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, \textit{in vitro} protein digestibility and \textit{in vitro} enzymatic hydrolysis of starch, glucose and starch content, as well as the most relevant bioactive compounds (GABA, $\gamma$-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 \textit{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 et 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
the formulation of high quality of health products. In this scenery, GBR is used as a raw material for obtaining different food products, like GBR balls, soup, bread, doughnuts, cookies and rice burger (Ito and Ishikawa, 2004).

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

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

2. Materials and Methods

2.1. Materials

Commercial certified BR cultivar INIAP 15 was provided by the National Institute of Agricultural 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 °C. 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).
2.4. **Nutritional composition**

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

2.5. **Determination of phytic acid**

An accurate photometrical Haug and Lantzsch’s determination of phytic acid phosphorus was used (Reichwald and Hatzack, 2008) with some modifications. 1 mL of HCl 1M was added to 50 mg of sample in an airtight stopper vial and heated for 1 hour in glycerol bath at 80°C under constant agitation at 10 x g. The mixture was then cooled to room temperature and centrifuged at 10,621 x g for 5 min and 0.250 mL of the supernatant was diluted with 1 mL of distilled water. An aliquot of 0.4 mL of sample, standard (phytic acid solution in 0.2 M HCl) or blank (0.2M HCl) were added to 0.8 mL of ferric solution (0.05 g of FeCl₃ in 500 mL of 0.2 M HCl) in an airtight stopper vial and was heated for 1 hour in glycerol bath at 80 °C with agitation at 10 x g. The mixture was cooled in ice bath for 15 minutes and centrifuged at 10,621 x g for 5 minutes at room temperature. Aliquot of 0.6 mL of the supernatant was added to 0.8 mL of the complexing reagent (0.5 g of 2,2’-bipiridine and 65 µL of thioglycolic acid dissolved in 50 mL of 0.2 M HCl) and absorbance was read at 540nm using a microplate reader (BioTek Instruments, Winooski, VT, USA) controlled by the Gene 5TM software version 1.1. (BioTek Instruments).

2.6. **In vitro protein digestibility**
The *in vitro* protein digestibility of the samples was determined by the modified method of Hsu et al. (1977). Briefly, 50 ml of aqueous protein suspension having 6.25 mg protein/ml was prepared. Then, samples were placed in a 37 °C water bath and the pH was adjusted to 8.00 using 0.1 M NaOH and/or 0.1 M HCl, while stirring. Trypsin at a concentration of 1.6 mg/ml was maintained in an ice bath and the pH was adjusted to 8.00 with 0.1M NaOH and/or 0.1M HCl. Five millilitres of enzyme solution were then added to the protein suspension, which was kept stirred at 37 °C. The trypsin had an activity of 13,766 BAEE units/mg proteins. The pH drop was recorded along 15 s after enzyme addition and at one minute intervals for 10 min. The enzyme solution was always freshly prepared before each series of experiments. The percent protein digestibility (Y) was calculated by using Eq. (1) (Hsu et al., 1977): 

\[
Y = 210.464 - 18.1x \quad (1)
\]

where x is the change in pH after 10 min.

### 2.7. *In vitro* starch digestibility and expected glycaemic index

Starch digestibility of bread was determined by dried samples, following the method described by (Dura et al., 2014) with minor modifications. Briefly, for free sugars removal, powder sample (0.1 g) suspended in 2 mL of 80% ethanol was kept in a shaking water bath at 85 °C for 5 min, and then centrifuged for 10 min at 1000×g. The remaining pellet was incubated with porcine pancreatic α-amylase (6 U/mL) (Type VI-B, ≥10 units/mg solid, Sigma Chemical, St. Louis, USA) in 10 mL of 0.1 M sodium maleate buffer (pH 6.9) in a shaking water bath at 37 °C. Aliquots of 200 μL were withdrawn during the incubation period and mixed with 200 μL of ethanol (96%, w/w) to stop the enzymatic reaction and the sample was centrifuged at 10,000 × g for 5 min at 4 °C. The precipitate was washed twice with 50% ethanol (200 μL) and the supernatants were pooled together and kept at 4 °C for further glucose enzymatic release.
Supernatant (100 μL) was diluted with 850 μL of 0.1 M sodium acetate buffer (pH 4.5) and incubated with 50 μL amyloglucosidase (33 U/mL) at 50 °C for 30 min in a shaking water bath. After centrifuging at 2000 × g for 10 min, supernatant was kept for glucose determination.

The glucose content was measured using a glucose oxidase–peroxidase (GOPOD) kit (Megazyme, Dublin, Ireland). The absorbance was measured using an Epoch microplate reader (Biotek Instruments, Winooski, USA) at 510 nm. Starch was calculated as glucose (mg) × 0.9. The rate of starch digestion was expressed as a percentage of the total starch hydrolyzed at different times (30, 60, 90, 120, 150, and 180 min). Replicates (n = 4) were carried out for each determination. A non-linear model established by Goñi et al. (1997) was applied to describe the kinetics of starch hydrolysis. The first order equation (2) has the form: 

\[ C = C_\infty (1 - e^{-kt}) \]  

where \( C \) corresponds to the percentage of starch hydrolyzed at time \( t \), \( C_\infty \) is the equilibrium percentage of starch hydrolyzed after 180 min, \( k \) is the kinetic constant and \( t \) is the time (min). The parameters \( C_\infty \) and \( k \) were estimated for each treatment.

Using the hydrolysis curve (0–180 min), hydrolysis index (HI) was obtained by dividing the area under the hydrolysis curve of the sample by the area of standard material obtained for white bread. The expected glycemic index (eGI) was calculated using the equation described by Grandfeldt et al. (1992):

\[ eGI = 8.198 + 0.862HI. \]

2.8. **Determination of \( \gamma \)-aminobutyric acid (GABA)**

\( \gamma \)-Aminobutyric acid (GABA) content was determined by HPLC as described in Caceres at al. (2014). 50 μL aliquot of concentrated water-soluble extract and 10 μL allyl-L-glycine solution (Sigma-Aldrich) used as internal standard were derivatized with 30 μL phenyl isothiocyanate (PITC 99%, Sigma-Aldrich) and dissolved in mobile phase A for GABA analysis. An Alliance Separation Module 2695 (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 °C. 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 wavelength 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.
2.10. **Determination of total phenolic content**

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

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

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

2.12. **Statistical Analysis**

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

3. **Results and Discussion**

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

The chemical composition of gluten free bread from BR and non-germinated BR showed no significant difference, with exception of ash content that was significantly lower in the bread from soaked flour 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
(Albarracin 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
increasing evidences that phytic acid may display health benefits reducing cholesterol levels in the diabetic KK mice (Lee et al., 2005) and exerting antioxidant and anticancer effects (Schlemmer et al., 2009).

3.2. Effect of soaking and germination time on in vitro protein digestibility of BR bread

Considering that germination activates enzymes like amylases, proteases and so on, protein digestibility was tested to determine if germination might improve protein digestibility of the resulting breads. Germination affected in vitro protein digestibility (Figure 1, panel A), inducing an increase that was significant in breads obtained from rice after 12 hours germination (12h GBR), but further germination led to a significant reduction in protein digestibility. Bread samples 24h GBR and 48h GBR showed slower decline in pH compared with other treatments (Figure 1, panel A). It has been reported that BR germination increases the albumin and decreased the globulin and gliadin content, improving the protein bioavailability (Zheng et al., 2007). In addition, germination increases the amount of free amino acid, especially GABA content (Veluppillai et al., 2009). Divergences with the results obtained in the present study might be attributed to the participation of lysine containing proteins in the non-enzymatic browning (Maillard) reaction during baking that is more accentuated in breads obtained from flours with extended germination (Cornejo & Rosell, 2014). In addition, the high temperature during baking could produce crosslinks between amino acids forming more rigid structures that reduce protein digestibility. Indeed, Lamberts et al. (2012) demonstrated that GABA was largely involved in Maillard reactions during baking, resulting in GABA trace levels in wheat bread samples.

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 ($C_\infty$) obtained in the hydrolyzed process reflected the concentration at the equilibrium point. The $C_\infty$ 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 $C_\infty$ 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 $\alpha$-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.
granule as digestion proceeds. However, these $k$ values are even lower than the ones reported by Matos & Rosell (2011) obtained in some commercial gluten free breads.

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

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

The content of $\gamma$-oryzanol, GABA and TPC in BR bread (control) was 3.98, 5.92, 121.23 mg/100g d.m., respectively (Figure 2A). Breads from pre-germinated BR and GBR showed lower $\gamma$-oryzanol content than control breads ($P \leq 0.05$). Comparison of GBR breads showed that extended germination time (24 and 48 h) brought about increased $\gamma$-oryzanol content in breads although levels reached were lower than those found in control bread ($P \leq 0.05$). Our results agree with studies showing a reduced $\gamma$-oryzanol concentration in pre-GBR and GBR (Kiing et al., 2009). This effect could be attributed to increased feruloyl esterase activity involved in the hydrolysis of esters of phenolic acids such as $\gamma$-oryzanol (esters of trans-ferulic acid) that results in the release of ferulic acid as it has been previously reported in barley.
(Sancho et al., 1999). On the contrary, several studies have shown that pre-germination for 48 h and germination of BR bring about increased levels of γ-oryzanol (Moongngarm and Khomphiphatkul, 2011). These differences indicate that effect of soaking and germination processes on γ-oryzanol content depends on many factors such as BR cultivar and processing conditions (time, temperature, water pH) (Kiing et al., 2009). The content of γ-oryzanol in breads from pre-GBR and GBR was lower than that found in their respective flours (11 and 14 mg/100g d.m., respectively) (unpublished data). These results indicate that baking led to noticeable γ-oryzanol losses likely due to its thermal degradation and its hydrolysis during dough fermentation by feruloyl esterase activity of *Sacharomyces cerevisiae* that results in the release of ferulic acid (Coghe et al., 2004). γ-Oryzanol is also hydrolyzed upon gastrointestinal digestion into free sterol and ferulic acid by cholesterol esterases (Mandak and Nyström, 2012). Therefore, the reported biological activity of γ-oryzanol is likely due to free ferulic acid released during digestion. Few clinical studies has been performed so far to support the beneficial effect of ferulic acid in humans, however, results from these studies confirmed the potentially important role of ferulic acid in free radical-induced diseases (Alzheimer’s disease, cancer, cardiovascular diseases, diabetes mellitus and skin disease) observed in preclinical research (Mancuso and Santangelo, 2014).

Regarding GABA content, breads from pre-GBR were similar to control bread (Figure 2A). Germination markedly improved GABA content in bread, this effect being significantly greater with extended germination time (*P*≤0.05). Breads from 48h GBR showed 6 times higher GABA than control bread (*P*≤0.05). These results agree with a previous study reporting a time-dependent GABA accumulation during germination of BR (Caceres et al., 2014; Charoenthaikij et al., 2010). GABA accumulation initiates in the soaking process (Caceres et al., 2014; Charoenthaikij et al., 2010) and continues during germination due to the increased activity of glutamate decarboxilase that catalyses the 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 °C for 24h) and germinated (28 °C 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≤0.05) (Figure 2A). Similarly to GABA, TPC was noticeably improved in GBR breads with germination time (P≤0.05). Breads from GBR for 48 h showed 1.5 times higher total phenolic concentration than control bread (P≤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≤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.
Interestingly, increased antioxidant activity was observed in bread compared with control when BR was germinated for longer time (24 and 48h). These results could be ascribed to the biosynthesis of compounds with antioxidant activity to keep a balance of the redox homeostasis during germination and to the hydrolysis of bound phenolics due to polysaccharide cell-wall degradation (He et al., 2011). TPC and γ-oryzanol content were positively correlated with ORAC ($r^2 = 0.8614$ and 0.7627, respectively) which supports this hypothesis. Besides radical-scavenging activity, several studies have demonstrated that phenolic compounds and γ-oryzanol may also display their antioxidant effects acting as hydrogen and electron donors and through indirect antioxidant mechanisms such as up-regulation of antioxidant genes and down-regulation of oxidative stress genes markers (Ismail et al., 2010). The use of 48 h GBR as raw material for bread making is recommended as it provides higher antioxidant activity for a better protection against oxidative stress which is linked with the development of several chronic diseases.

**Conclusions**

This study shows that germination of BR is a natural way of improving the nutritional quality of gluten-free rice breads. Brown-rice germination for 48 h provides 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.

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(SENESCYT, Ecuador). National Autonomous Institute of Agricultural Research from Ecuador (INIAP) is thanked for providing the BR cultivars.

References


FIGURE CAPTIONS

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

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

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

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.

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

$C_\infty$: equilibrium concentration of starch hydrolysed after 180 min, $k$: kinetic constant, $H_{90}$: starch hydrolysis at 90 min, Values with different letters in the same column are significantly different ($P<0.05$).
Figure 1

A

B

<table>
<thead>
<tr>
<th>Breads</th>
<th>Protein digestibility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BR</td>
<td>72.54±0.51^b</td>
</tr>
<tr>
<td>Pre-GBR</td>
<td>73.03±0.28^d</td>
</tr>
<tr>
<td>12h GBR</td>
<td>73.31±0.37^a</td>
</tr>
<tr>
<td>24h GBR</td>
<td>71.04±0.31^c</td>
</tr>
<tr>
<td>48h GBR</td>
<td>71.23±0.63^c</td>
</tr>
</tbody>
</table>