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Research article

Effect of whey protein and riceberry flour on quality and antioxidant activity under gastrointestinal transit of gluten-free cookies

Araya Ranok* and Chanida Kupradit

Department of Applied Biology, Faculty of Science and Liberal Arts, Rajamangala University of Technology Isan, Nakhon Ratchasima, 30000, Thailand

* **Correspondence:** Email: araya7799.bc@gmail.com; Tel: +6 694 281 5588; Fax: +6 644 233 052.

Abstract: The objective of this study was to develop nutritionally enriched gluten-free cookies by using whey protein concentrate (WPC) and rice (var. riceberry) flour (RB). The effects of WPC and/or RB on physicochemical properties, antioxidant activity under simulated gastrointestinal (GI) transit, and sensorial acceptability of cookies were evaluated and compared to those with wheat flour-based cookies. The bioactive contents, total polyphenols and gamma amino-butyric acid, significantly increased with increasing RB ($P < 0.05$). The physical properties showed that colour parameters (L*, a* and b*), water activity, and hardness values of cookies significantly increased as the amount of WPC increased up to a level of 50%. Incorporation of 50% WPC showed markedly the highest antioxidant activity under GI digestion with the values of 4.72 ± 0.02 mg Trolox eq./g (ABTS), 3.12 ± 0.06 mg Trolox eq./g (FRAP), and 26.57 ± 0.66 mg EDTA eq./g (metal chelating activity). The overall results showed that cookies with acceptable quality and improved nutrition and antioxidant activity could be produced by complete replacement of wheat flour with the WPC and/or RB. However, in order to obtain the desired cookie characteristics, the ratio of WPC to RB should be developed.

Keywords: antioxidants activity; whey protein; riceberry flour; gluten-free cookie; gastrointestinal transit

1. Introduction

Currently, consumers have considerable interest in functional foods with added health benefits as well as avoidance of certain foods which their bodies cannot properly process. Therefore, there is a great need for creating food products that could present a better alternative to dietary supplements

in terms of safety, consumption, delivery and effectiveness of bioactive compounds *in vivo* [1]. Cookies are one of the most popular snacks and are consumed by worldwide consumers. However, their calorie content is high because their main ingredients contain a high concentration of flour, fat and sugar. Moreover, wheat flour containing gluten proteins is a major ingredient of almost all snacks that have negative effects on the many people suffering from gluten intolerance, coeliac disease [2]. Hence, the development of gluten-free products as the prevalence of celiac disease has increased. Rice is one of the most suitable cereal crops for gluten-free products because it has a low level of sodium, high digestibility and hypoallergenic properties [3].

Riceberry rice, black-purple rice variety, is of interest to the consumer because it provides health benefits and contains several nutrients. It contains high levels of anthocyanin (cyanidin-3-glucoside, pelargonidin-3-glucoside), vitamin E and γ-Oryzanol, which exhibit strong antioxidant effects [4]. The pigment of rice has been attributed to anthocyanins and proanthocyanins, which have been reported to have many beneficial effects on human health, such as anti-inflammation, anti-ageing, antioxidants effects, inhibition of the growth of cancer cells and diabetic prevention [5–7]. Moreover, the digestion of grain protein results in amino acids, peptides, and also in an accumulation of many nutrients such as γ-oryzanol, tocopherol, tocotrienol, and γ-aminobutyric acid (GABA). GABA is a neurotransmitter in the brain and spinal cord of mammals and induces hypotensive, diuretic and tranquillizing effects. Hence, riceberry flour could be a good raw material for food products in order to improve the dietary fibre and bioactive contents, as well as also being a cheaper raw material [8].

Along with gluten-free products derived from rice flour, the development of a high protein-containing cookie is a worthwhile challenge to improve their nutritional value by increasing the content of protein and lowering carbohydrates [9,10]. Among proteins, whey proteins, a natural by-product of cheese production, are of interest because of their nutritional and functional properties. Whey protein and its derivatives are used as common ingredients in various products in the food industry because they are an inexpensive source of desirable nutrient-rich residues with high protein, including branched-chain amino acids (leucine, isoleucine, and valine), essential amino acids (cysteine) and minerals [11]. Furthermore, the bioactive peptides encrypted in their sequences have been a particular focus as a source of antioxidants. These peptides are inactive within the sequence of the parent protein but can be released during enzyme hydrolysis. Once released, the peptides have been shown to possess antioxidant properties [12,13]. For the food industry, whey proteins were used for food texture modifications such as gelling, foaming and emulsifying. Many researchers have studied the effect of supplemental whey protein on the physical and chemical properties of cookies, and have shown various results based on the content of whey protein and other ingredients [9,10,14]. It has been proven that the protein and starch proportion in cookies plays an important role in cookie quality, because of their water absorption capacity, their effect in dough rheology and their spread in the baking process [15]. However, the replacement of wheat flour with whey protein in combination with riceberry flour to develop the high nutritional value and their antioxidant activity under gastrointestinal transit has not been studied. It may affect the decomposition or conversion of the active compound, resulting in changes in its antioxidant properties.

In this study, whey protein concentrate and riceberry flour were incorporated into gluten-free cookies to improve nutritional value and function. The effect of whey protein and/or riceberry flour on physicochemical properties, antioxidant activity (under simulated GI transit) and sensorial acceptability of gluten-free cookies was examined.

2. Materials and methods

2.1. Preparation of cookies

Different formulations of cookies were obtained by making composite flour with riceberry flour (RB) and whey protein concentrate (WPC) containing 87.82% protein. Four mixes of flour and/or WPC were 50% RB, 25% RB and 25% WPC, 50% WPC and 50% wheat flour (WF) as control. In all recipes, flour and/or WPC base kept constant at 50.0%, the amounts of other ingredients were as follows; egg (17.0%), sugar (15.0%), sodium bicarbonate (0.25%), and salted butter (17.75%). Cookies were prepared by cream-up phase where salted butter and sugar were mixed together by a mixer at a medium speed for 4 min. The other ingredients including egg and sodium bicarbonate were added at the same speed for 2 min with flour and/or WPC to form dough for 10 min at room temperature. Cookie dough was flattened and then cut into a fixed shape with a circular cookie cutter of 4 cm diameter and 0.7 cm height. Then, they were baked at 130 ℃ for 25 min. After cooling for 1 h, cookies were stored in sealed air-tight plastic bags under ambient conditions until evaluation.

2.2. Phytochemical composition

2.2.1. Total phenolic compound (TPC)

Total phenolic compound content was measured by the method described by Matthäus, (2002) with some modifications [16]. One microliter of sample was added to 2 mL of 2% Na_2CO_3, followed by standing for 2 min. Then, 100 µL of Folin-Ciocalteau reagent that was diluted with methanol (1:1) was added. After allowing to stand at room temperature for 30 min, the absorbance was measured at 750 nm. TPC content was calculated based on the gallic acid standard curve and expressed as micrograms of gallic acid equivalent per gram of sample (µg GAE/g sample).

2.2.2. GABA content

GABA content of cookies was evaluated by the method of Karladee & Suriyong (2012) with some adaptation [17]. One gram of sample was dissolved with 80% ethanol (1:4), followed by mixing. Samples were filtered and then 1 mL of filtered solution was aliquoted and boiled in a water bath (80 ℃) to evaporate the ethanol. Each sample was diluted with 0.5 mL distilled water, followed by centrifugation at 10,000 rpm for 10 min. The floating portion on top was separated and evaporated, and then 0.2 mL of 0.2 M borate buffer and 1.0 mL of 6% phenol were added. The solutions were mixed and cooled on ice for 5 min. Then, 0.4 mL of 10% NaOCl was added and mixed for 1 min, followed by allowing to stand on ice for 5 min. At last, the solution was boiled for 10 min, and allowed to cool. The absorbance of sample was measured at a wavelength of 630 nm. GABA content was calculated and was expressed as micrograms of GABA per gram of sample (µg GABA/g sample).

2.3. Proximate composition

Cookies were analysed for chemical composition including moisture, ash, protein and fat

according to AOAC (*2000*). The content of total carbohydrates was determined by the difference after subtracting the moisture, protein, fat and ash content from the total matter.

2.4. Physical properties

Water activity (a_w) was determined in triplicate using an AQUALAB instrument (Series 3, Decagon, USA), calibrated in the range 0.1–0.95 with distilled water.

The L^*, a^* and b^* colour values of the different cookies were measured using a colour measurement spectrophotometer (Hunter Lab Colour measuring Labscan XE system, USA) with $L^*a^*b^*$ scale coordinates. L^* indicates degree of lightness or darkness; a^* indicates degree of redness (+) and greenness (−); whereas b^* indicates degree of yellowness (+) and blueness (−). Measurements were taken after 1 day of storage.

The texture analysis of sample was performed using a TA-CT3 texture analyser (Brookfield Texture Analyzer, Germany) equipped with 12.7 mm diameter cylinder probe. The test speed was fixed at 1 mms^{-1} with two penetration cycles. The force exerted on the sample was recorded, and the parameters of hardness (g) and chewiness (mJ) were evaluated. The test was replicated six times.

2.5. In vitro pepsin–pancreatin simulated GI digestion

Gluten-free cookies were subjected to *in vitro* simulated gastrointestinal conditions as described by Helal & Tagliazucchi (2018) with some modifications [18]. Initially, the samples were homogenized in 0.5% w/v NaCl solution. During gastric phase simulation, the pH of the samples was adjusted to 2.5 with 0.5 M HCl. Pepsin was added to samples (2,000 U/mL). The mixture was incubated at 37 ℃ for 2 h. The samples were then subjected to the simulated intestinal phase. The pH of sample was adjusted to 7.5 with 20% Na_2CO_3 and then pancreatin and bile salts were added (final concentration: 0.8 g/L and 10 mM, respectively), followed by incubation at 37 ℃ for 3 h. Aliquots of the samples were collected before and after each simulated peptic and pancreatic digestion for determination of α-amino acid content and antioxidant activity. To terminate the digestion, the sample aliquots were kept in boiling water for 10 min, and then cooled to room temperature and centrifuged at 11,000 g for 15 min to eliminate the insoluble material. The supernatants were stored at 4 ℃ before use.

2.6. Determination of α-amino acid content

In vitro digestibility of cookies in the GI tract was determined by measuring the α-amino acid content by the TNBS method, described by Adler-Nissen (1979) [19]. Briefly, 50 μL of each sample was mixed with 0.5 mL of 0.2 M sodium phosphate buffer, pH 8.2 and 0.5 mL of 0.005% TNBS reagent. The reaction mixture was incubated at 50°C for 1 h, then 1 mL of 0.1 N HCl was added for terminating reaction and the mixture was left standing at room temperature for 30 min. The absorbance of the sample was then measured at 420 nm. The results were expressed as milligram leucine equivalent per gram of sample.

2.7. Determination of antioxidant activities

2.7.1. ABTS radical cation ($ABTS^{\bullet+}$) scavenging activity assay

The $ABTS^{\bullet+}$ radical scavenging activities of samples were determined as described by Wiriyaphan et al. (2012) with a slight modification [20]. The twenty microliter of the samples were added to 1.980 mL of diluted $ABTS^{\bullet+}$ solution. The mixture was shaken for 30 s and left in the dark for 5 min. The absorbance of the solution was measured at 734 nm. The degree of $ABTS^{\bullet+}$ radical scavenging activity of samples was calculated on the basis of the trolox standard curve and was expressed as milligram trolox equivalent per gram of sample.

2.7.2. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay of samples was performed according to the methods reported by Wiriyaphan et al. (2012) with a slight modification [20]. One millilitre of FRAP reagent containing 10 mM 2,4,6-tripryridyl-s-triazine (TPTZ) in 40 mM HCl, 20 mM $FeCl_3$ and 0.3 M acetate buffer (pH 3.6) at a ratio of 1:1:10 (v:v:v) and 0.1 mL samples were added and immediately mixed. After standing at 37 ℃ for 20 min, the absorbance was measured at 593 nm. The ferric reducing activity of the test samples was expressed as microgram trolox equivalent per gram of sample.

2.7.3. Metal chelating capacity

The chelation of ferrous ions (Fe^{2+}) by digested gluten-free cookies was estimated by the method of Conway et al. (2013) with some adaptation [21]. Briefly, 50 μL of 2 mM $FeCl_2$ was added to 100 μL of different conditions of the sample that were mixed with 2.4 mL of distilled water. The reaction was initiated by the addition of 100 μL of 5 mM ferrozine solution. The mixture was shaken and left to stand at room temperature for 20 min prior to measuring the absorbance at 562 nm. EDTA standard solutions were used to determine the calibration curves. The chelation of ferrous ions of the test samples was expressed as milligram EDTA equivalent per gram of sample.

2.8. Sensorial analysis of cookies

A total of 30 volunteer panellists participated in the test. All were consumers of products and declared no food allergies or gluten intolerance. They were instructed to rinse their mouths with water between samples. For each sample, the panellists were asked to rate their preference for its appearance, odour, taste, texture and overall liking on three separate 9-box structured hedonic scales (scale 1, dislike to scale 9, like very much).

2.9. Statistical analysis

All data were expressed as mean ± standard deviation and analysed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range tests. A significance level of $P < 0.05$ was used.

3. Results and discussions

3.1. Phytochemical content

Total phenolic compounds (TPC) and GABA content of cookie were significantly enhanced when the addition of RB increased ($P < 0.05$) as shown in Table 1. The addition of 50% RB exhibited the highest of TPC and GABA content with the values of 1,015 ± 19.6 μg GAE/g and 280.1 ± 25.3 μg GABA/g, respectively, while the addition of 50% WPC fortified cookie gave the lowest TPC and GABA contents. These results suggested that purple or black pigmented in riceberry rice is the main substance of phenolic compounds, which has been reported as anthocyanin [7,22,23]. This is consistent with the study by Settapramote et al. (2018), which reported that the total phenolic compounds in riceberry rice were 179.16 to 327.61 mg GAE/100 g [24]. For GABA content, it was also found increased with increasing of RB. A study conducted by Thao and Niwat (2017) also reported that RB increased the GABA content in noodles, which is higher than that of white rice flour [25].

Table 1. Phytochemical and proximate composition of cookies substituted with various levels of riceberry flour (RB) and whey protein concentrate (WPC).

Formulation	TPC μg GAE/g	GABA μg /g	Proximate composition (%)				
			Protein	Lipid	Total Carbohydrate	Ash	Moisture
50% RB	1,015 ± 19.6^{a}	280.1 ± 25.3^{a}	9.2 ± 0.1^{c}	23.4 ± 1.7^{ab}	57.0 ± 3.1^{b}	2.2 ± 0.0^{c}	6.98 ± 0.04^{a}
25% RB, 25% WPC	934.7 ± 6.7^{b}	187.5 ± 11.1^{b}	22.9 ± 2.0^{b}	25.0 ± 1.0^{ab}	45.4 ± 2.4^{c}	2.3 ± 0.2^{b}	4.44 ± 0.21^{d}
50% WPC	292.7 ± 14.8^{d}	54.7 ± 7.7^{d}	38.3 ± 2.2^{a}	26.3 ± 1.5^{a}	27.5 ± 1.2^{d}	2.7 ± 0.0^{a}	5.20 ± 0.03^{b}
50% WF	365.9 ± 7.4^{c}	95.5 ± 10.9^{c}	9.0 ± 1.1^{c}	22.7 ± 2.0^{b}	62.1 ± 3.8^{a}	1.1 ± 0.0^{d}	5.02 ± 0.40^{c}

Notes: Values are expressed as mean ± standard deviation (n = 3). Means in the same column with different letters were significantly different at $P < 0.05$.

3.2. Proximate composition

The chemical composition of cookies substituted with flour and/or WPC are presented in Table 1. The addition of whey protein at inclusion levels of 25% and 50% significantly promoted an increase in protein and ash content ($P < 0.05$) when compared with RB and WF based cookies. The protein content of 25% and 50% WPC fortified cookies were 22.9 ± 2.0 and 38.3 ± 2.2%, respectively. This increase may be due to the addition of whey protein in the product that was rich in protein and minerals as expected [11]. Cookies fortified with WPC had a fat content slightly greater than the RB and WF cookie due to the fact that WPC had a higher fat content than wheat flour [9]. As the increase of protein occurred, it corresponded to a decrease in carbohydrate content of the cookies with increasing replacement of flour. These results are in agreement with Parate et al. (2011) who reported that the addition of whey protein in biscuits resulted in high protein and minerals and a

slight increase of fat but carbohydrate decreased significantly from the control [9]. The moisture content ranged from 4.44% to 6.98%, which the RB based cookie showed the highest moisture.

3.3. Physical properties of cookies

Physical properties (water activity, colour and texture properties) of flour and/or whey protein substituted cookies are shown in Table 2. The water activity (a_w) was affected by the amount of protein or RB added to replace the flour in the cookies. The water activity of cookies, an indicator of the keeping quality of the cookies, ranged from 0.38 to 0.48 which increased with an increase in the amount of whey protein. The increase in water activity value may be due to their water absorption capacity.

Colour is one of the most important factors for the consumer's consideration when purchasing products. The results of the hunter L^*, a^* and b^* values corresponding to lightness, redness, and yellowness, respectively, showed variation depending on the proportions of RB and WPC. L^*, a^* and b^* values significantly increased with increasing WPC level ($P < 0.05$) which showed yellowness as expected. The incorporation of RB showed a dark brown colour, resulting in a significant reduction in L^*, a^* and b^* values. This result is due to RB containing dark purple pigments which present phenolic acids and anthocyanins being main constituents [23]. Additionally, the oxidation reaction of phenolic compounds in RB during the thermal process is also involved in colour changes of the product [26]. This agrees with many researchers who have observed that the substitution with brown rice flour in products resulted in dark brown coloration, reduction of L^*, a^* and b^* values [8,27,28]. L* value of 50% WPC substituted cookies (64.45) was lower than the control (67.86), suggesting its high lactose and protein contents likely influenced the higher browning rate. Gallagher et al. (2003) described the influence of whey powders in gluten-free bread, finding lower L* values in batches formulated with whey powders than in bread made without whey [29].

Textural characteristics are some of the most important factors affecting the quality of products and purchasing by consumers. In this study, two properties including hardness and chewiness were determined. Hardness is measured as the peak force needed to penetrate the products, while chewiness refers to the energy required to turn a biscuit into a state in which it can be swallowed [8,27]. The results revealed a trend of increasing hardness and chewiness as the amount of WPC increased (Table 2). The increase in chewiness indicated a corresponding increase in the hardness of the cookies with increasing WPC and showed significantly higher hardness than both RB and WF based cookies. The incorporation of 25% RB and 25% WPC in cookies revealed the highest values of hardness (1.19 ± 0.17 Kg) and chewiness (3.33 ± 0.07 mJ), while the incorporation of 50% RB revealed the lowest values according to the texture of incorporated RB cookie was crumbly and brittle. This was due to rice flour lack of gluten, which is the most important protein for contributing water retention, forming elastic structures [27,31]. The decrease in hardness as the content of purple and brown rice flour in the product increased was observed by Chung et al. (2014) and Klunklin and Savage (2018) [8,27]. Sarabhai et al. (2015) observed that when rice flour and WPC were added to the biscuits the hardness value was highest [30]. As a result of Gani et al. (2015) who showed that the fracture force of cookies blended with protein concentrates and hydrolysates increased significantly with increased levels of protein concentrates [10]. The different texture characteristics of cookies could be attributed to the changes in gluten content and the degradation of

macromolecules and low bulk density of flour showing that the proportional ingredients and thermal process are important [27,31].

Table 2. Physical properties of cookies substituted with various levels of riceberry flour (RB) and whey protein concentrate (WPC).

Formulation	a_w	Color			Hardness (kg)	Chewiness (mJ)
		L*	a*	b*		
50% RB	0.43 ± 0.01^{c}	36.72 ± 0.00^{d}	5.79 ± 0.00^{d}	1.15 ± 0.02^{d}	0.27 ± 0.07^{d}	0.05 ± 0.06^{c}
25% RB, 25% WPC	0.45 ± 0.01^{b}	41.57 ± 0.02^{c}	9.47 ± 0.00^{c}	2.63 ± 0.01^{c}	1.19 ± 0.17^{a}	3.33 ± 0.07^{a}
50% WPC	0.48 ± 0.00^{a}	64.45 ± 0.00^{b}	14.25 ± 0.02^{a}	25.98 ± 0.01^{a}	0.86 ± 0.22^{b}	1.15 ± 0.31^{b}
50% WF	0.38 ± 0.00^{d}	67.86 ± 0.18^{a}	11.28 ± 0.06^{b}	22.11 ± 0.05^{b}	0.41 ± 0.05^{c}	0.25 ± 0.17^{c}

Notes: Values are expressed as mean ± standard deviation (n = 6). Means in the same column with different letters were significantly different at $P < 0.05$.

3.4. Changes in α-amino acid content during GI digestion

A two-phase *in vitro* digestion (a pepsin treatment for 2 h followed by a pancreatin treatment for 3 h) was used to determine the α-amino acid content and antioxidant activities. An increase in α-amino acid content indicated the extent of proteolysis during digestion as it reflected the formation of oligopeptides and/or amino acids [19]. The α-amino acid content of cookies significantly increased with increasing WPC concentration and sequential simulated GI digestive phase ($P < 0.05$), as shown in Figure 1. The α-amino acid content of 50% WPC substituted cookies exhibited the highest values in origin, gastric and pancreatic phase of digestion with values of 4.03 ± 0.03, 13.03 ± 0.33, and 48.51 ± 2.44 mg leucine Eq./g, respectively. This finding suggests that pepsin broke the peptide bonds of proteins in cookies into smaller fractions. The pancreatin may have hydrolysed some of the peptides into even smaller peptides and possibly amino acids, leading to the protein and/or peptides being more completely broken down [21,32].

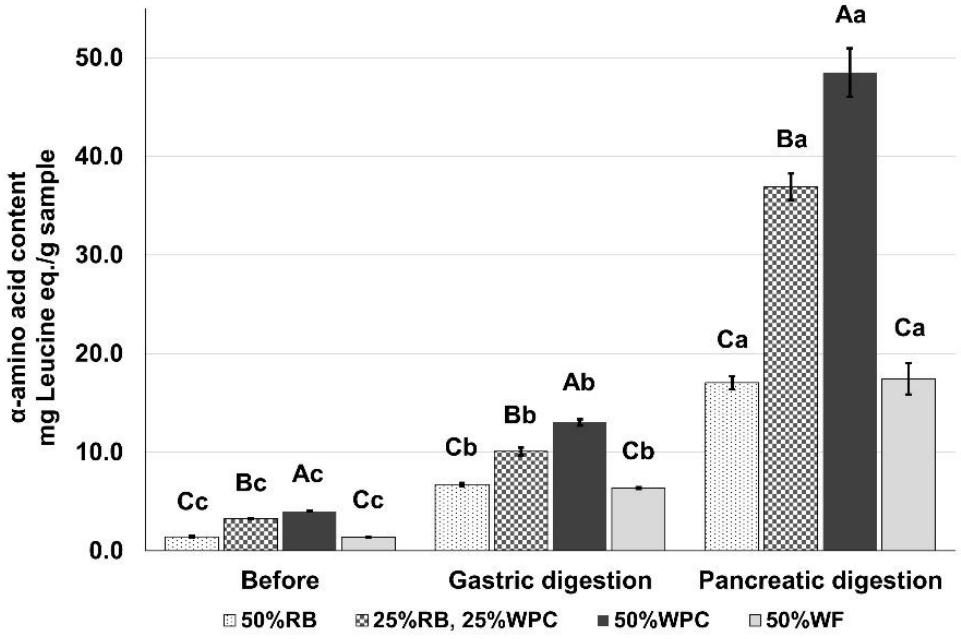

Figure 1. Changes in α-amino acid content during simulated *in vitro* digestion by pepsin–pancreatin. Data are expressed as mean ± standard deviation (n = 3). ^{A,B} different superscript capital letters denote significant differences between formulation of cookies for the same sampling period of the *in vitro* assay ($P < 0.05$). ^{a,b} different lowercase superscript letters denote significant differences between different sampling periods of the *in vitro* assay for the same formulation of cookie ($P < 0.05$).

3.5. Effect of simulated GI conditions on the antioxidant activity of cookie

3.5.1. ABTS radical scavenging activity

The ABTS radical scavenging activity of cookies incorporated with RB and/or WPC are shown in Figure 2a. Scavenging activity of cookies incorporated with RB and/or WPC was significantly higher than WF control ($P < 0.05$). Radical scavenging activity of cookies significantly increased with increasing WPC concentration and the sequential simulated GI digestion phase. Initial scavenging activity of RB and/or WPC based cookies were slightly differed but markedly different after digestion. This changing trend of scavenging activity correlated with the increasing α-amino acid content, indicating that the bioactive peptides formed during digestion could partly contribute to higher scavenging of $ABTS^{\bullet+}$. The disruption of the WPC structure by enzyme hydrolysis may have unfolded the protein, releasing of peptide and/or free amino acids, and exposed certain amino acid residues that were electron donors and could react with free radicals to become more stable products and eliminate the free radical [12]. These results are similar to those of Corrochano et al. (2019), who showed ABTS radical inhibition of whey protein hydrolysate after the simulated GI digestion was significantly higher than that of the intact protein and Peng et al. (2010) reported that the WPI hydrolysates had higher scavenging abilities on $ABTS^{\bullet+}$ when compared with non-hydrolysed WPI and increased with an increase in protein concentration [12,32]. The scavenging activity of RB based

cookies might be influenced by the high amount of polyphenols anthocyanin (cyanidin-3-glucoside, pelargonidin-3-glucoside), vitamin E and γ-Oryzanol which have strong antioxidant effects [24]. These compounds are able to capture free radicals by donation of phenolic hydrogen atoms; this is the reason for its antioxidant capacity [33].

3.5.2. Ferric reducing antioxidant power (FRAP)

The effect of RB and/or WPC substituted cookies on ferric reducing power is presented in Figure 2b. The trend of reducing power revealed the same ABTS radical scavenging which increased with an increase in WPC concentration. For *in vitro* assay, the results showed significant increases after the gastric phase and then showed a significant decline after the pancreatin digestive phase ($P < 0.05$). The highest reducing power was also observed in 50% WPC fortified cookies with values of 1.10 ± 0.08, 1.67 ± 0.04, and 1.22 ± 0.07 mg trolox Eq./g sample, by pepsin and pancreatin treatments, respectively. The strong reducing power of the cookies under gastric digestion may be due to the peptides possibly contained substances that were electron donors and could react with free radicals to terminate the radical chain reaction, such as Cys and Met residues containing nucleophilic sulfur side chains and Trp, Tyr, and Phe residues containing aromatic side chains that can easily donate hydrogen atoms [34]. Contrarily, a decrease in reducing power after pancreatic digestion suggests that the peptides were more completely hydrolysed, thus making them more hydrophilic and changing the length and structure of the peptide, which may not be suitable for donating hydrogen atoms [35].

3.5.3. Metal chelating capacity

The results of Fe^{2+}-chelation ability of cookies also increased with increasing WPC concentration and exhibited a decline after the gastric phase and then dramatically increased after pancreatic phase ($P < 0.05$) (Figure 2c). The influence of metal chelating capacity depended on net anionic charge of substance which digestive condition may affect. For WPC substituted cookies, it suggests that hydrolysis of protein increased peptide solubility, possibly due to the increased carboxyl and amino groups exposure which enhanced metal chelation. In addition, a net anionic charge will be established on a protein at pH values above its pI, causing the Fe^{2+}-chelating ability to decline after the gastric phase (pH 2.5) and increase markedly after the pancreatin phase (pH 7.5) [35]. These results are similar to those of Conway et al. (2013), who showed the metal-chelating capacity of WPC increased with an increasing WPC concentration after peptic and tryptic digestion [21]. Peng et al. (2010) reported that an increase in enzyme hydrolysed whey protein significantly enhanced antioxidant activities, which can act as a hydrogen donor, a metal ion chelator, and a radical stabiliser to inhibit lipid oxidation [12]. For anthocyanin-enrich RB cookies, the colour and stability of anthocyanin are influenced by pH, light, temperature, and structure. At low pH, the flavylium cation structure is formed and become an anionic quinoidal base structure at high pH [36]. This effect may affect the Fe^{2+}-chelating ability after the gastric phase decreases, and increases significantly after the pancreatic phase. These data indicate that the fortified WPC and RB cookies can be used as a functional food that can inhibit oxidative reactions by changing the physical location of transition metals in the human body.

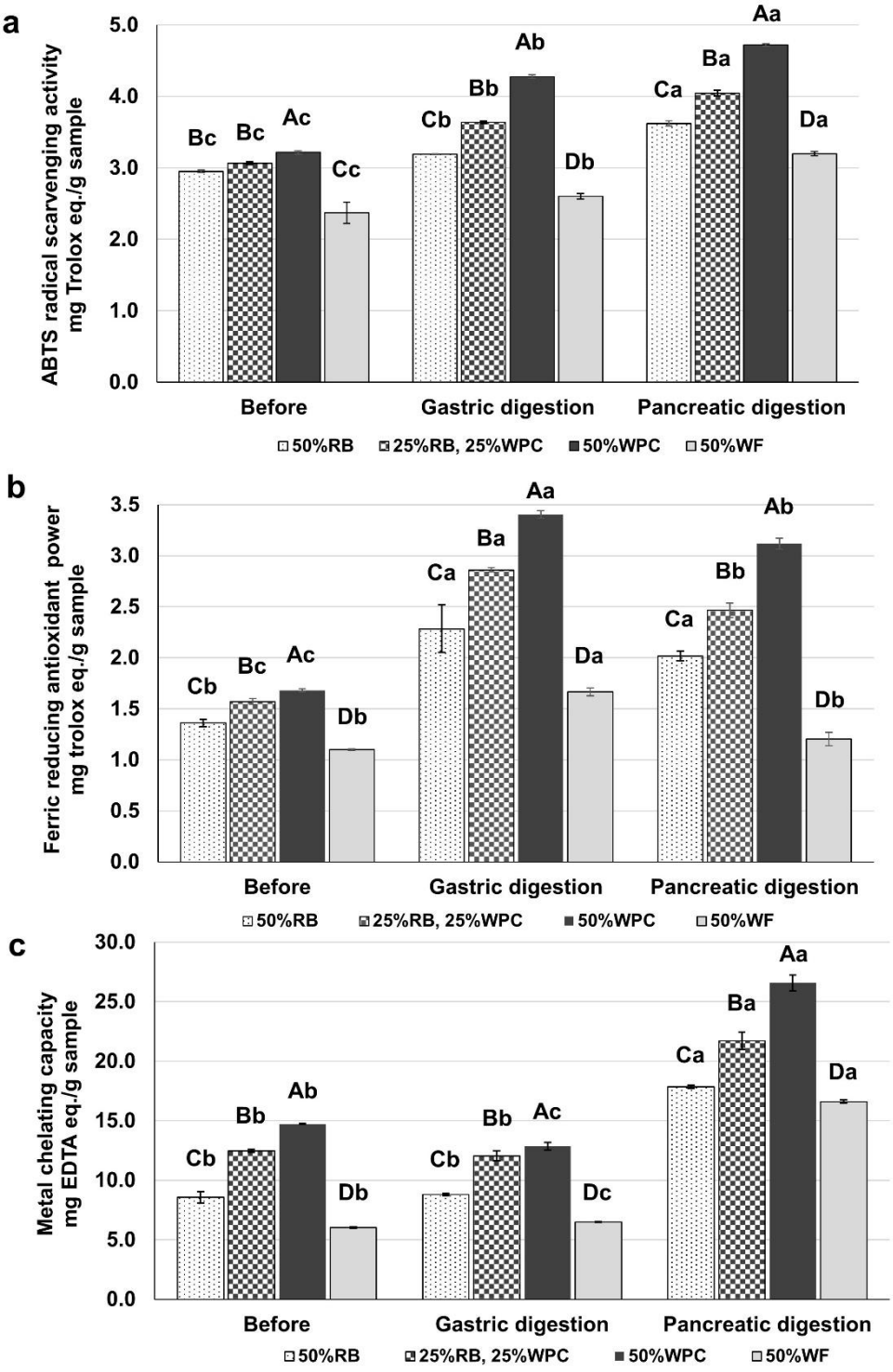

Figure 2. Antioxidant activity of cookies. Changes in ABTS radical scavenging activity (a), ferric reducing antioxidant power (b), and metal chelating activity (c) of cookies during sequential *in vitro* gastric and intestinal conditions. Data are expressed as mean ± standard deviation (n = 3). ^{A,B} different superscript capital letters denote significant differences between formulation of cookies for the same sampling period of the *in vitro* assay ($P < 0.05$). ^{a,b} different lowercase superscript letters denote significant differences between different sampling periods of the *in vitro* assay for the same formulation of cookie ($P < 0.05$).

3.6. Sensorial acceptability of cookies

Sensory evaluation results are shown in Table 3. The results revealed that sensory scores of cookies showed significant positive increases with increasing WPC in all the parameters. The substitution with RB in cookies decreased the sensorial acceptance when compared to the WF control. The control WF based cookies had the highest sensory values, reaching an average score of about 7.0. However, cookies incorporating 50% WPC had average acceptability scores for all the parameters that did not differ significantly from the control. These results indicate that cookies with higher WPC concentration showed higher acceptability than cookies with higher RB concentration. The scores relating to the measurements derived from the physical properties such as texture and colour associated with incorporation of cookies with 50% RB, revealed a crumbly texture and darkening, resulting in the low overall acceptability.

Table 3. Sensorial analysis of cookies using different proportion of RB and/or WPC.

Formulation	Appearance	Odour	Taste	Texture	Overall Acceptability
50% RB	4.85 ± 2.03^{c}	4.18 ± 1.84^{c}	3.80 ± 1.84^{c}	3.90 ± 1.85^{c}	4.38 ± 1.90^{c}
25% RB, 25% WPC	5.78 ± 1.83^{b}	6.00 ± 1.40^{b}	5.55 ± 1.89^{b}	5.53 ± 1.77^{b}	5.75 ± 1.74^{b}
50% WPC	7.00 ± 1.46^{a}	5.95 ± 1.65^{b}	5.90 ± 1.65^{ab}	5.85 ± 1.32^{ab}	6.43 ± 1.48^{ab}
50% WF	7.25 ± 1.46^{a}	6.93 ± 2.03^{a}	6.45 ± 1.85^{a}	6.45 ± 1.75^{a}	6.73 ± 1.48^{a}

Notes: Values are expressed as mean ± standard deviation (n = 30). Means in the same column with different letters were significantly different at $P < 0.05$.

4. Conclusions

The results of this study show that the partial or complete replacement of wheat flour with the WPC and/or RB can help modify the characteristics of gluten-free cookies with acceptable quality and improved nutrition and antioxidant activity. The products developed with WPC had nutritional enrichment related to protein and ash content, while those developed with RB exhibited a high level of bioactive compounds and GABA content. Adding WPC to cookies can significantly improve the hardness, brightness and yellowness of the product. The antioxidant activity during the GI transit increased with an increase in WPC, and the addition of 50% WPC showed the highest value. Compared with RB based cookies, the higher WPC content showed higher acceptability. This result indicated that by enhancing antioxidant activity and providing high nutritional value, cookies supplemented with WPC and RB can be produced as functional foods. However, in order to obtain the desired cookie characteristics, the ratio of WPC to RB should be developed.

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Conflict of interest

There are no conflicts of interest to declare.

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