WAF50 | | | |
| | | | | C | FP | PS | IN | C | FP | PS | IN | C | FP | PS | IN |
| Ins$P_6$ | $\mu$mol g dm$^{-1}$ | | n.d. | 2.3 | 0.6 | 0.3 | 0.3 | 1.8 | 1.3 | 0.2 | 0.2 | 6.8 | 3.4 | 2.0 | 3.5 |
| Ca[a] | mg 100 g dm$^{-1}$ | | 48 ± 8[a] | 43 ± 3[a] | | | | 92 ± 15[b] | | | | 125 ± 13[c] | | | |
| DRI contribution[b] | % | Adults (1,000)** | 9.01 | 8.16 | | | | 17.6 | | | | 23.8 | | | |
| Ins$P_6$/Ca[c] | mol mol$^{-1}$ | >0.24 | 0.00 | 0.22 | 0.06 | 0.03 | 0.03 | 0.08 | 0.06 | 0.01 | 0.01 | 0.22 | 0.11 | 0.06 | 0.11 |
| Fe[a] | mg 100 g dm$^{-1}$ | | 1.1 ± 0.1[a] | 2.1 ± 0.1[b] | | | | 2.0 ± 0.1[b] | | | | 3.2 ± 0.6[c] | | | |
| DRI contribution[b] | % | Man (8) | 25.6 | 51.1 | | | | 49.0 | | | | 76.3 | | | |
| | | Woman (18)* | 11.6 | 22.7 | | | | 21.8 | | | | 33.9 | | | |
| Ins$P_6$/Fe[c] | mol mol$^{-1}$ | >1 | 0.0 | 6.0 | 1.6 | 0.8 | 0.8 | 4.9 | 3.5 | 0.5 | 0.5 | 11.7 | 5.9 | 3.4 | 6.0 |
| Zn[a] | mg 100 g dm$^{-1}$ | | 2.3 ± 0.2[a] | 3.6 ± 0.2[b] | | | | 2.3 ± 0.2[a] | | | | 3.3 ± 0.7[b] | | | |
| DRI contribution[b] | % | Man (11) | 38.8 | 62.3 | | | | 40.1 | | | | 57.0 | | | |
| | | Woman (8) | 53.4 | 85.7 | | | | 55.2 | | | | 78.4 | | | |
| Ins$P_6$/Zn[c] | mol mol$^{-1}$ | >5 | 0.0 | 4.7 | 1.1 | 0.6 | 0.6 | 9.1 | 3.7 | 0.6 | 0.6 | 16.2 | 6.8 | 4.0 | 7.0 |

*WF* 100 % wheat flour, *WWF* 100 % whole wheat flour,*WAF25* 25 % whole amaranth flour/75 % wheat flour, *WAF50*50 % whole amaranth flour/50 % wheat flour, *C* control without phytase, *FP* treatment with fungal phytase, *PS* treatment with *B. pseudocatenulatum* phytase, *IN* treatment with *B. longum* spp. *infantis* phytase

[a] Values followed by the same letter in the same row are not significantly different at 95 % confidence level; *dm* dry matter, *n.d.* not detected

[b] DRI (dietary reference intakes) contribution (%) for a daily average intake of 250 g of bread if the mineral absorption inhibitors are absent. The values in parenthesis are recommended dietary allowances and adequate intakes for individuals between 19 and >70 years, except for: *(between 31 and >70 years), and **(men between 19 and 70 years, women between 19 and 50 years); Food and Nutrition Board, Institute of Medicine, National Academy of Science, 2004

[c] Threshold ratios (Ins$P_6$/mineral) for mineral availability inhibition; Ins$P_6$, *myo*-inositol hexakisphosphate; minerals, Ca, Fe or Zn

amaranth leads to Ins$P_6$ levels that are below the threshold of inhibition of mineral availability. Furthermore, due to the food-grade nature of bifidobacteria, the use of their phytases during food manufacture represents an attractive strategy to produce whole grain products (from cereals and pseudocereals) with enhanced nutritional quality.

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**Conflict of interest** None.

**Compliance with Ethics Requirements** This article does not contain any studies with human or animal subjects.

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