Running title: Mineral availability of whole rye-wheat sour-bread with bifidobacteria

Myo-inositol hexakisphosphate degradation by *Bifidobacterium pseudocatenulatum* ATCC 27919 improves mineral availability of high fibre rye-wheat sour bread

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ABSTRACT

The goal of this investigation was to develop baking products using *Bifidobacterium pseudocatenulatum* ATCC27919, a phytase producer, as starter in sourdough included in whole rye-wheat mixed bread. This bifidobacterial strain contributed to *myo*-inositol hexakisphosphate (phytate) hydrolysis, resulting in breads with higher mineral availability as was predicted through phytate/mineral molar ratios, which remained below the inhibitory threshold values for Ca and Zn intestinal absorption. The products with sourdough showed similar technological quality as their homologous without sourdough, being the levels of acetic and D/L lactic acids in dough and bread significantly higher with the use of sourdough. Overall acceptability scores showed that breads with 25% of whole rye flour were highly accepted regardless of the inclusion of sourdough. This work emphasizes that the *in situ* production of phytase during fermentation by GRAS/QPS microorganisms constitutes a strategy particularly appropriated to reduce phytate contents in products for human consumption.

Keywords: whole rye flour, sourdough, phytates, phytase, *Bifidobacterium,* dietary fibre, mineral availability, dietary reference intakes

Chemical compounds studied in this article

Myo-inositol hexakisphosphate (PubChem CID: 890); *myo*-inositol pentakisphosphate (PubChem CID 482); D-lactic acid (PubChem CID: 61503); L-Lactic acid (PubChem CID: 107689); acetic acid (PubChem CID 176).

1. Introduction

Whole grain cereal foods provide significant health benefits. 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 (Pereira et al., 2002; McIntosh, Noakes, Royle, & Foster, 2003; Laaksonen et al., 2005). In addition to dietary fibre, whole grains are source of a wide range of vitamins, minerals and bioactive compounds such as lignans, phenolic acids, phytosterols, tocotrienols and phytic acid (Katina et al., 2005). Compared to wheat, rye is a better source of dietary fibre. It contains a remarkable amount of soluble fibre, due to its high content in soluble arabinoxylan (Lappi et al., 2010), and it has been shown that rye fibre consumption is more effective than wheat fibre in overall improvement of biomarkers of bowel health (Gråsten et al., 2000; McIntosh et al., 2003). In addition, whole grain rye is characterized by a well-balanced composition of macro and micronutrients. However, whole grains also contain phytic acid, an antinutrient that impairs mineral absorption (Greiner & Konietzny, 1999; Lopez et al., 2001).

Processing is a prerequisite for consumption of whole grains and it is also important because it may modify the amount and bioavailability of nutrients and antinutrients. Sourdough fermentation is a traditional process employed in wheat and rye baking. Beneficial effects of sourdough fermentation on bread quality include an increased bread flavour, prolonged self life and delayed staling (Gänzle, Loponen, & Gobbetti, 2008). A sourdough fermentation process also improves texture and palatability due to peptide, lipid and carbohydrate metabolism (Gänzle et al., 2008). Regarding nutritional quality, the use of sourdough may decrease the glycemic index, because of its potential to modify the digestibility of starch, owing to increased lactic and acetic acid levels (Katina et al., 2005). Furthermore, the sourdough could enhance the bioavailability of minerals and the microbial metabolism during sourdough fermentation may also

produce new nutritionally active compounds (Katina et al., 2005). As was mentioned above, in addition to many nutritional components, whole grain cereals also contain significant amounts of phytic acid (*myo*-inositol (1,2,3,4,5,6)-hexakisphosphate or InsP_6) or its salts (phytates), a well-known inhibitor of mineral, proteins and trace elements bioavailability. Aside from this negative effect, phytic acid is a precursor for the generation of bioactive compounds (Haros et al., 2009). The phytate hydrolysis decreases the negative effects on mineral absorption and generates lower *myo*-inositol phosphates with potential benefits to human health. Phytases are the enzymes that catalyse this hydrolysis and several strategies exist to increase their activity. Enzymatic phytate degradation during breadmaking process depends on many factors including pH, temperature, water content, bran content, leavening agent, fermentation time, process and exogenous phytase addition (generally from microbial sources). The endogenous phytase from cereal has optimal pHs around 4.5 and therefore the use of sourdough improves the degradation of phytates, due to the decrease of pH (Fretzdorff & Brümmer, 1992; Lopez et al., 2001; Reale et al., 2004). *Lactobacillus* strains typically responsible for sourdoughs fermentation lack phytase activity and their phytate degrading capacity is limited and based on non-specific acid phosphatases able hydrolise phytates at a low rate (Haros et al., 2009). However, phytase activity has been described for food-grade strains of the *Bifidobacterium* genus, which are endogenous inhabitants of the gastrointestinal tract, suggesting their utility in producing fermented cereal based products. In fact, phytase-producing bifidobacteria have been applied in both direct and indirect breadmaking processes (Sanz-Penella, Tamayo-Ramos, Sanz, & Haros, 2009; Sanz-Penella, Tamayo-Ramos, & Haros, 2012). Results showed that bifidobacterial strains presented a good adaptation to the dough ecosystem and contributed to acidification resulting in whole wheat breads with significantly lower levels of phytates (Palacios, Haros, Rosell, & Sanz, 2008). The aim in the present study

was to develop whole rye-wheat mixed bread, with increased nutritional quality, by using *Bifidobacterium pseudocatenulatum* ATCC27919 from human origin as a sourdough starter for improving phytate hydrolysis and the production of organic acids.

2. Materials and methods

2.1 Materials

Commercial flours were purchased from the local market. The characteristics of wheat and whole rye flours were: moisture, 13.79±0.07 and 12.58±0.02 %; protein, 11.32±0.09 and 10.13±0.02 % in dry basis; fat, 1.02±0.06 and 1.17±0.12 % in dry basis; ash, 0.63±0.01 and 1.56±0.01 % in dry basis; and phytate contents were: 1.02±0.03 and 7.64±0.05 μmol/g in dry basis, respectively. Compressed yeast (*Saccharomyces cerevisiae*, Levamax, Spain) was used as starter for the breadmaking process and *Bifidobacterium pseudocatenulatum* ATCC 27919, originally isolated from infant faeces, was used as starter in sourdough fermentation.

2.2 Microbial growth conditions

Bifidobacteria were grown in Garche broth in which inorganic phosphate (KH_2PO_4 and NaH_2PO_4) was replaced by 0.74 g/L phytic acid dipotassium salt (Sigma-Aldrich, St. Louis, MO, USA) and 0.1 M of 3-[N-Morpholino] propanesulphonic acid buffer (MOPS, Sigma-Aldrich, St. Louis, MO, USA) (Haros, Bielecka, Honke, & Sanz, 2007). The medium was inoculated at 5 % (v/v) with 18-hour old cultures, previously propagated under the same conditions (AnaeroGenTM , Oxoid, England) until the beginning of the stationary phase of growth (~14-18 hours). Bacterial growth was monitored by measuring optical density at 600 nm.

The inoculum was prepared by harvesting the bacterial cells by centrifugation (21.000x*g*, 20 min, 4 ºC, SLC 1500, Sorvall Evolution), washing the pellets twice and suspending them in 0.085% NaCl solution (Sanz-Penella et al., 2009). The bacterial suspensions were used to inoculate the sourdough. Microbial counts in sourdough and dough samples were determined by plate count on selective media. In the case of bifidobacteria counts, Garche agar was employed by using the double layer technique and a temperature of incubation of 37 ºC for 48 h (Haros et al., 2007). Yeast counts were determined in Rose Bengal Agar (Scharlau Chemie, Barcelona, Spain) after aerobic incubation at 30 ºC for 72 h (Sanz-Penella et al., 2009).

2.3 Breadmaking process

Five formulations were used for making bread dough: 100% wheat flour, 25% whole rye-wheat flour, 50% whole rye-wheat flour, 75% whole rye-wheat flour and 100% whole rye flour. The bread dough formula consisted of one of these formulations (750g), compressed yeast (2% flour basis), sodium chloride (1.8% flour basis), tap water (up to optimum absorption, 500 Brabender Units). The ingredients were mixed for 10 min, rested for 10 min, divided into 100-g pieces, kneaded and rested again for 10 min. Doughs were manually sheeted, rolled and fermented up to the optimum volume increase at 28 ºC and 85% of relative humidity. The samples were backed at 160-180 ºC/ 20-30 min according to formulation and cooled at room temperature for 2 hours. The formulation samples were done in duplicate. Wheat sourdoughs without yeast inoculated with *B. pseudocatenulatum* were prepared and added in three levels to bread doughs formula for replacement of flour at 0, 10 and 20%.

2.4 Sourdoughs preparation

154 The sourdough formulation consisted in a mixture of flour and water (1:2, w/w) with an
155 inoculum of 3.2 x 10^9 CFU of *B. pseudocatenulatum* per gram of flour. Incubation was
156 carried out at 37 ºC for 18 hours under anaerobic conditions. The control acid
157 sourdough consisted in the same formulation and conditions, but instead of the inclusion
158 of the bifidobacterial strain, a mixture of antibiotics at 1% v/v (Penicillin, 50 U/ml;
159 Streptomycin, 0.05 mg/ml; Neomycin, 0.1 mg/ml and Cycloheximide, 0.5 mg/ml from
160 Sigma-Aldrich Steinheim, Germany) was included. The pH of this control sourdough
161 was adjusted to the pH level reached in sourdoughs acidified with bifidobacteria by
162 using similar proportions of lactic and acetic acid and it was included at 20% for
163 preparation of dough for breadmaking.
164
165 **2.5 pH, total titratable acidity and D/L-lactic and acetic acids determination**
166 Sourdough, dough and bread pH was determined electrometrically during sampling.
167 Measurements were done in triplicate using a pH meter. For determination of titratable
168 acidity ten grams of sourdough, dough and bread was mixed and blended with 100 ml
169 of acetone:water (5:95, v/v). Later, they were titrated against 0.1 N NaOH up to pH 8.5.
170 The results were expressed as the volume (ml) of NaOH 0.1 N needed for titrating 10 g
171 of sourdough, fermented dough or bread. Concentrations of D-lactic acid, L-lactic acid
172 and acetic acid were analysed using a specific coupled enzymatic reaction (Boehringer
173 Mannheim/R-Biopharm) measured at 340 nm (PolarStar Omega BMG Labtech,
174 Germany). The results were expressed as μmoles of D/L lactic or acetic acid per gram of
175 sourdough, fermented dough or bread (Sanz-Penella et al., 2012).
176
177 **2.6 Bread composition**
178 2.6.1 Determination of dietary fibre

179 The dietary fibre content was measured by the total dietary fibre assay procedure
180 (AOAC, 1991).
181
182 2.6.2 Determination of *myo*-inositol phosphates
183 InsP_6 present in flours and the remaining InsP_6 and lower *myo*-inositol phosphates
184 generated during the breadmaking process (InsP_5, InsP_4 and InsP_3) were purified by
185 ion-exchange chromatography and measured by the HPLC method described by Türk
186 and Sandberg (1992), later modified by Sanz-Penella et al. (2008). Identification of the
187 *myo*-inositol phosphates was achieved by comparison with standards of phytic acid di-
188 potassium salt (Sigma-Aldrich, St. Louis, MO). Samples were analyzed in triplicate.
189
190 2.6.3 Determination of minerals
191 The total Fe, Ca and Zn concentrations in bread samples were determined using a flame
192 atomic absorption spectrometer at the Servei Central de Suport a la Investigació
193 Experimental from the Universitat de València. Previously, samples were placed in a
194 Teflon perfluoroalkoxy (PFA) vessel and treated with 1 ml HNO_3 (14 M, Merck) and 1
195 ml of H_2O_2 (30 % v/v, Panreac Quimica, Spain). The Teflon PFA vessel was irradiated
196 at 800 W (15 min at 180ºC) in a microwave accelerated reaction system (MARS) from
197 CEM (Vertex, Spain). At the end of the digestion program, the digest was placed in a
198 tube and made up to volume with 0.6 M HCl (Merck). Samples were analyzed in
199 triplicate.
200
201 **2.7 Bread quality**
202 The technological parameters analyzed were moisture (%), loaf specific volume (ml/g),
203 width/height ratio of the central slice (cm/cm) and crumb hardness (N) using texture
204 analyzer TA-XT plus. Determinations were carried out 10 times per sample for each

baking. The colour 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) (Sanz-Penella et al., 2009). From the colour parameters the total colour difference (ΔE*) was calculated by the equation: $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$. Samples were analyzed at least in triplicate. Digital image analysis was used to measure bread crumb structure. Images were previously squared at 240 pixels per centimetre with a flatbed scanner (HP ScanJet 4400C, Hewlett Packard, USA) supported by the HP PrecisionScan Pro 3.1 Software. Two 10 mm x 10 mm square fields of view of the central slice (10 mm thick) of each loaf were used, thereby yielding six digital images per each baking. Data was processed using Sigma Scan Pro Image Analysis Software (version 5.0.0, SPSS Inc., USA). The chosen parameters were number of cells per cm^2 and mean cell area (mm^2). Measurements were done 9 times per sample per each baking (Sanz-Penella et al., 2009).

Sensory analysis was carried out with the participation of 50 tasters and untrained volunteers who are usual consumers of bread. The product overall acceptability was assessed using a hedonic scale of 9 points (Popper, Rosenstock, Schraidt, & Kroll, 2004).

2.8 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 $p<0.05$.

3. Results and discussion

3.1 Characteristics of sourdough

The characteristics of the sourdough prepared with bifidobacteria are shown in Table 1. The sourdough was inoculated with $1.3x10^9$ CFU of *Bifidobacterium pseudocatenulatum* ATCC 27919 per gram of flour. This bacterium adapted well to the sourdough medium and bacterial counts after 18 h of incubation at 37 ºC in anaerobic conditions reached 10^{11} CFU/g. Microbial examination after Gram-staining confirmed that these bacterial counts were derived from the inoculated bifidobacteria (data not shown). The cell counts (Garche medium) in control sourdoughs without inoculation and supplemented with antibiotics were only 10^4 CFU/g throughout the fermentation, excluding a contribution of microbial metabolism to any analytical parameters observed in these samples. The sourdough inoculated with bifidobacteria showed an acidification from an initial pH of 5.9 to final pH of 4.3. These final pH values reached at the end of the fermentation showed that the bifidobacteria were metabolically active and fermented the carbohydrate sources of the flour producing organic acids and increasing the TTA to 5.2 ml of NaOH. The L-lactic and D-lactic acids production were 41.0 and 15.7 µmoles/g, respectively, while the acetic acid production was 76.8 µmoles/g. The higher values for acetic acid compared to lactic acid (molar ratio lactic/acetic of 0.73) was characteristic for the fermentative metabolism of bifidobacteria on the carbohydrates which are typically present in wheat flour (maltose and maltodextrins) (Van der Meulen, Adriany, Verbrugghe, & De Vuyst, 2006). The control acid sourdough was adjusted at 4.2 and reached a TTA of 5.4 ml of NaOH.

3.2 Characteristics of the dough

The sourdough fermented with bifidobacteria was used for making dough for breadmaking with different rye proportions and sourdough levels (Table 1). The yeast

fermentation process did not produce any significant decrease in the dough pH. However, the dough volume increased during the fermentation until the optimal volume was reached, being significantly influenced by the addition of whole rye flour and the inclusion of sourdough (data not shown). The inclusion of sourdough to the formulation significantly increased the bacterial counts, which ranged from 2.9 to 10.4 log CFU/g, while the yeast counts remained constant. As expected, pH values decreased whereas TTA values showed a significant increase with the raise in the proportion of sourdough. However, the inclusion of the whole rye flour did not have a significantly effect in any of these parameters. The increase of sourdough proportion in the formulations mainly affected the acidic properties of the bread dough due to production of organic acids. Data showed a constant and significant increase of acetic and L-lactic acid with the increase of sourdough proportion, whereas the D-lactic acid content decreased when raising the proportion of sourdough. Sanz-Penella et al. (2012) reported a similar behaviour when the same bifidobacterial strain was inoculated in doughs made with whole wheat flour. The molar ratio between lactic and acetic acids ranged from 0.25 and 0.61 in all the formulations with sourdough inoculated with bifidobacteria. These values were still typical according to sugar catabolism of bifidobacteria, which varies depending on the strain, the type of soluble sugars and the processing conditions (Sanz-Penella et al., 2012).

3.3 Evaluation of bread

The pH value of bread was modified with the addition of sourdough in the bread formula and they ranged from 6.21 to 5.52, whereas the TTA values increased ranging from 2.03 to 4.75 ml of NaOH (Table 2). These results are comparable with those reported in the literature for breads made with sourdough inoculated with lactobacilli

and can vary over a wide range, depending not only on the strain, but on the variety of cereal used (Katina et al., 2005; Liukkonen et al., 2003).

During the bread baking step there was a weight loss mainly due to water evaporation followed by organic acid loss. The residual amount of acetic acid after baking was 2.8-3.8 μmoles/g of bread for samples without sourdough and 4.9-5.9 μmoles/g of bread for samples with 20% of sourdough. On the other hand, D/L lactic acid remained in concentration 1.0-2.4 μmoles/g of bread without sourdough and 7.8-8.3 μmoles/g of bread with 20% of sourdough. The molar ratio between D/L-lactic and acetic acids registered an increase from 0.27-0.84 mol/mol in the control samples without sourdough up to 1.40-1.64 with the addition of 20% of sourdough.

A decrease in the bread quality was observed by the raise of whole rye flour proportion in the bread formula. The inclusion of whole rye flour produced significant changes in specific volume and in crumb firmness. Samples with more than 50 % of whole rye flour content were the most affected with a 2.4- to 2.6-fold decrease in specific volume and a 3.5- to 8.5-fold increase in firmness (Table 2). These changes are mainly due to the lower proportion of gluten in whole rye flour in comparison to wheat flour (Bodroža-Solarov, Filipčev, Kevrešan, Mandić, & Šimurina, 2008). The effect of sourdough resulted in a decrease in the specific volume, whereas samples with 20 % sourdough content showed significantly higher crumb firmness than control sample. The application of sourdough has been reported to either increase (Corsetti et al., 2000; Crowley, Schober, Clarke, & Arendt, 2002) or decrease (Barber, Bfiguena, Barber, & Martinez-anaya, 1991; Salovaara & Valjakka, 2007) bread volume, and the type of effect depends on the acidification level obtained and the microbial strain employed (Katina et al., 2005). Furthermore, during sourdough fermentation, pH drop may favour amylolytic or proteolytic reactions, leading to an impact on structure-forming components like gluten and starch. Thiele et al. (2004) demonstrated that gluten

macropolymers are solubilised and degraded during sourdough fermentation, which resulted in a less elastic texture of bread dough containing sourdough (Clarke, Schober, Dockery, Sullivan, & Arendt, 2004; Thiele, Grassl, & Gänzle, 2004)

A weaker gluten network might result in breads with a worse technological quality. The crumb softness as well as the mean cell area and number of cells per cm^2 presented a significant decrease with the increase of the proportion of whole rye flour in bread formula (from 1.6±0.2 mm^2 to 0.58±0.09 mm^2 and from 121±17 cells/cm^2 to 96±13 cells/cm^2, respectively). Nevertheless, all these parameters in samples added with sourdough did not show significant differences compared to the control without sourdough (data not shown). The effect of the percentage of rye flour used in the formulations on crust and crumb colour was also analysed. The CIEL*a*b* parameters were significantly affected by the increase of whole rye flour. As was expected, samples with higher rye flour amount showed an enhance in the darkness (lower L*), values ranged from 59±7 to 47±3, a higher redness, values ranged from 11±1 to 12±1 and a lower yellowness, values ranged from 37.1±0.8 to 28.2±0.8. The total colour difference between control sample and bread with rye and sourdough, ΔE, were higher than five units, indicating that significant differences are perceptible to consumers by visual observation (Sanz-Penella et al., 2012).

3.4 Effect of formulation on nutritional value of bread

3.4.1 Dietary Fibre and contribution to adequate dietary intake

As was expected, the incorporation of whole rye flour in the formulation, gradually and significantly increased the total dietary fibre (Table 3). An increase in whole rye flour content resulted in breads with soluble/insoluble fibre ratios closer to the recommended ratio value of 1:3 (Salas-Salvadó, Bulló, Pérez-Heras, & Ros, 2007). Table 3 also shows

334 the adequate intakes (AIs) for dietary fibre given by the Food and Nutrition Board of the
335 Institute of Medicine, National Academy of Science (NAS, 2005), taking into account
336 the World Health Organization´s recommendation of a consumption of 250 g of bread
337 per day. For example, the substitution of 50% of wheat flour by whole rye flour
338 contributed to an increase in the intakes of total dietary fibre, reaching values of 52 to
339 56% for men and 79 to 85% for women of AIs. Furthermore, soluble arabinoxylans are
340 the main dietary fibre polysaccharides in rye -around 55% of the total polysaccharides-
341 which could provide a positive influence on the post-prandial glycaemic response
342 (Knudsen & Lærke, 2010). This fact, together with the organic acids derived from the
343 sourdough process could contribute to lowering the glycaemic index in these bread
344 formulations.
345
346 **3.4.2. Minerals and contribution to dairy dietary reference intake**
347 Table 4 shows the contribution to mineral intake of the five formulations used in this
348 study based on the dietary reference intakes (DRIs) given by the Food and Nutrition
349 Board of the Institute of Medicine, National Academy of Science (NAS, 2004) and
350 assuming a consumption of 250 g of bread per day. The substitution of wheat flour by
351 whole rye flour contributed to higher intakes of Ca, which is increased from 5.5 up to
352 16.5 % of DRIs. The contribution to DRIs for Fe and Zn was also significantly
353 increased. Regarding Fe, the inclusion of whole rye flour resulted in an increase in the
354 percentages of the DRI from 38 % to 94 % for men and 17 % to 42 % for women. For
355 Zn the percentages of the DRI increased from 34 % to 76 % for men and 47 % to 104%
356 for women. Despite, the mineral bioavailability depends on the presence of phytates,
357 which affects their absorption due to its chelating effect, which gives raise to insoluble
358 complexes (Sandström & Sandberg, 1992; Sanz-Penella et al., 2012). In this context, the

359 predicted intakes are obviously overestimated due to the presence of phytates (Sanz-
360 Penella, Wronkowska, Soral-Smietana, & Haros, 2013).
361
362 **3.4.1 Effect of sourdough on the *myo*-inositol phosphates levels**
363 In order to determine the content of the anti-nutritional *myo*-inositol phosphates in
364 breads made with phytase-producing bifidobacteria, phytate and lower *myo*-inositol
365 phosphates were measured (Table 4). As expected, the phytate content in the final
366 product was increased by the addition of whole rye flour. However, the inclusion of
367 sourdough inoculated with bifidobacteria caused a significant decrease in the phytate
368 content in breads at the two different proportions investigated (10 and 20%). The
369 contents of InsP_5, which also has a strong chelating potential on minerals, were also
370 significantly reduced. In addition, lower *myo*-inositol phosphates, like InsP_3, showed
371 accumulation. Nevertheless, the lower *myo*-inositol phosphate levels did not present
372 significant differences between the control made with a chemically acidified sourdough
373 and samples with fermented sourdoughs. Therefore, InsP_6 reduction and generation of
374 lower *myo*-inosytol phosphates can be primarily correlated to the activation of the cereal
375 endogenous phytase by the acidic pH, as seen in previous works (Sanz-Penella et al.,
376 2012). However, hydrolysis of InsP_6 in samples containing 20% bifidobacterial
377 sourdough and 50, 75 or 100% whole rye flour was significantly higher (between 5 to
378 11% higher), than values obtained using a chemically acidified sourdough in the same
379 proportion, indicating that the additional hydrolysis is due to the phytase activity of *B.*
380 *pseudocatenulatum*. These results were in the line to those obtained when bifidobacteria
381 treatment was applied to whole wheat breads (Sanz-Penella et al., 2012), showing the
382 versatility of these microorganisms in the fermentation and phytate reduction in
383 different types of whole grain flours.
384

3.4.2 Human mineral availability estimation

The phytate/mineral molar ratios are used to predict the inhibitory effect of InsP_6 on the bioavailability of minerals (Ma et al., 2005, García-Mantrana, Monedero, & Haros, 2014). Mineral bioavailability was predicted for calcium, iron and zinc in the bread samples. In the case of Ca, a phytate/mineral molar ratios value higher than 0.24 starts compromising this mineral bioavailability. For Fe the phytate/Fe molar ratio could impair Fe bioavailability in humans at values higher than 1, whereas for Zn, if the phytate/Zn molar ratio is higher than 5 the bioavailability of Zn could be less than 50% (Ma et al., 2005). These ratios were decreased by the raise in sourdough proportion, which resulted in higher InsP_6 degradation. Due to the Ca content and the hydrolysis effect of sourdough on phytates, the InsP_6/Ca ratios were below the critical value for all the samples. For Zn, inhibitory effects (more than 50% of the mineral) were predicted in samples with 75 and 100% rye and this effect was efficiently avoided by the inclusion of sourdough at both 10 and 20%. Although the inclusion of sourdough reduced the InsP_6/Fe ratios close to the threshold in samples with 25% rye, the high phytate content of rye resulted in ratios still above the critical threshold in all samples.

3.5 Sensory analysis

Sensory evaluation study using a hedonic scale showed that the main factor for the approval by consumers was the proportion of whole rye flour added to the formulation. As the percentage of whole rye flour increased, the product acceptability decreased, while the proportion of sourdough did not influence significantly. Thus, breads made with 25% rye flour were the most accepted. However, it is important to underline that samples with up to 50% rye reached about 85% acceptance, with the inclusion of sourdough inoculated with bifidobacteria having no effect in the acceptability.

4. Conclusions

Whole rye flour contributes to an increase in the nutritional value of bread products. Whole rye flour used until 50% of wheat substitution only resulted in a slight depreciation in bread performance and contributed to an increase in the intakes of total dietary fibre, reaching values close to 50% and 80% of AIs for men and women, respectively, with high consumer acceptance. Sourdough inoculated with bifidobacteria was able to increase the phytate hydrolysis and raised organic acid levels that modify the starch digestibility, which could contribute to lowering the glycaemic index. The reduction of phytates in all the formulations with rye and sourdough up to 10% led to InsP_6 levels below the threshold of inhibition for Ca and Zn availability. Previous research has demonstrated that recombinant purified phytases from bifidobacteria are very effective, and superior than a commercial fungal phytase, in reducing InsP_6 contents to non-inhibitory levels in breads made with whole amaranth flour or in infant cereals (Sanz-Penella et al., 2012; García-Mantrana et al., 2014). Although direct inoculation of bifidobacteria may not be as effective as the use of their purified phytases, the benefits obtained in the sourdough fermentation with these phytase-producing microorganisms reinforce the idea that they could be used as starters in sourdough formulations. Their inclusion does not affect the bread quality and results in an increased nutritional value.

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557 regeneration of NAD+ through its growth-associated production. *Applied and*
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559

560 **Table 1.** Values of pH, TTA, concentration of organic acids and cell counts of
561 sourdough, dough[ab]

Formulation		Parameter						
Rye[c] %	Sour Dough[b] %	pH	TTA[b] ml NaOH	Acetic Acid μmols/g	L-lactic Acid μmols/g	D-lactic Acid μmols/g	Bacteria Log CFU/g	Yeast Log CFU/g
Sourdough								
0	**100**	4.3±0.2	5.2±0.4	76.8±1.8	41.0±2.1	15.7±0.9	10.2±0.7	4.4±0.1
Dough								
0	**0**	6.1±0.1^{e}	2.5±0.0^{a}	3.2±1.1^{a}	1.8±0.2^{c}	1.4±0.1^{i}	3.2±0.6^{a}	8.2±0.2^{a}
	10	5.7±0.1^{cd}	3.5±0.1^{b}	8.8±0.5^{de}	3.1±0.1^{g}	1.0±0.2^{gh}	10.4±0.3^{d}	8.9±0.1^{d}
	20	5.7±0.0^{c}	4.8±0.1^{f}	11.7±0.3^{g}	5.2±0.1^{h}	0.8±0.1^{defg}	10.4±0.3^{d}	8.8±0.1^{bcd}
25	**0**	6.1±0.0^{e}	2.5±0.1^{a}	4.3±0.8^{a}	1.5±0.1^{c}	1.2±0.2^{hi}	2.9±0.2^{a}	8.5±0.3^{abc}
	10	5.8±0.1^{cd}	3.7±0.0^{c}	8.1±0.2^{cd}	3.0±0.2^{fg}	0.9±0.1^{efg}	8.5±0.7^{bc}	9.0±0.1^{d}
	20	5.7±0.1^{c}	4.5±0.1^{e}	10.5±0.3^{f}	5.0±0.2^{h}	0.6±0.1^{cdef}	8.2±0.5^{bc}	8.4±0.1^{a}
50	**0**	6.3±0.1^{f}	2.5±0.1^{a}	3.5±0.1^{a}	1.1±0.1^{b}	0.9±0.1^{fg}	2.9±0.4^{a}	8.2±0.2^{a}
	10	5.9±0.1^{d}	3.6±0.1^{bc}	7.6±0.1^{bc}	2.6±0.1^{ef}	0.6±0.1^{cdef}	9.6±1.3^{cd}	8.8±0.1^{bcd}
	20	5.5±0.0^{b}	4.5±0.1^{e}	9.6±0.5^{ef}	5.2±0.1^{h}	0.4±0.1^{abc}	7.9±0.1^{b}	8.4±0.1^{a}
75	**0**	6.1±0.0^{ef}	2.6±0.1^{a}	3.8±0.1^{a}	0.7±0.1^{a}	0.6±0.2^{cde}	3.7±0.7^{a}	8.2±0.1^{a}
	10	5.3±0.1^{a}	4.3±0.1^{de}	7.5±0.1^{bc}	2.4±0.1^{de}	0.5±0.1^{bcd}	8.2±1.0^{bc}	8.5±0.3^{ab}
	20	5.7±0.0^{c}	4.4±0.1^{de}	9.7±0.1^{ef}	5.6±0.1^{h}	0.2±0.1^{a}	7.5±0.8^{b}	8.1±0.1^{a}
100	**0**	5.8±0.0^{cd}	4.2±0.1^{d}	4.0±0.1^{a}	0.5±0.1^{a}	0.8±0.1^{defg}	3.9±0.7^{a}	8.2±0.1^{a}
	10	5.8±0.1^{cd}	3.4±0.1^{b}	6.7±1.0^{b}	2.20±0.29^{d}	0.8±0.2^{defg}	10.1±0.1^{d}	8.9±0.2^{cd}
	20	5.8±0.0^{cd}	4.2±0.1^{d}	10.2±0.9^{f}	5.75±0.16^{h}	0.2±0.1^{ab}	9.6±0.9^{cd}	8.1±0.1^{a}

562 [a]Mean±SD n=3; values followed by the same letter in the same column are not significantly different at 95%
563 confidence level.
564 [b]TTA: total titratable acidity.
565 [c]Bread formulations Rye 0%: 100% wheat flour, Rye 25%: 25% whole rye flour/75% wheat flour, Rye 50%: 50%
566 whole rye flour/50% wheat flour, Rye 75%: 75% whole rye flour/25% wheat flour and Rye 100%: 100% whole rye
567 flour, sourdough 0%, 10% and 20%: formulations dough with 0%, 10% and 20% of sourdough inoculated with
568 bifidobacteria, respectively.
569

570 **Table 2.** Values of pH, TTA, technological parameters and acceptability of bread with

571 different proportions of whole rye flour and sourdough[ab]

Formulation		Parameter				
Rye[c]	Sour-dough[c]	pH	TTA[b]	Specific Volume	Firmness	Acceptability
%	%		ml NaOH	ml/g	N	
0	**0**	6.21±0.06^{h}	2.03±0.09^{a}	5.06±0.37^{j}	0.57±0.05^{a}	7.89±0.03^{d}
	10	5.89±0.13^{ef}	2.73±0.20^{d}	3.86±0.15^{g}	0.80±0.02^{a}	7.91±0.05^{d}
	20	5.76±0.04^{cde}	2.70±0.04^{d}	4.85±0.05^{i}	0.75±0.01^{a}	7.87±0.06^{d}
25	**0**	6.08±0.02^{fg}	3.01±0.25^{e}	4.11±0.09^{h}	0.95±0.03^{ab}	8.89±0.02^{e}
	10	5.99±0.07^{fg}	2.86±0.42^{de}	3.51±0.01^{f}	0.96±0.03^{ab}	8.94±0.03^{e}
	20	5.52±0.03^{a}	4.75±0.09^{h}	3.37±0.05^{f}	1.46±0.14^{b}	8.95±0.01^{e}
50	**0**	5.99±0.02^{fg}	2.34±0.02^{bc}	2.59±0.09^{e}	2.10±0.05^{c}	6.69±0.06^{c}
	10	5.91±0.01^{fg}	2.20±0.07^{ab}	2.46±0.08^{e}	2.61±0.01^{c}	6.77±0.05^{c}
	20	5.54±0.12^{ab}	4.52±0.15^{h}	2.02±0.10^{d}	4.30±0.01^{de}	6.79±0.02^{c}
75	**0**	6.08±0.01^{g}	2.06±0.13^{a}	1.89±0.04^{cd}	3.85±0.50^{d}	2.24±0.01^{b}
	10	5.62±0.09^{abc}	4.48±0.02^{h}	1.55±0.03^{a}	4.51±0.01^{e}	2.23±0.05^{b}
	20	5.73±0.10^{bcd}	3.77±0.04^{g}	1.79±0.10^{bc}	6.34±0.14^{f}	2.21±0.03^{b}
100	**0**	5.83±0.06^{de}	3.46±0.18^{f}	1.83±0.01^{bcd}	4.55±0.44^{e}	1.36±0.01^{a}
	10	6.03±0.04^{g}	2.58±0.11^{cd}	1.66±0.03^{ab}	7.36±0.21^{g}	1.36±0.02^{a}
	20	5.79±0.06^{de}	3.46±0.18^{f}	1.83±0.01^{bcd}	6.40±0.04^{f}	1.35±0.01^{a}

572

573 [a]Mean±SD n=3; values followed by the same letter in the same column are not significantly different at

574 95% confidence level.

575 [b]TTA: total titratable acidity

576 [c]Bread formulations Rye 0%: 100% wheat flour, Rye 25%: 25% whole rye flour/75% wheat flour, Rye

577 50%: 50% whole rye flour/50% wheat flour, Rye 75%: 75% whole rye flour/25% wheat flour and Rye

578 100%: 100% whole rye flour, sourdough 0%, 10% and 20%: formulations dough with 0%, 10% and 20%

579 of sourdough inoculated with bifidobacteria, respectively.

Table 3. Effect of bread formulation on dietary fibre content and contribution to adequate dietary intake

Parameter[a]	Units[b]	Sour-dough[c], %	Rye[c], %				
			0	25	50	75	100
Total Dietary Fibre	g/100g d.m.	0	4.68±0.17[b]	8.10±0.08[c]	10.89±0.13[e]	13.69±0.25[f]	16.46±0.21[j]
		10	4.43±0.11[ab]	8.29±0.20[c]	10.42±0.13[d]	14.24±0.21[g]	15.88±0.08[i]
		20	4.25±0.02[a]	8.31±0.06[c]	10.40±0.11[d]	14.56±0.07[h]	14.63±0.16[h]
Soluble Fibre	g/100g d.m.	0	2.51±0.03[b]	3.67±0.07[c]	3.89±0.05[d]	4.03±0.07[d]	4.70±0.08[gh]
		10	2.56±0.06[b]	3.60±0.12[c]	3.93±0.05[d]	4.44±0.08[f]	4.82±0.05[i]
		20	2.37±0.01[a]	3.60±0.01[c]	4.19±0.06[e]	4.68±0.04[g]	4.92±0.08[hi]
Insoluble Fibre	g/100g d.m.	0	2.17±0.14[b]	4.43±0.01[c]	6.96±0.08[g]	9.66±0.18[h]	11.77±0.13[k]
		10	1.87±0.16[a]	4.70±0.08[d]	6.49±0.10[f]	9.80±0.13[hi]	10.97±0.04[j]
		20	1.88±0.04[a]	4.71±0.06[d]	6.22±0.05[e]	9.89±0.04[hi]	9.81±0.08[i]
Soluble/Insoluble Fibre Ratio 1:3[d]	g/g	0	1:0.9	1:1.2	1:1.8	1:2.4	1:2.5
		10	1:0.7	1:1.3	1:1.7	1:2.2	1:2.2
		20	1:0.8	1:1.3	1:1.5	1:2.1	1:2.0
AI[e] Contribution	%	0	23/35	39/60	56/85	69/104	75/115
		10	24/37	43/65	54/82	71/108	71/109
		20	22/34	43/65	52/79	74/113	72/109

[a]Mean±SD n=3; values followed by the same letter in the same column are not significantly different at 95% confidence level.

[b]Dry matter, d.m.

[c]Bread formulations sourdough 0%, 10% and 20%: formulations dough with 0%, 10% and 20% of sourdough inoculated with bifidobacteria, respectively; Rye 0%: 100% wheat flour, Rye 25%: 25% whole rye flour/75% wheat flour, Rye 50%: 50% whole rye flour/50% wheat flour, Rye 75%: 75% whole rye flour/25% wheat flour and Rye 100%: 100% whole rye flour.

[d]1:3 ratio of soluble/insoluble fibre (Salas-Salvadó, Bulló, Pérez-Heras, & Ros, 2007)

[e]AI (adequate intake) contribution (%) for a daily average intake of 250 g of bread. AI in g per day for dietary fibre in adults man/woman is (38/25). The values in parenthesis are recommended dietary allowances and adequate intakes for adults for each gender between 19 and 50 years; Food and Nutrition Board, Institute of Medicine (2005)

595 **Table 4.** Effect of bread formulation on mineral dietary reference intake contribution
596 and mineral availability prediction

Parameter[ab]	Units[c]	Sour-dough[d] %	Rye[d], %				
			0	25	50	75	100
InsP_6	μmoles/g	0	0.02±0.01[a]	1.26±0.02[c]	2.45±0.13[g]	3.26±0.08[j]	4.15±0.01[k]
		10	0.01±0.01[a]	0.74±0.26[b]	1.88±0.05[e]	2.73±0.12[hi]	3.30±0.03[j]
		20	0.02±0.01[a]	0.68±0.15[b]	1.56±0.04[d]	2.24±0.09[f]	2.64±0.06[h]
		CA-20	0.02±0.01[a]	0.72±0.02[b]	1.76±0.07[e]	2.83±0.01[i]	3.31±0.02[j]
InsP_5	μmoles/g	0	0.01±0.01[a]	0.28±0.01[cd]	0.53±0.01[f]	0.78±0.07[hi]	1.19±0.08[j]
		10	0.01±0.01[a]	0.20±0.01[bc]	0.45±0.02e	0.70±0.05[g]	0.86±0.08[i]
		20	0.03±0.01[a]	0.20±0.01[b]	0.35±0.03d	0.59±0.06[f]	0.74±0.01[e]
		CA-20	0.01±0.01[a]	0.18±0.01[b]	0.33±0.01d	0.44±0.02[gh]	0.77±0.04[gh]
InsP_4	μmoles/g	0	0.01±0.01[a]	0.12±0.01[cde]	0.20±0.02[efg]	0.25±0.06[gh]	0.41±0.05[ij]
		10	0.05±0.01[abc]	0.14±0.01[def]	0.22±0.03[fg]	0.41±0.13[j]	0.33±0.08[hi]
		20	0.03±0.01[ab]	0.14±0.02[de]	0.20±0.01[efg]	0.26±0.01[gh]	0.32±0.01[h]
		CA-20	0.03±0.01[ab]	0.10±0.01[bcd]	0.20±0.03[efg]	0.18±0.01[efg]	0.32±0.03[h]
InsP_3	μmoles/g	0	0.07±0.01[a]	0.16±0.02[b]	0.16±0.01[b]	0.17±0.02[b]	0.18±0.01[bc]
		10	0.19±0.01[bcdef]	0.22±0.02[cdefg]	0.25±0.01[gh]	0.29±0.04[h]	0.23±0.02[efg]
		20	0.18±0.01[bcd]	0.22±0.02[cdefg]	0.30±0.01[h]	0.23±0.03[fg]	0.23±0.01[efg]
		CA-20	0.18±0.01[bcde]	0.17±0.02[b]	0.23±0.01[fg]	0.20±0.01[bcdef]	0.22±0.03[defg]
Ca	mg/100g	0/10/20	27.7±0.1[a]	45.1±0.8[b]	62.5±1.8[c]	80.90±1.4[d]	97.32±3.7[e]
Ca-DRI Contribution[e] %		0/10/20	5.5	8.8	12.4	15.4	16.5
InsP_6/Ca[f] >0.24	mol/mol	0	0.01	0.11	0.16	0.16	0.17
		10	0.00	0.07	0.12	0.14	0.15
		20	0.00	0.06	0.10	0.11	0.12
Fe	mg/100g	0/10/20	1.5±0.1[a]	2.3±0.2[ab]	3.0±0.2[bc]	3.7±0.3[cd]	4.1±0.4[d]
Fe-DRI Contribution[e] %		0/10/20	38/17	55/24	73/32	88/39	94/42
InsP_6/Fe[f] >1.0	mol/mol	0	0.15	3.13	4.60	4.95	5.25
		10	0.04	1.84	3.54	4.14	4.48
		20	0.07	1.69	2.94	3.40	3.54
Zn	mg/100g	0/10/20	1.9±0.2[a]	2.9±0.5[b]	3.6±0.3[bc]	4.1±0.1[cd]	4.9±0.1[d]
Zn DRI Contribution[e] %		0/10/20	34/47	51/70	63/87	72/99	76/104
InsP_6/Zn[f] >5.0	mol/mol	0	0.14	2.85	4.51	5.17	5.58
		10	0.03	1.67	3.48	4.33	4.72
		20	0.07	1.54	2.89	3.55	3.73

597 [a]Mean±SD n=3; values followed by the same letter in the same column are not significantly different at 95%
598 confidence level.
599 [b]InsP_3 to InsP_6: *myo*-inositol phosphate containing 3-6 phosphates per inositol residue.
600 [c]In dry matter
601 [d]Bread formulations: sourdough 0%, 10% and 20% formulations dough with 0%, 10% and 20% of sourdough
602 inoculated with bifidobacteria, respectively; CA-20: dough with 20% acid control sourdough with antibiotics; Rye
603 0%: 100% wheat flour, Rye 25%: 25% whole rye flour/75% wheat flour, Rye 50%: 50% whole rye flour/50%
604 wheat flour, Rye 75%: 75% whole rye flour/25% wheat flour and Rye 100%: 100% whole rye flour.
605 [e]DRI (dietary reference intakes) contribution (%) for a daily average intake of 250 g of bread if the mineral
606 absorption inhibitors are absent. DRI in mg per day for Ca in adults is (1,000)**, for Fe in man/woman is (8/18*)
607 and for Zn in man/woman is (11/8), respectively. The values in parenthesis are recommended dietary allowances
608 and adequate intakes for individuals between 19 and >70 years, except for: *(between 31 and >70 years), and
609 **(men between 31 and 70 years, women between 19 and 50 years); NAS (2014)
610 [f]Threshold ratios (InsP_6/mineral) for mineral availability inhibition; mineral: Ca, Fe or Zn