Effects of germination on the nutritive value and bioactive compounds of brown rice breads

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List of up to 10 names of chemical compounds:

- γ-Aminobutyric acid (GABA)
- γ-oryzanol
- phytic acid
- 2,2´-bipiridine
- thioglycolic acid
- gallic acid

ABSTRACT

The effect of germination conditions on the nutritional benefits of germinated brown rice flour (GBR) bread has been determined. The proximate composition, phytic acid, *in vitro* protein digestibility and *in vitro* enzymatic hydrolysis of starch, glucose and starch content, as well as the most relevant bioactive compounds (GABA, γ-oryzanol and total phenolic compounds) and antioxidant activity of breads prepared with GBR at different germination conditions was determined. When comparing different germination times (0 h, 12 h, 24 h, 48 h), germination for 48 h provides GBR bread with nutritionally superior quality on the basis of its higher content of protein, lipids and bioactive compounds (GABA and polyphenols), increased antioxidant activity and reduced phytic acid content and glycaemic index, although a slight decrease in *in vitro* protein digestibility was detected. Overall, germination seems to be a natural and sustainable way to improving the nutritional quality of gluten-free rice breads.

***Keywords*:** Brown rice, germination, nutritive value, gluten free.

1. Introduction

In the last decade, the use of brown rice (BR) has broadened not only in the common diet, but also in diet of people with celiac disease or allergies to typical cereals. In addition, the germination of BR grains provides higher nutritional and functional values since they are associated with the quality and quantity of their nutrients, biologically active compounds and antioxidant potential. Currently consumers demand natural foods and sprout products have become increasingly popular among people interested in improving and maintaining their health status by changing dietary habits. In this scenario, sprouted BR grains are excellent examples of functional food, because besides their nutritive value they lower the risk of various diseases and/or exert health promoting effects.

Germinated brown rice (GBR) is considered as gluten-free grain characterized by an excellent nutrient profile and germination enhances sharply the content of bioactive compounds such as GABA (γ-aminobutyric acid), phenolic compounds, γ-oryzanol and the antioxidant activity (Caceres el al., 2014). For instance, while the consumption of rice is associated with diabetes mellitus due to its high glycaemic index, GBR takes a leading role against diabetics and at the same time, a reduction on phytic acid is achieved enhancing mineral availability (Kim et al., 2012).

Scientific research supports the beneficial effects of these bioactive compounds, which includes regulation of blood pressure and heart rate, alleviation of pain and anxiety, improves sleeplessness and the autonomic disorder associated to menopausal or presenile period, suppresses liver damage, inhibits cancer cell proliferation and protects against oxidative stress (Oh & Oh 2004). In Japan, GBR was launched to the market in 1995. Since then, GBR is increasing its popularity within the Japanese population and, simultaneously, numerous derived food products have increased. Consequently, the use of GBR as a functional ingredient has focused the attention of researchers addressing the study on changes in nutritional composition and bioactivity. Thus, an increasing trend is focusing on their use in

63 the formulation of high quality of health products. In this scenery, GBR is used as a raw material for
64 obtaining different food products, like GBR balls, soup, bread, doughnuts, cookies and rice burger (Ito
65 and Ishikawa, 2004).

66 Bread is a staple food in many parts of the world providing most calories of the diet. Bread is
67 mostly prepared from wheat flour that it is the constraint for celiac patients, lifelong disorder with a
68 prevalence of 1% of the world population. The only acceptable treatment is the restriction of gluten from
69 the diet and, therefore, GBR bread is an attractive healthy alternative for this group of patients. The
70 availability of palatable BR-containing gluten-free products would represent a significant advance
71 towards ensuring an adequate intake of nutrients and bioactive compounds mostly in subjects with celiac
72 disorder but also in general consumers. Accordingly, developing bread based on GBR with desirable
73 nutritional quality providing bioactive compounds is worthy of investigation.

74 To date, experimental GBR breads have been characterized with adequate instrumental and
75 sensory attributes (Cornejo & Rosell, 2014). However, to our knowledge, investigations on the effect of
76 germination conditions on the nutritive composition of bread-made BR are very limited. Therefore, the
77 aim of the present study was to assess the proximate composition, phytic acid, *in vitro* protein
78 digestibility and *in vitro* enzymatic hydrolysis of starch, glucose and starch content, as well as the most
79 relevant bioactive compounds (GABA, γ-oryzanol and total phenolic compounds) and antioxidant
80 activity of breads prepared with GBR at different germination conditions.

81

82 **2. Materials and Methods**

83 *2.1. Materials*

84 Commercial certified BR cultivar INIAP 15 was provided by the National Institute of Agricultural
85 Research from Ecuador (INIAP). Seeds were harvest between May and December 2011. The gluten-free

bread formulations also contained compressed yeast (LEVAPAN, Lessaffre, Valladolid, Spain) and hydroxypropylmethylcellulose (Methocel K4M) obtained from Dow Chemical Company (Michigan, USA).

2.2. Germination and flour preparation

Brown rice was sterilized with 0.1% sodium hypochlorite solution (1:5 w/v) for 30 min, and then rinsed with distilled water. Afterwards, rice was soaked in distilled water (seed water ratio, 1/5, w/v) for 24 h at 28±1 ºC. Soaking water was drained and rinsed seeds were placed in plastic trays containing moist filter and covered with moist filter paper. The filter papers were kept wet by capillarity. Germination was carried out at 28 ±1°C and 100% relative humidity under darkness for 12, 24 and 48 hours. Germination period was selected on the basis of preliminary assays where nutritional pattern was followed in parallel to technological functionality of flours. After germination, seeds were dried at 50±1°C for 24 hours. Once dried, seeds were ground with a diameter inferior to 1mm with cyclone mill (UDY Corporation, USA). Brown rice flour was also obtained for comparison purposes, besides flour from soaked rice without germination. Two sets of samples were prepared for each treatment.

2.3. Bread preparation

The dough was performed using the recipe of Marco & Rosell (2008). Half of the rice flour was mixed with boiling water (half of the water) and mixed for five minutes. The dough was left to rest until the temperature decreased to 30 °C. Then, the rest of the flour, the other ingredients and water were added and mixed for 5 min. Later, the dough was put into pans and fermented for 40 min at 35 °C and 85% RH. Finally, the fermented dough was baked for 35 min at 175 °C. The bread was analysed after 24h of baking. Bread samples were coded BR for breads made with unprocessed BR flour, Pre-GBR for breads made with soaked brown rice and GBR preceded with germination time for those germinated brown rice flour (as example, 12h GBR for GBR germinated for 12 h).

109 *2.4. Nutritional composition*

110 Chemical composition of gluten-free breads was determined following AOAC (2005) methods and they
111 include: moisture (method 925.10), ash (method 923.03), fat (method 922.06) and protein (method
112 920.87). The carbohydrate content of the samples was calculated by difference, subtracting 100 g minus
113 the sum of grams of moisture, protein, fat and ash. The components were converted to food energy using
114 conversion factors (4.0 kcal g-1 for proteins and carbohydrates and 9.0 kcal g-1 for fats) (FAO, 2003).

115 *2.5. Determination of phytic acid*

116 An accurate photometrical Haug and Lantzsch´s determination of phytic acid phosphorus was used
117 (Reichwald and Hatzack, 2008) with some modifications. 1 mL of HCl 1M was added to 50 mg of
118 sample in an airtight stopper vial and heated for 1 hour in glycerol bath at 80°C under constant agitation
119 at 10 x *g*. The mixture was then cooled to room temperature and centrifuged at 10,621 x *g* for 5 min and
120 0.250 mL of the supernatant was diluted with 1 mL of distilled water. An aliquot of 0.4 mL of sample,
121 standard (phytic acid solution in 0.2 M HCl) or blank (0.2M HCl) were added to 0.8 mL of ferric
122 solution (0.05 g of $FeCl_3$ in 500 mL of 0.2 M HCl) in an airtight stopper vial and was heated for 1 hour
123 in glycerol bath at 80 °C with agitation at 10 x *g*. The mixture was cooled in ice bath for 15 minutes and
124 centrifuged at 10,621 x *g* for 5 minutes at room temperature. Aliquot of 0.6 mL of the supernatant was
125 added to 0.8 mL of the complexing reagent (0.5 g of 2,2´-bipiridine and 65 µL of thioglycolic acid
126 dissolved in 50 mL of 0.2 M HCl) and absorbance was read at 540nm using a microplate reader (BioTek
127 Instruments, Winooski, VT, USA) controlled by the Gene 5TM software version 1.1. (BioTek
128 Instruments).

129

130 *2.6. In vitro protein digestibility*

131 The *in vitro* protein digestibility of the samples was determined by the modified method of Hsu et al.
132 (1977). Briefly, 50 ml of aqueous protein suspension having 6.25 mg protein/ml was prepared. Then,
133 samples were placed in a 37 ºC water bath and the pH was adjusted to 8.00 using 0.1 M NaOH and/or
134 0.1 M HCl, while stirring. Trypsin at a concentration of 1.6 mg/ml was maintained in an ice bath and the
135 pH was adjusted to 8.00 with 0.1M NaOH and/or 0.1M HCl. Five millilitres of enzyme solution were
136 then added to the protein suspension, which was kept stirred at 37 ºC. The trypsin had an activity of
137 13,766 BAEE units/mg proteins. The pH drop was recorded along 15 s after enzyme addition and at one
138 minute intervals for 10 min. The enzyme solution was always freshly prepared before each series of
139 experiments. The percent protein digestibility (Y) was calculated by using Eq. (1) (Hsu et al., 1977): Y
140 = 210.464 – 18.1x (1), where x is the change in pH after 10 min.
141
142 *2.7. In vitro starch digestibility and expected glycaemic index*
143 Starch digestibility of bread was determined by dried samples, following the method described by (Dura
144 et al., 2014) with minor modifications. Briefly, for free sugars removal, powder sample (0.1 g)
145 suspended in 2 mL of 80% ethanol was kept in a shaking water bath at 85 °C for 5 min, and then
146 centrifuged for 10 min at 1000× *g*. The remaining pellet was incubated with porcine pancreatic α-
147 amylase (6 U/mL) (Type VI-B, ≥10 units/mg solid, Sigma Chemical, St. Louis, USA) in 10 mL of 0.1 M
148 sodium maleate buffer (pH 6.9) in a shaking water bath at 37 °C. Aliquots of 200 μL were withdrawn
149 during the incubation period and mixed with 200 μL of ethanol (96%, w/w) to stop the enzymatic
150 reaction and the sample was centrifuged at 10,000 × *g* for 5 min at 4 °C. The precipitate was washed
151 twice with 50% ethanol (200 μL) and the supernatants were pooled together and kept at 4 °C for further
152 glucose enzymatic release.

153 Supernatant (100 μL) was diluted with 850 μL of 0.1 M sodium acetate buffer (pH 4.5) and
154 incubated with 50 μL amyloglucosidase (33 U/mL) at 50 °C for 30 min in a shaking water bath. After
155 centrifuging at 2000 × *g* for 10 min, supernatant was kept for glucose determination.
156 The glucose content was measured using a glucose oxidase–peroxidase (GOPOD) kit (Megazyme,
157 Dublin, Ireland). The absorbance was measured using an Epoch microplate reader (Biotek Instruments,
158 Winooski, USA) at 510 nm. Starch was calculated as glucose (mg) × 0.9. The rate of starch digestion
159 was expressed as a percentage of the total starch hydrolyzed at different times (30, 60, 90, 120, 150, and
160 180 min). Replicates (n = 4) were carried out for each determination. A non-linear model established by
161 Goñi et al (1997) was applied to describe the kinetics of starch hydrolysis. The first order equation (2)
162 has the form: $C=C_{\infty}(1-e^{-kt})$ (2) , where C corresponds to the percentage of starch hydrolyzed at time *t*,
163 C_{∞} is the equilibrium percentage of starch hydrolyzed after 180 min, *k* is the kinetic constant and *t* is the
164 time (min). The parameters C_{∞} and *k* were estimated for each treatment.
165 Using the hydrolysis curve (0–180 min), hydrolysis index (HI) was obtained by dividing the area under
166 the hydrolysis curve of the sample by the area of standard material obtained for white bread. The
167 expected glycemic index (eGI) was calculated using the equation described by Grandfeldt et al. (1992):
168 eGI = 8.198 + 0.862HI.
169
170 *2.8. Determination of γ-aminobutyric acid (GABA)*
171 γ-Aminobutyric acid (GABA) content was determined by HPLC as described in Caceres at al. (2014).
172 50 μL aliquot of concentrated water-soluble extract and 10μL allyl-L-glycine solution (Sigma-Aldrich)
173 used as internal standard were derivatized with 30 μL phenyl isothiocyanate (PITC 99%, Sigma-
174 Aldrich) and dissolved in mobile phase A for GABA analysis. An Alliance Separation Module 2695
175 (Waters, Milford, USA), a photodiode array detector 2996 (Waters) and an Empower II

chromatographic software (Waters) were used as chromatographic system. 20µL of sample were injected into a C18 Alltima 250 x 4.6 mm i.d., 5 µm size (Alltech) column equipped with a same filling guard column (Alltech), both thermostatted at 30 ºC. The chromatogram was developed at a flow rate of 1.0 mL/min by eluting the sample with mobile phase A (0.1 M ammonium acetate pH 6.5) and mobile phase B (0.1 M ammonium acetate, acetonitrile, methanol, 44/46/10, v/v/v, pH 6.5) as in Caceres et al. (2014). Samples were independently analyzed in triplicate and results were expressed as mg GABA/100 g.

2.9. Determination of γ-oryzanol

The analysis of γ-oryzanol in rice samples was performed according to Moongngarm et al. (2010) by extraction in methanol, filtration, concentration and ulterior recovering in methanol to be analysed by HPLC. The system consisted in an Alliance Separation Module 2695 (Waters, Milford, USA), a photodiode array detector 2996 (Waters) setted at 325 nm wavelengh and Empower II software (Waters). 20µL were injected into a C18 column (150 x 3.9 mm i.d., 5 µm size, Waters) and mobile phase (1.0 mL/min) was eluted consisting in solvent A (acetonitrile), solvent B (methanol) and solvent C (bi-distilled water) for 50 min as follows: isocratic flow 60% A, 35% B and 5% C for first 5 min, gradient flow 60% A and 40% B to 8 min keeping it at isocratic flow to 10 min, and then gradient flow 22% A and 78% B to min 20 to maintain isocratically to 35 min, changing to initial conditions to 45 min, isocratic conditions that were kept to equilibrate column to 50 min. γ-Oryzanol in rice samples was identified by retention time and spiking the sample with a standard solution of γ-oryzanol from bran rice (Cymit, Spain) and the purity of peaks was confirmed comparing the spectra and by MS analysis. γ-Oryzanol content was quantified by percentage of peak area according to the calibration curve prepared γ-oryzanol standard solutions. Replicates were independently analyzed and results were expressed in mg γ-oryzanol/100 g.

199 *2.10. Determination of total phenolic content*

200 The Folin-Ciocalteu method was used for determination of total phenolic content (TPC) according to
201 Caceres et al., (2014). The absorbance was measured at 739 nm using a microplate reader (Synergy HT,
202 BioTek Instruments) and TPC were quantified by external calibration using gallic acid (Sigma-Aldrich)
203 as standard. Samples were independently analyzed in triplicate and results were expressed as mg of
204 gallic acid equivalents (GAE) per 100g.

205 *2.11. Determination of oxygen radical absorbance capacity (ORAC)*

206 Antioxidant activity was determined by the method of oxygen radical absorbance capacity by
207 fluorescence using an automatic multiplate reader (BioTek Instruments) at λ_{exc} 485 nm and λ_{em} 520 nm
208 as described recently in Caceres at al., (2014). Individual samples were analysed in triplicated and
209 results were expressed as mg of Trolox equivalents (TE)/100g.

210 *2.12. Statistical Analysis*

211 Standardized skewness and standardized kurtosis analyses were made to verify normal distribution of
212 the data. Multiple sample comparison was conducted to evaluate significant differences among samples
213 by analysis of variance (ANOVA) and multiple range tests. Fisher's least significant differences (LSD)
214 test was used to describe means with 95% confidence (P<0.05). All statistical analyses were performed
215 using Statgraphics Centurion 16 (Statistical Graphics Corporation, UK).

216

217 **3. Results and Discussion**

218 *3.1. Effect of soaking and germination time on nutritional properties of BR bread*

219 The chemical composition of gluten free bread from BR and non-germinated BR showed no significant
220 difference, with exception of ash content that was significantly lower in the bread from soaked flour
221 likely due to the loss of minerals during washing (Table 1). The chemical composition of the gluten free

breads agrees with values reported by Matos & Rosell (2011) in commercial gluten free breads. It can be seen that germination increased the protein content and decreased the carbohydrate, but that effect was independent on the germination time of the grains. In addition, a progressive reduction of ash content was observed with the germination time. Regarding the fat content, it was observed a progressive decrease up to 24 hours germination, but after that a significant increase was observed. There was a significant increase of free glucose content as germination proceeded, likely due to sugars released during germination. In fact, some researches had found a reduction of starch content and an increase of reducing sugar content during germination due to degradation of the starch by the enzyme activity (Charoenthaikij et al 2012, Xu et al 2012). During germination, enzymes become active and the α-amylase activity increases, acting on starch degradation, and in consequence increasing the amount of small dextrin and fermentable sugars. Despite fermentable sugars are used by yeast during bread fermentation, results revealed that significant differences were observed ascribed to the flour used.

A reduced phytic acid content was observed in bread when BR was submitted to steeping and germination processes ($P \leq 0.05$) (Table 1). A higher phytic acid reduction was reached at 12 and 48 h of BR germination (25%) than at 24 h (13%) ($P \leq 0.05$). Lower phytic acid content observed in bread from pre-germinated and GBR could be explained by leaching of this compound into the soaking water and activation of endogenous phytase activity during germination that provides myoinositol and phosphoric acid for seedling growth (Albarracín et al., 2013). Phytic acid has the ability to chelate minerals (iron, zinc, magnesium and calcium) and affects negatively the absorption of amino acids, proteins, and starch (Oatway et al., 2001). Previous studies have demonstrated that reduced phytic acid content achieved by rice soaking and germination treatment lead to improved protein digestibility and mineral diazability (Albarracín et al., 2013). Therefore, germination of BR provides bread with better nutritional quality on the basis of its reduced phytic acid content compared to control bread. On the other hand, there has been

245 increasing evidences that phytic acid may display health benefits reducing cholesterol levels in the
246 diabetic KK mice (Lee et al., 2005) and exerting antioxidant and anticarcinogen effects (Schlemmer et
247 al., 2009).
248
249 *3.2. Effect of soaking and germination time on in vitro protein digestibility of BR bread*
250 Considering that germination activates enzymes like amylases, proteases and so on, protein digestibility
251 was tested to determine if germination might improve protein digestibility of the resulting breads.
252 Germination affected *in vitro* protein digestibility (Figure 1, panel A), inducing an increase that was
253 significant in breads obtained from rice after 12 hours germination (12h GBR), but further germination
254 led to a significant reduction in protein digestibility. Bread samples 24h GBR and 48h GBR showed
255 slower decline in pH compared with other treatments (Figure 1, panel A). It has been reported that BR
256 germination increases the albumin and decreased the globulin and gliadin content, improving the protein
257 bioavailability (Zheng et al., 2007). In addition, germination increases the amount of free amino acid,
258 especially GABA content (Veluppillai et al., 2009). Divergences with the results obtained in the present
259 study might be attributed to the participation of lysine containing proteins in the non-enzymatic
260 browning (Maillard) reaction during baking that is more accentuated in breads obtained from flours with
261 extended germination (Cornejo & Rosell, 2014). In addition, the high temperature during baking could
262 produce crosslinks between amino acids forming more rigid structures that reduce protein digestibility.
263 Indeed, Lamberts et al. (2012) demonstrated that GABA was largely involved in Maillard reactions
264 during baking, resulting in GABA trace levels in wheat bread samples.
265
266 *3.3. Effect of soaking and germination time on in vitro starch digestibility of BR bread*

The *in vitro* starch digestibility curves of gluten free breads are shown in Figure 1 (panel B). In general, it can be observed that soaking and germination influenced the starch hydrolysis of the gluten free bread. Presumably, germination gives some resistance to starch granules likely due to the annealing that could undergo during soaking and drying. This result agrees with Xu et al. (2012) findings in germinated BR flour. They attributed the reduction of the digestion of starch to the presence of more crystalline starch structure after germination, due to the fact that enzymes hydrolyses first the amorphous region that are ease to digest (Dura et al., 2014). In addition, considering that baking is a thermal treatment, Chung et al. (2012) demonstrated that hydrothermal treatment in GBR, reduce the starch digestibility. They attributed this effect to structural changes induced by heat-moisture treatment that provoked rigidity of starch granules and molecules, which are less susceptible to the action of digestive enzymes. No significant difference could be observed between 12h GBR and 24h GBR, but the effect was even more accentuated after 48 hour of germination, slowing down the starch hydrolysis.

The parameters extracted from the regression curves of the recorded *in vitro* starch digestibility are shown in Table 2. The end point values (C_∞) obtained in the hydrolyzed process reflected the concentration at the equilibrium point. The C_∞ value of BR gluten free bread was within the values reported in other gluten free breads (Matos & Rosell, 2011; de la Hera et al., 2014). A significant reduction of C_∞ where found with germination, which reflected decreased digestibility of starch granules, indicating that germination led to less accessible or more resistant starch granules. In addition, k value significantly increased as germination time increases, reflecting structural differences (Butterworth et al., 2012; Dura et al., 2014). Presumably, the action of α-amylase during germination changes the internal structure of the starch molecule making it more difficult to digest, as suggested Xu et al. (2012) and Chung et al. (2012). An increase of k value by germination could be nutritionally unfavourable due to low k values are related to a slow diffusion of pancreatic amylase into the starch

290 granule as digestion proceeds. However, these *k* values are even lower than the ones reported by Matos
291 & Rosell (2011) obtained in some commercial gluten free breads.

292 The hydrolysis index (HI) as well as the estimated glycaemic index (eGI) were significantly
293 reduced with germination (Table 2), leading to breads with medium to low eGI. Indeed, the values of HI
294 and eGI were lower than the ones reported for gluten free breads (Matos & Rosell, 2011; de la Hera et
295 al., 2014). Usually, rice gluten free breads are expected to have higher GI (>70), due to the fact that this
296 kind of breads are mainly starchy foodstuff (Matos & Rosell, 2011). However, the varieties of the rice,
297 as well as dough preparation, influence the *in vitro* starch digestibility (Frei et al., 2003; de la Hera et al.,
298 2014). The significant reduction of glycaemic index induced by the rice germination might be associated
299 to the internal changes in the starch granules during germination. Low glycaemic index values are
300 considered favourable to health, especially as a tool to prevent diseases where glycaemic control plays
301 an important role, such as obesity, diabetes and hyperlipidemia.

302

303 *3.4. Effect of soaking and germination time on the content of bioactive compounds and antioxidant*
304 *activity of BR bread*

305 The content of γ-oryzanol, GABA and TPC in BR bread (control) was 3.98, 5.92, 121.23 mg/100g d.m.,
306 respectively (Figure 2A). Breads from pre-germinated BR and GBR showed lower γ-oryzanol content
307 than control breads ($P \leq 0.05$). Comparison of GBR breads showed that extended germination time (24
308 and 48 h) brought about increased γ-oryzanol content in breads although levels reached were lower than
309 those found in control bread ($P \leq 0.05$). Our results agree with studies showing a reduced γ-oryzanol
310 concentration in pre-GBR and GBR (Kiing et al., 2009). This effect could be attributed to increased
311 feruloyl esterase activity involved in the hydrolysis of esters of phenolic acids such as γ-oryzanol (esters
312 of trans-ferulic acid) that results in the release of ferulic acid as it has been previously reported in barley

313 (Sancho et al., 1999). On the contrary, several studies have shown that pre-germination for 48 h and
314 germination of BR bring about increased levels of γ-oryzanol (Moongngarm and Khomphiphatkul,
315 2011). These differences indicate that effect of soaking and germination processes on γ-oryzanol content
316 depends on many factors such as BR cultivar and processing conditions (time, temperature, water pH)
317 (Kiing et al., 2009). The content of γ-oryzanol in breads from pre-GBR and GBR was lower than that
318 found in their respective flours (11 and 14 mg/100g d.m., respectively) (unpublished data). These results
319 indicate that baking led to noticeable γ-oryzanol losses likely due to its thermal degradation and its
320 hydrolysis during dough fermentation by feruloyl esterase activity of *Sacharomyces cerevisiae* that
321 results in the release of ferulic acid (Coghe et al., 2004). γ-Oryzanol is also hydrolyzed upon
322 gastrointestinal digestion into free sterol and ferulic acid by cholesterol esterases (Mandak and Nyström,
323 2012). Therefore, the reported biological activity of γ-oryzanol is likely due to free ferulic acid released
324 during digestion. Few clinical studies has been performed so far to support the beneficial effect of
325 ferulic acid in humans, however, results from these studies confirmed the potentially important role of
326 ferulic acid in free radical-induced diseases (Alzheimer´s disease, cancer, cardiovascular diseases,
327 diabetes mellitus and skin disease) observed in preclinical research (Mancuso and Santangelo, 2014).

328 Regarding GABA content, breads from pre-GBR were similar to control bread (Figure 2A).
329 Germination markedly improved GABA content in bread, this effect being significantly greater with
330 extended germination time ($P \leq 0.05$). Breads from 48h GBR showed 6 times higher GABA than control
331 bread ($P \leq 0.05$). These results agree with a previous study reporting a time-dependent GABA
332 accumulation during germination of BR (Caceres et al., 2014; Charoenthaikij et al., 2010). GABA
333 accumulation initiates in the soaking process (Caceres et al., 2014; Charoenthaikij et al., 2010) and
334 continues during germination due to the increased activity of glutamate decarboxilase that catalyses the
335 decarboxilation of L-glutamic via GABA shunt pathway (Scott-Taggart y col., 1999). GABA

concentration of pre-GBR and GBR breads was lower than that observed by our group in pre-germinated (28 ºC for 24h) and germinated (28 ºC for 48 h) flours from Ecuadorian BR cultivars (8.0-16.7 mg/100 g d.m. and 70.8-83.1 mg/100 g d.m., respectively) (Caceres et al., 2014). This observation indicate that GABA concentration decreases during BR bread making in consistency with previous studies (Watanabe et al., 2004). GABA losses during bread making are attributed to its consumption during yeast fermentation or amino acid degradation in Maillard browning reactions during baking as reported by Lamberts et al. (2012). Human intervention studies have shown that a daily intake of 10-20 mg of GABA is able to prevent pre-hypertension (Inoue et al., 2003). Therefore, a daily consumption of 100 g of bread from GBR for 48 h containing 37.5 mg of GABA would provide enough GABA to display the health benefits observed in previous studies (Inoue et al., 2003).

Total phenolic content was higher in breads from pre-GBR and GBR than control bread ($P \leq 0.05$) (Figure 2A). Similarly to GABA, TPC was noticeably improved in GBR breads with germination time ($P \leq 0.05$). Breads from GBR for 48 h showed 1.5 times higher total phenolic concentration than control bread ($P \leq 0.05$). These results agree with previous studies on grains germination (Caceres et al., 2014; Moongngarm & Saetung, 2010) and are directly related to the induction of enzymes involved in the phenylpropanoid pathway and in the degradation of the cell wall polysaccharides and proteins that cause the release of bound phenolics (He et al., 2011). This is supported by Tian, Nakamura, and Kayahara (2004) who showed a significant increase in free ferulic, *p*-coumaric and sinapic acids and as well as insoluble but hydrolysable phenolic compounds, together with decreases in the hydroxycinnamate sucrose esters in GBR.

Antioxidant activity of bread was (583 μg TE /100 g d.m.) was reduced when BR was submitted to the steeping process ($P \leq 0.05$) (Figure 2B). BR germination for 12 h slightly increased ORAC values of pre-GBR bread although antioxidant activity was not improved compared to control bread.

359 Interestingly, increased antioxidant activity was observed in bread compared with control when BR was
360 germinated for longer time (24 and 48h). These results could be ascribed to the biosynthesis of
361 compounds with antioxidant activity to keep a balance of the redox homeostasis during germination and
362 to the hydrolysis of bound phenolics due to polysaccharide cell-wall degradation (He et al., 2011). TPC
363 and γ-oryzanol content were positively correlated with ORAC (r^2 =0.8614 and 0.7627, respectively)
364 which supports this hypothesis. Besides radical-scavenging activity, several studies have demonstrated
365 that phenolic compounds and γ-oryzanol may also display their antioxidant effects acting as hydrogen
366 and electron donors and through indirect antioxidant mechanisms such as up-regulation of antioxidant
367 genes and down-regulation of oxidative stress genes markers (Ismail et al., 2010). The use of 48 h GBR
368 as raw material for bread making is recommended as it provides higher antioxidant activity for a better
369 protection against oxidative stress which is linked with the development of several chronic diseases.

370 **Conclusions**

371 This study shows that germination of BR is a natural way of improving the nutritional quality of gluten-
372 free rice breads. Brown-rice germination for 48 h provides bread with nutritionally superior quality on
373 the basis of its higher content of protein, lipids and bioactive compounds (GABA and polyphenols),
374 increased antioxidant activity and reduced phytic acid content and glycaemic index.

375

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480

481 **FIGURE CAPTIONS**

482 **Figure 1.** *In vitro* digestibility of proteins (A) and starch (B) of gluten free bread from raw
483 (BR), pre-germinated (Pre-GBR) and germinated brown rice (GBR) at different times (12,
484 24 and 48 h). BR (*), Pre-GBR (■),12h GBR (▲), 24h GBR (♦),48h GBR (●). Values with
485 different letters in the table inset are significantly different ($P \leq 0.05$).

486

487 **Figure 2**. GABA, γ-oryzanol and total polyphenols content (TPC) (A); and antioxidant
488 activity (B) of gluten free breads from brown rice (BR), pre-germinated brown rice (Pre-
489 GBR) and germinated brown rice for 12 (12h GBR), 24 (24h GBR) and 48 h (48h GBR).
490 Error bars indicate standard deviation. Different letters indicate significant differences ($P \leq$
491 0.05, LSD test)

Table 1. Proximate composition, energy, free glucose and phytic acid content of gluten-free bread from raw (BR), pre-germinated (Pre-GBR) and germinated brown rice (GBR) at different times.

Treatment	Moisture (g/100g)	Total Protein (g/100g)	Fat (g/100g)	Carbohydrates (g/100g)	Ash (g/100g)	Energy (Kcal)	Free Glucose (g/100g)	Phytic acid (g/100g)
BR	49.77±2.15a	6.03±0.05c	6.96±0.05b	74.19±0.91a	2.85±0.01a	214±9b	0.29±0.02d	1.09±0.05c
Pre-GBR	50.08±1.40a	6.12±0.04c	6.74±0.04c	74.20±1.01a	2.42±0.03d	213±6b	0.31±0.02d	0.82±0.06a
12h GBR	50.46±1.72a	8.14±0.21a	6.50±0.06d	72.45±1.18b	2.65±0.04b	210±7b	0.39±0.03c	0.82±0.08a
24h GBR	49.98±0.75a	8.01±0.08ab	5.58±0.03e	73.74±0.55ab	2.52±0.03c	209±3b	0.52±0.04b	0.95±0.02b
48h GBR	44.45±1.49b	7.81±0.12b	7.72±0.04a	72.49±0.51b	2.35±0.05e	230±1a	0.97±0.02a	0.81±0.02a

Values with different letters in the same column are significantly different (P<0.05).

Table 2. Kinetics parameters of the in vitro starch digestibility and estimated glycemic index of gluten-free bread from raw (BR), pre-germinated (Pre-GBR) and germinated brown rice (GBR) at different times.

Treatment	C_∞ (g/100g)	k (min^{-1})	H_{90} (g/100g)	HI	eGI
BR	96.81±1.58a	0.006±0.001c	36.66±1.56b	60.21±3.89a	60.10±3.35a
Pre-GBR	81.23±4.56b	0.007±0.001c	44.84±1.05a	56.63±1.93a	57.01±1.66a
12h GBR	39.29±4.84c	0.025±0.005b	32.65±3.68b	47.04±5.53b	48.74±4.77b
24h GBR	40.88±5.46c	0.022±0.001b	32.86±6.03b	46.42±5.61b	48.22±4.84b
48h GBR	25.27±1.63d	0.041±0.006a	26.15±0.68c	34.30±0.91c	37.76±0.79c

C_∞: equilibrium concentration of starch hydrolysed after 180 min, K: kinetic constant, H90: starch hydrolysis at 90 min, Values with different letters in the same column are significantly different (P<0.05).

Figure 1

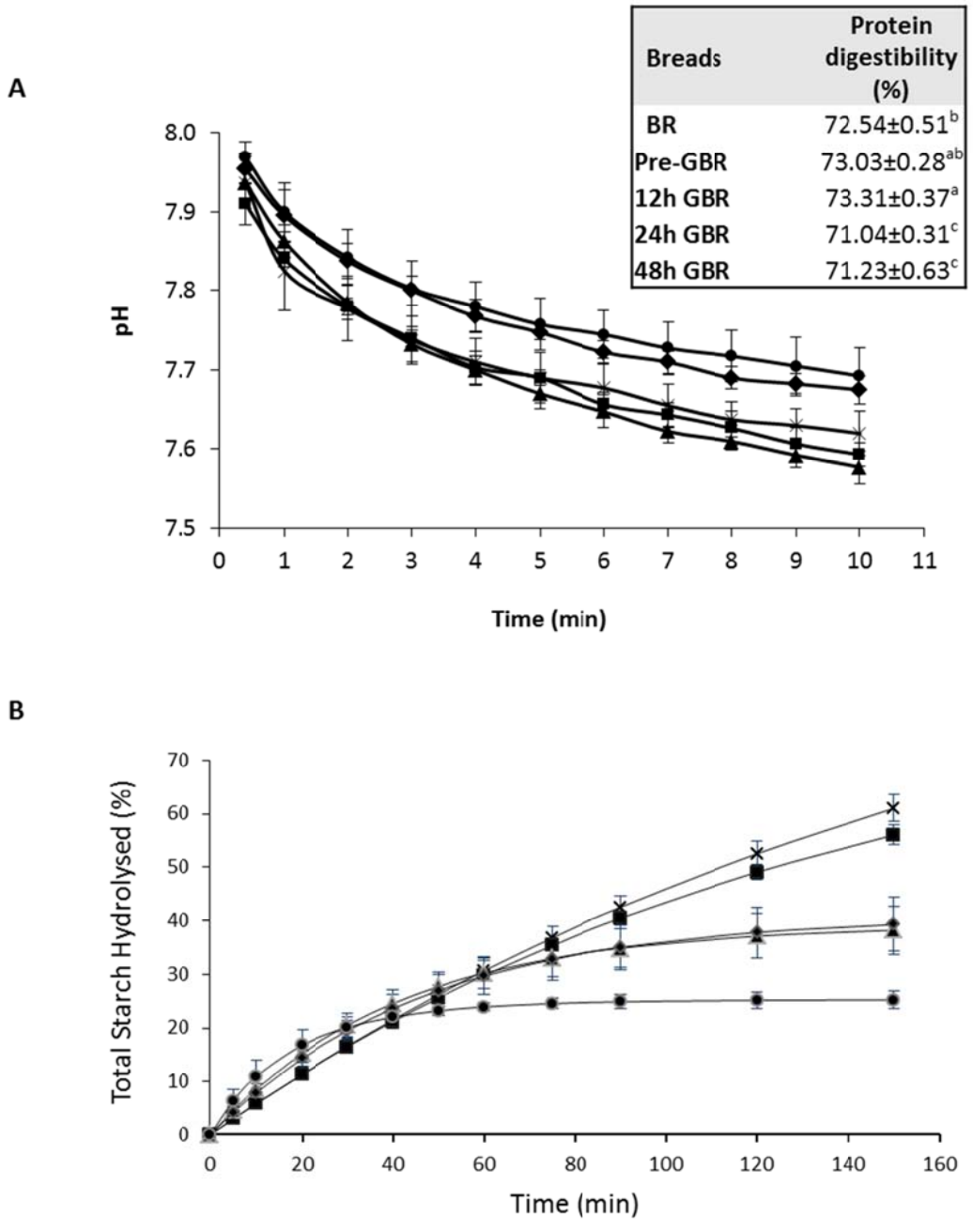
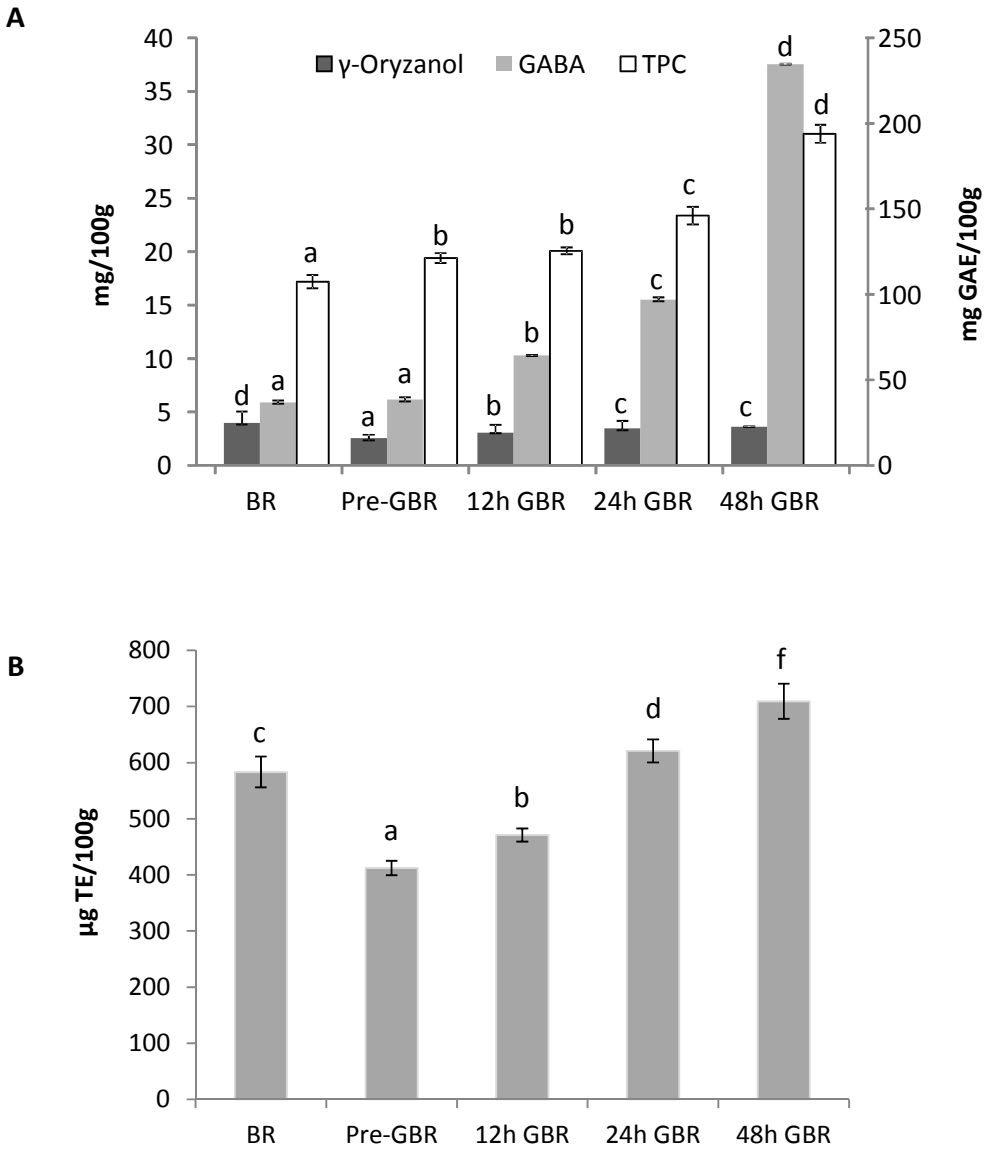

A
Breads
Protein digestibility (%)
BR
72.54±0.51b
Pre-GBR
73.03±0.28ab
12h GBR
73.31±0.37a
24h GBR
71.04±0.31c
48h GBR
71.23±0.63c
pH
Time (min)
B
Total Starch Hydrolysed (%)
Time (min)

Figure 2


A
γ-Oryzanol
GABA
TPC
mg/100g
mg GAE/100g
BR
Pre-GBR
12h GBR
24h GBR
48h GBR
B
µg TE/100g
BR
Pre-GBR
12h GBR
24h GBR
48h GBR