Journal of Applied Biology & Biotechnology 2026. Article in Press
Available online at http://www.jabonline.in
DOI: 10.7324/jabb.2027.303513

Development and characterization of cookies partially substituted with retrograded black rice flour as a potential functional snack

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ARTICLE INFO

Article history:
Received on: 17 December 2025
Accepted on: 23 September 2026
Available Online: XX

Key words:
Black rice, dietary fiber, functional cookies, retrograded black rice flour, sensory evaluation

ABSTRACT

Cookies are widely consumed snacks across various social groups, although their nutritional value is generally considered limited. This study aimed to determine the optimal formulation of retrograded black rice flour (RBRF) in cookies by evaluating their chemical, physical, bioactive compound, and sensory characteristics. Composite flours were formulated at wheat flour-to-RBRF ratios of 100:0, 90:10, 80:20, 70:30, and 60:40. The physicochemical, nutritional, bioactive compound, and sensory properties of the resulting cookies were evaluated. Substitution with RBRF significantly ($p < 0.05$) decreased moisture (3.83%–2.41%) and protein (8.58%–5.32%) contents while increasing ($p < 0.05$) ash (1.22%–1.75%) and total dietary fiber (8.09%–36.99%). Water-holding capacity increased from 0.43 to 1.98 g/g with increasing RBRF substitution. Bioactive compound analysis showed significant increases ($p < 0.05$) in total anthocyanins (30.04–94.29 mg C3GE/100 g), flavonoids (2.95–6.54 mg QE/g), and total phenolics [62.95–119.41 mg gallic acid equivalents (GAE)/100 g] compared with the control. Sensory evaluation showed that 10% RBRF substitution achieved the highest overall acceptability score (6.61), slightly exceeding that of the control (6.50), whereas higher substitution levels negatively affected color and flavor attributes. Overall, partial substitution with up to 10% RBRF produced cookies with increased total dietary fiber and higher concentrations of anthocyanins, flavonoids, and phenolic compounds while maintaining acceptable sensory quality. These findings demonstrate the potential of RBRF as a functional ingredient for improving the nutritional and bioactive compound profiles of cookies.

1. INTRODUCTION

Cookies are among the most widely consumed bakery products, appreciated as convenient snacks due to their pleasant taste, textural appeal, and extended shelf life. Their popularity spans various age groups, resulting in a steadily increasing global demand [1,2]. Wheat flour is commonly used as the main ingredient in cookie formulations because of its gluten-forming properties, which provide desirable crispness and structural integrity. However, wheat-based cookies are nutritionally limited, mainly due to their low fiber and antioxidant content. This has raised concern amid growing consumer awareness of functional foods that help regulate blood glucose levels, prevent obesity, and support immune function. Consequently, low-glycemic-index foods are increasingly preferred not only by individuals with metabolic disorders but also by health-conscious consumers adopting preventive dietary habits [3]. In parallel, the gluten-free diet trend has gained momentum, driven by the rising prevalence of celiac disease and the search for digestively safer alternatives [4].

Rice flour, particularly from black rice (*Oryza sativa* L.), has attracted considerable attention as a promising ingredient for developing low-gluten bakery products such as cookies [5]. Black rice flour has been investigated as a total or partial substitute for wheat flour in various food systems, including cakes, breads, noodles, and fermented dairy products [6]. It is a rich source of anthocyanins, natural polyphenolic pigments responsible for its deep purple coloration and notable antioxidant capacity. Moreover, its starch-rich composition provides a functional carbohydrate matrix suitable for bakery applications [7]. Nonetheless, replacing wheat flour with black rice flour remains technologically challenging, often resulting in suboptimal texture, volume, or sensory properties. Recent advances in composite flour formulation have demonstrated that an appropriate ratio of rice and wheat flours can mitigate these drawbacks, where starch gelatinization plays a decisive role in defining cookie texture and structural attributes [5].

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Complete substitution of wheat flour in cookies is still difficult, particularly regarding dough rheology and structural formation. Therefore, incorporating dietary fiber or starch-based ingredients with improved functionality has emerged as a viable approach to enhance the nutritional and technological quality of cookies [8]. Starch modification techniques, such as retrogradation, have shown potential in this regard. The retrogradation process promotes the recrystallization of amylose and amylopectin chains, leading to increased resistant starch content classified as dietary fiber and altered water-binding properties and thermal stability [9]. These molecular rearrangements can improve dough handling, texture, and product stability. Retrograded starch granules also exhibit greater resistance to mechanical shear during mixing and baking, thereby contributing to improved physical properties in cookies. Several studies have reported that using retrograded flours can substantially enhance flavor, texture, and overall quality of bakery products [10]. For instance, a formulation containing 90% retrograded banana flour and 10% wheat flour yielded cookies with a low glycemic index, though sensory evaluation was not conducted [11].

Changes in ingredient composition can markedly affect the physicochemical and sensory characteristics of cookies, as each raw material exhibits distinct functional properties [3,12,13]. Therefore, optimization of flour composition is essential to achieve desirable product quality. Previous studies have investigated the incorporation of black rice flour, resistant starch, and retrograded flours from other sources, such as banana flour, into cookie formulations. However, the effects of incorporating retrograded black rice flour (RBRF) derived from a specific black rice variety into wheat-based cookies, particularly in relation to physicochemical characteristics, bioactive compounds, and sensory properties, remain insufficiently characterized. Therefore, this study aimed to develop and characterize cookies formulated with composite flours containing wheat flour and RBRF, with particular emphasis on their physicochemical properties, bioactive compounds, and sensory characteristics. This approach provides further insight into the potential application of RBRF as a functional ingredient in bakery products.

2. MATERIAL AND METHODS

2.1. Materials

Black rice (*Oryza sativa* L. var. Sirampog, Indonesia) was used as the primary raw material in this study. The cookie ingredients consisted of wheat flour (Segitiga Biru, Indonesia), margarine (Blue Band, Indonesia), butter (Wijsman, Netherlands), powdered sugar (Point, Indonesia), dark chocolate (Colatta, Indonesia), cocoa powder (Windmolen, Indonesia), and fresh chicken eggs obtained from local farmers in Semarang, Indonesia. All chemicals and enzymes used were of analytical grade and procured from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of Retrograded Black Rice Flour

Retrograded black rice flour was prepared following a microwave treatment method. Briefly, 30 g of black rice was mixed with 100 ml of deionized water and heated at 75°C for 15 minutes to form a paste. The paste was subsequently heated in a microwave at 900 W for 60 seconds and then cooled to room temperature. The cooled paste was oven-dried at 40°C for 24 hours and ground into a fine powder to obtain RBRF with a uniform particle size.

2.3. Cookies Preparation

The study began with the preparation of composite flours consisting of wheat flour (WF) and RBRF at ratios of 100:0 (WF), 90:10 (CF1), 80:20 (CF2), 70:30 (CF3), and 60:40 (CF4), based on research by Diza *et al.* [14] with modification. Each flour formulation was then used to prepare cookie dough according to the formulation presented in Table 1. The selection of a 0%–40% substitution range was informed by preliminary trials (data not shown), which revealed that substitution levels beyond 40% led to a marked loss of dough cohesiveness. Furthermore, this range aligns with substitution levels commonly employed in previous studies on comparable pigmented-rice or fiber-rich composite flours [5,6,14].

The composite flour was first blended until homogeneous and then mixed with melted margarine and butter using a high-speed mixer for 2 minutes. Powdered sugar was then added, and mixing was continued at high speed for 5 minutes. Egg yolk was incorporated and mixed for another 3 minutes at high speed. Subsequently, water was added, and the dough was mixed at low speed for 2 minutes until a soft and homogeneous consistency was obtained. Finely chopped dark chocolate and cocoa powder were then incorporated and evenly distributed using a spatula. Approximately 12 g of dough was molded with a stainless-steel cutter (4.5 cm diameter) and baked in a preheated oven at 170°C for 15 minutes. After baking, the cookies were cooled to room temperature and stored in sealed polyethylene pouches until further analysis.

2.4. Determination of Proximate Composition of Cookies

The proximate composition of the cookies, including moisture, ash, crude protein, and crude fat, was determined according to the AOAC methods [15]. Moisture content was measured by oven-drying the samples at 105°C to a constant weight. Ash content was determined by incineration in a muffle furnace at 550°C until a white residue was obtained. Crude protein was analyzed using the Kjeldahl method (N × 6.25), while crude fat content was determined by Soxhlet extraction using petroleum benzene as a solvent.

2.5. Determination of Dietary Fiber Profile of Cookies

The dietary fiber profile of the cookies was analyzed using the Megazyme Total Dietary Fiber Assay Kit (K-TDFR; Megazyme International, Ireland). Cookie samples (1.0 g) were defatted with petroleum ether and dried prior to analysis. The samples were sequentially digested enzymatically to remove starch and protein by incubation with heat-stable α-amylase (100°C, 30 minutes), protease (60°C, pH 7.5, 30 minutes), and amyloglucosidase (60°C, pH 4.5,

Table 1. Formulation of cookie dough from retrograded black rice flour.

Ingredients (g)	WF	CF1	CF2	CF3	CF4
Wheat flour	100	90	80	70	60
Retrograded black rice flour	0	10	20	30	40
Margarine	38	38	38	38	38
Butter	38	38	38	38	38
Water	7	7	7	7	7
Powdered sugar	50	50	50	50	50
Egg yolk	17	17	17	17	17
Dark chocolate compound	25	25	25	25	25
Cocoa powder	5	5	5	5	5
Total ingredients	280	280	280	280	280

WF, cookie from wheat flour; CF1 – CF4, cookie from composite flour. Composite flour made from wheat flour/retrograded black rice flour at 90:10 (CF1), 80:20 (CF2), 70:30 (CF3), and 60:40 (CF4).

30 minutes). Following enzymatic digestion, the mixtures were filtered to separate the insoluble dietary fiber (IDF) residue, which was washed with distilled water, 95% ethanol, and acetone and then dried at 105°C to a constant weight. The filtrates containing soluble dietary fiber (SDF) were precipitated with 95% ethanol (4× sample volume, preheated to 60°C), allowed to stand for 60 minutes, and then filtered, washed, and dried in the same manner. The total dietary fiber (TDF) content was calculated as the sum of IDF and SDF, corrected for protein and ash contents determined in duplicate residues. Results were expressed as grams per 100 g of dry sample.

2.6. Determination of Water-Holding Capacity of Cookies

Water-holding capacity (WHC) was determined according to the method of Santiago-García *et al.* [16] with slight modification. Briefly, 0.5 g of ground sample was mixed with 10 ml of distilled water and stirred for 1 minute. The mixture was allowed to stand at room temperature for 24 hours and then centrifuged at 3,000 rpm for 10 minutes. The supernatant was discarded, and the residue was weighed. WHC was expressed as grams of water retained per gram of sample.

2.7. Determination of Total Anthocyanin Content of Cookies

Approximately 1.0 g of cookie powder was accurately weighed and extracted with ethanol. The sample was transferred into a test tube, and ethanol was added to obtain a final extraction volume of 10 ml. The mixture was thoroughly homogenized and allowed to equilibrate before the extract was used for anthocyanin determination. Aliquots of the extract were then separately mixed with 1 ml of potassium chloride buffer (pH 1.0) and 1 ml of sodium acetate buffer (pH 4.5). Each mixture was allowed to stand at room temperature (approximately 25°C) for 15 minutes. Absorbance was measured at 520 and 700 nm using a UV–Vis spectrophotometer. The absorbance difference (A) was calculated as the difference between ($A_{520} - A_{700}$) at pH 1.0 and ($A_{520} - A_{700}$) at pH 4.5 [17].

Total anthocyanin content (TAC), expressed as mg cyanidin-3-glucoside equivalents (C3GE) per 100 g of sample, was calculated using the following equation:

$$TAC\ (mg\ C3GE/100\ g) = [A \times MW \times DF \times 1000 / (\varepsilon \times l)] \times [V/m] \times 100$$

where A is the absorbance difference, MW is the molecular weight of cyanidin-3-glucoside (448.8 g/mol), DF is the dilution factor, ε is the molar absorptivity of cyanidin-3-glucoside (26,900 L/mol·cm), l is the optical path length (1 cm), V is the total volume of extract (0.010 L), and m is the mass of sample (1.0 g). In the present extraction procedure, 1.0 g of cookie powder was extracted to a final volume of 10 ml (0.010 L). Because an aliquot of the resulting extract was used directly for the pH differential assay without any additional dilution, the DF was 1. Thus, the DF accounts only for any additional dilution performed after extraction; if the extract is further diluted before spectrophotometric measurement, the corresponding dilution factor should be incorporated into the calculation.

2.8. Determination of Total Flavonoid Content of Cookies

The total flavonoid content (TFC) was determined using the aluminum chloride colorimetric method with slight modifications. Cookie powder (0.5 g) was extracted with 5 ml of ethanol and filtered. Then, 1 ml of the filtrate was mixed with 4 ml of distilled water and 0.3 ml of 5% (w/v) $NaNO_2$ solution. After 5 minutes, 0.3 ml of 10% (w/v) $AlCl_3$ solution was added, followed by 2 ml of 1 M NaOH after 6 minutes. The mixture was then adjusted to a final volume of 10 ml with distilled water and thoroughly mixed. Absorbance was measured at 510 nm using a UV–Vis spectrophotometer. Quercetin dissolved in ethanol (10–100 ppm) was used to construct the standard calibration curve. The results were expressed as mg quercetin equivalents (QE) per g of sample [18].

2.9. Determination of Total Phenolic Content of Cookies

Cookie powder (0.5 g) was placed in a dark tube, and 5 ml of 10% (v/v) Folin–Ciocalteu reagent was added. The mixture was homogenized for 5 minutes, followed by the addition of 4 ml of 7.5% (w/v) Na_2CO_3 solution. The reaction mixture was incubated at room temperature (25°C ± 1°C) for 60 minutes. Ethanol was used as the blank solution. Gallic acid dissolved in ethanol (100–500 ppm) was used to construct the calibration curve. Absorbance was measured at 765 nm using a UV–Vis spectrophotometer. The TPC of cookie was expressed as mg GAE per 100 g of sample [18].

2.10. Sensory Evaluation of Cookies

The sensory attributes of the cookies were evaluated by a panel of 30 semi-trained members selected from the Department of Food Technology and Nutritional Sciences at Universitas Muhammadiyah Semarang, Indonesia. All panelists were familiar with the characteristics of cookie quality. The evaluation was conducted using a 7-point hedonic scale, ranging from 1 (dislike very much) to 7 (like very much). Each cookie sample was assigned a random three-digit code to avoid bias. The evaluated attributes included color, flavor, crispiness, and overall acceptability. Panelists were provided with mineral water to rinse their mouths between samples [19].

2.11. Statistical Analysis

All physicochemical and chemical analyses were performed in quintuplicate, with the five replicates corresponding to five independently prepared and baked cookie batches per formulation (biological replication), rather than repeated measurements of a single batch. Sensory evaluation involved 30 panelists ($n = 30$), and data from all panelists were complete and retained in the analysis. Data were expressed as mean ± standard deviation (SD). Statistical analyses were carried out using SPSS software (version 20.0; IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) was applied to determine significant differences among the samples. When significant differences were detected ($p < 0.05$), mean comparisons were performed using Duncan's multiple range test (DMRT).

3. RESULT AND DISCUSSION

3.1. Proximate Composition of Cookies

Substitution of wheat flour with RBRF had a significant effect on the proximate composition of the cookies, particularly moisture, ash, and protein contents. As presented in Table 2, moisture content declined progressively with increasing RBRF substitution, from 3.83% in the control WF to 3.49% in CF1 and 2.41% in CF4. This decline is likely related to differences in water retention capacity and matrix structure resulting from higher RBRF levels, a pattern consistent with previous reports on cookies formulated with dietary fiber-enriched flours [16,20]. Changes in starch structure during retrogradation have been linked in the literature to altered water-binding behavior [21]; however, resistant starch formation and the specific retrograded structures involved were not directly quantified in this study. Importantly, the moisture content of all samples remained below 4%, satisfying both the Indonesian National Standard and the United States standard for

cookies [22,23], and the CF3 and CF4 samples fell within the 1.5%–3.0% range reported to be optimal for maintaining textural crispness during storage [24].

Ash content increased significantly with higher RBRF substitution, rising from 1.22% in the control WF to 1.36%–1.75% in CF1–CF4 (Table 2). This trend is consistent with the higher mineral content typically associated with black rice compared with wheat flour [25], indicating that RBRF substitution directly contributed to the increased ash content observed. In contrast, fat content varied only slightly among formulations, with the control WF showing the highest value (30.81%) compared with RBRF-substituted samples (28.15%–28.49%). This modest reduction may reflect compositional differences and interactions within the flour matrix; however, lipid retention mechanisms during baking were not directly assessed in this study. The observed differences in fat content should therefore be interpreted as compositional outcomes rather than evidence of a specific resistant-starch-related mechanism.

Protein content ranged from 5.32% to 8.58%, with the highest value recorded in the control WF sample. Although protein content decreased significantly with increasing RBRF substitution, all formulations still met the minimum requirement of ≥4.5% specified by the Indonesian National Standard for cookies [22]. Notably, the protein levels observed in this study exceeded those reported by previous research for black rice-based cookies (3.9%) [26]. The decrease in protein content with increasing RBRF substitution can be attributed mainly to differences in the protein content of the raw materials, as black rice contains approximately 9.61% protein compared with up to 15% in wheat flour [27]. While retrogradation-related changes in starch–protein interactions and dough structure have been proposed as contributing factors [28,29], these mechanisms were not directly measured in the present study. Despite the decrease in protein content, RBRF-substituted cookies remain promising as low-gluten bakery products with added functional and nutritional value.

3.2. Dietary Fiber of Cookies

The TDF, SDF, and IDF contents of the cookie samples are presented in Table 3. Substitution of wheat flour with RBRF significantly influenced the TDF, SDF, and IDF contents ($p < 0.05$). The TDF content increased from 8.09% in the control formulation WF to 15.08%–36.99% in the RBRF-substituted formulations (CF1–CF4). This increase indicates a substantial contribution of RBRF to the dietary fiber fraction determined in the final cookie products. The magnitude of the increase in TDF was consistent with the incorporation of a fiber-containing raw material into the formulations. Although starch retrogradation may alter the proportion of starch fractions, including the formation of resistant starch under specific processing conditions [30,31], resistant starch was not independently quantified in the present study. Accordingly, the increase in TDF cannot be attributed specifically to resistant starch formation. Rather, the observed values represent the total dietary fiber fraction determined by the analytical procedure employed. Furthermore, potential physiological responses associated with dietary fiber consumption, including effects on satiety, gastrointestinal function, and postprandial glycemic response, were not assessed and therefore cannot be established from the present results [32].

Significant differences ($p < 0.05$) were likewise observed in the SDF and IDF fractions. The control cookies contained 1.93% SDF and 6.15% IDF, whereas the RBRF-substituted formulations exhibited SDF contents ranging from 1.90% to 6.84% and IDF contents ranging from 13.18% to 30.15%. The variation in SDF among formulations may reflect differences in the contribution of soluble components originating from RBRF and their distribution within the cookie matrix. Previous studies have reported that soluble dietary fiber can influence physicochemical characteristics of food systems, particularly water binding, viscosity, and textural attributes [33,34]. However, these functional relationships were not directly investigated in the present study and therefore should be regarded as potential implications rather than demonstrated effects. The marked increase in IDF may be associated with the incorporation of insoluble structural components present in RBRF, including cell-wall-associated constituents, as well as other fractions retained during the dietary fiber analysis. Since resistant starch was not separately determined, its quantitative contribution to the measured dietary fiber fractions could not be established. Collectively, these findings demonstrate that RBRF substitution significantly modified the dietary fiber profile of the cookies, with a more pronounced increase in the IDF fraction. While consumption of dietary fiber-rich foods has been associated in previous studies with

Table 2. Proximate composition of cookies.

Parameter	WF	CF1	CF2	CF3	CF4
Moisture (%)	3.83 ± 0.21^{e}	3.49 ± 0.20^{d}	3.15 ± 0.11^{c}	2.70 ± 0.07^{b}	2.41 ± 0.14^{a}
Ash (%)	1.22 ± 0.05^{a}	1.36 ± 0.02^{b}	1.50 ± 0.01^{c}	1.61 ± 0.04^{d}	1.75 ± 0.05^{e}
Lipid (%)	30.81 ± 0.59^{b}	28.49 ± 0.90^{a}	28.36 ± 0.56^{a}	28.25 ± 0.61^{a}	28.15 ± 0.76^{a}
Protein (%)	8.58 ± 0.20^{e}	7.55 ± 0.33^{d}	6.96 ± 0.44^{c}	6.19 ± 0.25^{b}	5.32 ± 0.36^{a}

Values are presented as means ± standard deviation. Values with different superscript letters within the same row are significantly different ($p < 0.05$). WF: dough/cookie from wheat flour formula; CF1 – CF4: dough/cookie from composite flour formula. Composite flour made from wheat flour/retrograded black rice flour of 90:10 (CF1), 80:20 (CF2), 70:30 (CF3), and 60:40 (CF4).

Table 3. Dietary fiber of cookies.

Parameter	WF	CF1	CF2	CF3	CF4
TDF	8.09 ± 0.11^{a}	15.08 ± 0.15^{b}	24.93 ± 0.44^{c}	29.09 ± 0.27^{d}	36.99 ± 0.12^{e}
SDF	1.93 ± 0.20^{a}	1.90 ± 0.25^{a}	4.99 ± 0.51^{b}	5.01 ± 0.22^{c}	6.84 ± 0.32^{d}
IDF	6.15 ± 0.10^{a}	13.18 ± 0.23^{b}	19.94 ± 0.17^{c}	24.09 ± 0.10^{d}	30.15 ± 0.22^{e}

Values are presented as means ± standard deviation. Values with different superscript letters within the same row are significantly different ($p < 0.05$). WF: dough/cookie from wheat flour formula; CF1 – CF4: dough/cookie from composite flour formula. Composite flour made from wheat flour/retrograded black rice flour at 90:10 (CF1), 80:20 (CF2), 70:30 (CF3), and 60:40 (CF4). TDF, Total Dietary Fiber; SDF, Soluble Dietary Fiber; IDF, Insoluble Dietary Fiber.

physiological outcomes such as increased satiety and attenuation of postprandial glycemic responses [32], such effects cannot be directly inferred from the dietary fiber composition determined in the present study and require confirmation through appropriate physiological or clinical assessments.

3.3. Water-Holding Capacity of Cookies

Substitution of wheat flour with RBRF significantly affected the WHC of the ground baked cookies, as shown in Figure 1. WHC reflects the ability of the ground baked cookie sample to absorb and retain water under the conditions of the assay. Compared with the control WF, which had a WHC of 0.43 g water/g, the RBRF-substituted formulations (CF1–CF4) exhibited significantly higher WHC values, ranging from 0.76 to 1.98 g water/g ($p < 0.05$). The increasing WHC with higher levels of RBRF substitution indicates that the resulting baked cookie matrix, after grinding, had a greater capacity to retain water under the experimental conditions. This increase may be associated with the hydrophilic components introduced through RBRF substitution and the structural characteristics of the processed rice flour. However, the specific contribution of resistant starch to the observed increase in WHC was not directly determined in this study. The observed trend is consistent with previous studies on retrograded/resistant starch systems, which showed changes in water-retention properties following starch modification [35,36]. Therefore, the present WHC results should be interpreted specifically as the water-retention capacity of the ground baked cookie samples and should not be extrapolated to dough functionality, dough rheology, storage behavior, or glycemic response, which were not evaluated in this study.

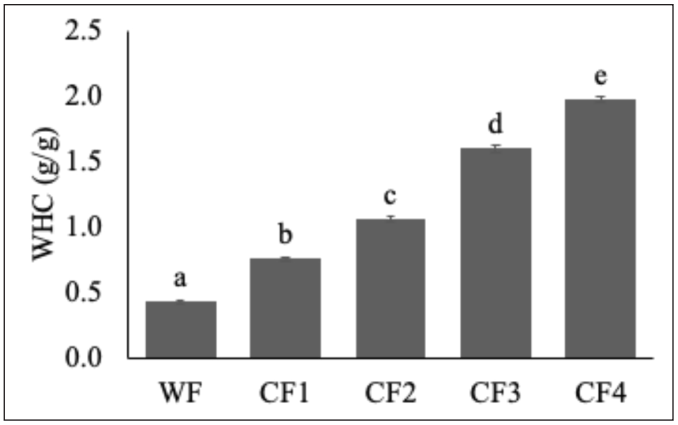

Figure 1. Water-holding capacity of cookies prepared with various flour formulas. Different superscript letters (a–e) indicate statistically significant differences ($p < 0.05$), as determined by Duncan's test. WF: Wheat Flour, CF1: Composite flour made from wheat flour: retrograded black rice flour (90:10), CF2: Composite flour made from wheat flour: retrograded black rice flour (80:20), CF3: Composite flour made from wheat flour: retrograded black rice flour (70:30), CF4: Composite flour made from wheat flour: retrograded black rice flour (60:40).

3.4. Total Anthocyanin Content of Cookies

The TAC of cookies substituted with RBRF (CF1–CF4) ranged from 30.04 to 94.29 mg C3GE/100 g (Fig. 2). These values were higher than those reported for muffins (27.54–46.11 mg C3GE/100 g) and biscuits (50.00 mg C3GE/100 g) formulated with black rice flour [37]. Cyanidin-3-glucoside has been identified as the predominant anthocyanin in black rice, accounting for approximately 88% of the total anthocyanin content [38]. In addition, black rice contains other anthocyanins, including cyanidin-3,5-diglucoside, cyanidin-3-rutinoside, malvidin-3-glucoside, and peonidin-3-glucoside [39,40]. The higher TAC observed in the RBRF-substituted cookies is consistent with the presence of anthocyanin compounds derived from black rice in the formulations.

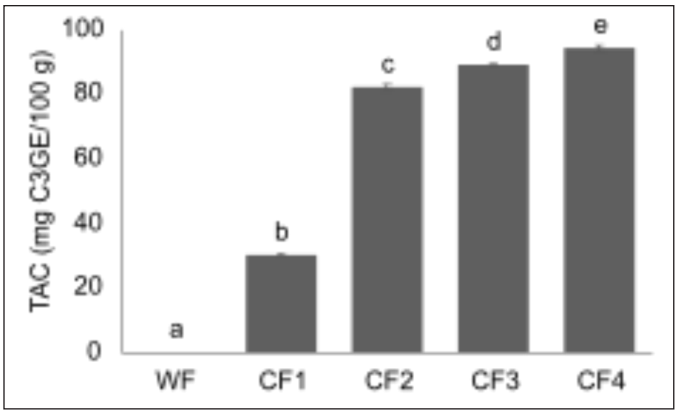

Figure 2. Total anthocyanin content of cookies prepared with various flour formulas. Different superscripts in (a-e) each figure indicate statistically significant differences ($p < 0.05$), as determined by Duncan's test. WF: Wheat Flour, CF1: Composite flour made from wheat flour: retrograded black rice flour (90:10), CF2: Composite flour made from wheat flour: retrograded black rice flour (80:20), CF3: Composite flour made from wheat flour: retrograded black rice flour (70:30), CF4: Composite flour made from wheat flour: retrograded black rice flour (60:40).

Previous studies have proposed that anthocyanins may interact with food proteins and potentially influence protein–protein interactions, including those associated with gluten structure [41,42]. Such interactions have been hypothesized to involve non-covalent interactions and, under certain conditions, possible effects on disulfide-bond-related protein network development. These mechanisms could represent a possible explanation for differences in dough or cookie characteristics observed with increasing RBRF substitution. However, this interpretation remains a hypothesis in the present study, as protein-network formation, rheological properties, disulfide bond changes, and microstructural characteristics were not directly evaluated. Therefore, the observed differences should not be attributed specifically to anthocyanin-mediated gluten weakening or disruption of disulfide bonds. Further studies incorporating rheological, protein-structural, and microstructural analyses would be required to test these proposed mechanisms.

Accordingly, the present results demonstrate an increase in anthocyanin content associated with RBRF substitution, but they do not establish antioxidant activity or a specific protective mechanism of anthocyanins during baking. Likewise, physiological benefits associated with the consumption of anthocyanin-rich foods reported in previous studies should not be interpreted as health outcomes demonstrated by the present study [43,44].

3.5. Total Flavonoid Content of Cookies

The TFC of cookies increased significantly with the incorporation of RBRF into the formulation (Fig. 3). The TFC of RBRF-substituted cookies ranged from 2.95 mg QE/g (CF1) to 6.54 mg QE/g (CF4), whereas the control sample (WF) contained only 0.18 mg QE/g. Flavonoids are among the phenolic compounds reported in black rice [26]. Tricin has been reported in black rice [44], and other flavonoids, such as luteolin, apigenin, quercetin,

isorhamnetin, kaempferol, and myricetin, have also been identified [45,46]. These compounds have been associated in the literature with various biological activities [47].

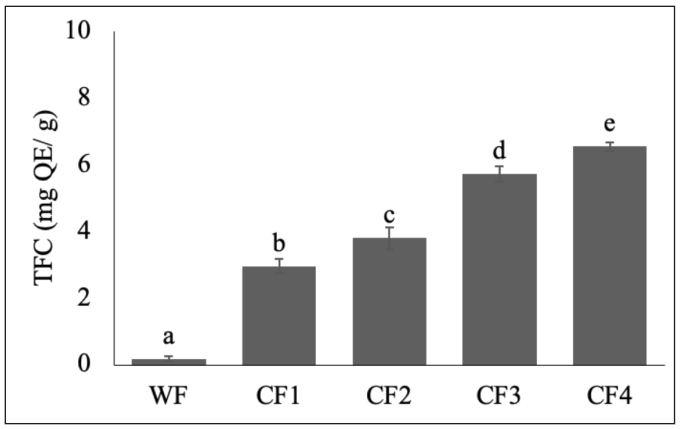

Figure 3. Total flavonoid content of cookies prepared with various flour formulas. Different superscripts in (a–e) each figure indicate statistically significant differences ($p < 0.05$), as determined by Duncan's test. WF: Wheat Flour, CF1: Composite flour made from wheat flour: retrograded black rice flour (90:10), CF2: Composite flour made from wheat flour: retrograded black rice flour (80:20), CF3: Composite flour made from wheat flour: retrograded black rice flour (70:30), CF4: Composite flour made from wheat flour: retrograded black rice flour (60:40).

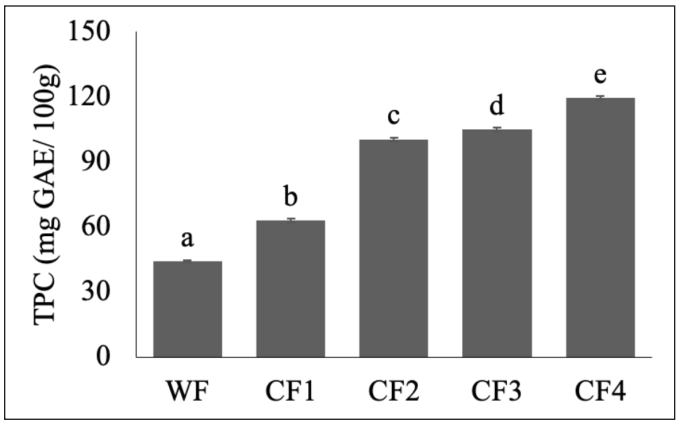

Figure 4. Total phenolic content of cookies prepared with various flour formulas. Different superscripts in (a–e) each figure indicate statistically significant differences ($p < 0.05$), as determined by Duncan's test. WF: Wheat Flour, CF1: Composite flour made from wheat flour: retrograded black rice flour (90:10), CF2: Composite flour made from wheat flour: retrograded black rice flour (80:20), CF3: Composite flour made from wheat flour: retrograded black rice flour (70:30), CF4: Composite flour made from wheat flour: retrograded black rice flour (60:40).

3.6. Total Phenolic Content of Cookies

The TPC of the control cookies WF was 44.16 mg GAE/100 g, as shown in Figure 4. In comparison, substitution with RBRF at levels of 10%–40% (CF1–CF4) significantly increased the TPC to 62.95–119.41 mg GAE/100 g, representing an enhancement of approximately 42.55%–170.40% compared to the control formulation. These values were higher than those reported by Mau [48], who observed a 30.01%–74.94% increase in the TPC of chiffon cakes supplemented with black rice flour at similar substitution levels. The elevated TPC in RBRF-substituted cookies is consistent with the contribution of phenolic acids, anthocyanins, and flavonoids naturally present in black rice [49]. The composition and concentration of phenolic compounds in black rice are strongly influenced by genotype and pericarp pigmentation, with approximately 81% of phenolics existing in bound form [50,51]. Overall, the substantial increases in measured anthocyanin, phenolic, and flavonoid contents indicate that RBRF acts as a source of bioactive compounds in the cookie formulations.

3.7. Sensory Properties of Cookies

Sensory evaluation is a key determinant influencing consumer acceptance and market potential for novel food products. In this study, cookies enriched with varying levels of RBRF were evaluated for color, flavor, crispiness, and overall acceptability. The mean scores are summarized in Table 4. The color attribute showed a gradual decline in panelists' preferences as RBRF substitution increased. The control cookies (100% wheat flour) obtained the highest score ($p < 0.05$), followed by those containing 10%, 20%, 30%, and 40%.

The decreased color preference was associated with the darker appearance of the cookies, attributed to anthocyanin pigments inherent in black rice. Although this darker hue indicates the presence of anthocyanin pigments, panelists preferred the lighter golden-brown appearance typical of conventional wheat cookies. Similar findings were reported in previous studies, in which substituting pigmented cereals reduced color preference due to decreased lightness and increased redness [52,53]. The brown coloration observed in cookies is a result of the Maillard reaction, a chemical reaction that occurs when sugars interact with amino acids during the baking process [54].

For the flavor attribute, the control cookies received the highest score (6.59) with a significant difference ($p < 0.05$), which gradually decreased (6.43–6.09) with increasing RBRF levels. This trend suggests that the characteristic aroma of black rice may have influenced flavor perception. Although low substitution levels (≤10%) remained well accepted, higher levels likely introduced earthy or grainy notes that were less preferred, particularly in darker samples. These findings are consistent with previous research on black rice flour–based snack crackers [53]. Interestingly, the crispiness attribute showed an opposite trend. Cookies containing 10% RBRF achieved a slightly higher score (7.00) than the control (6.92). This difference may be related to changes in the flour matrix and moisture

Table 4. Average level of cookies acceptability.

Parameter	WF	CF1	CF2	CF3	CF4
Color	6.29 ± 0.60^{d}	6.10 ± 0.79^{cd}	5.98 ± 0.69^{c}	4.79 ± 0.62^{b}	4.40 ± 0.95^{a}
Flavor	6.59 ± 0.72^{d}	6.43 ± 0.34^{c}	6.26 ± 0.85^{b}	6.17 ± 0.73^{ab}	6.09 ± 0.72^{a}
Crispiness	6.92 ± 0.59^{d}	7.00 ± 0.94^{e}	6.75 ± 0.27^{c}	6.26 ± 0.46^{b}	6.09 ± 0.81^{a}
Overall	6.50 ± 0.33^{c}	6.61 ± 0.65^{d}	6.38 ± 0.83^{b}	5.68 ± 0.77^{a}	5.63 ± 0.72^{a}

Values are presented as means ± standard deviation. Values with different superscript letters within the same row are significantly different ($p < 0.05$). WF: dough/cookie from wheat flour formula; CF1 – CF4: dough/cookie from composite flour formula. Composite flour made from wheat flour/retrograded black rice flour at 90:10 (CF1), 80:20 (CF2), 70:30 (CF3), and 60:40 (CF4).

interactions, but the specific contribution of starch retrogradation was not directly measured. However, at higher substitution levels (20%–40%), crispiness scores decreased progressively (6.75, 6.26, and 6.09), which may be associated with the higher fiber content and altered structural properties of the composite flour. Regarding overall acceptability, cookies with 10% RBRF achieved the highest mean score (6.61), slightly higher than the control (6.50), while higher substitution levels (20%–40%) resulted in a gradual decline (6.38, 5.68, and 5.63). These results indicate that 10% RBRF provided the highest sensory acceptability among the tested formulations.

4. CONCLUSION

Partial substitution of wheat flour with RBRF affected the physicochemical, functional, and sensory properties of the cookies. Increasing RBRF substitution resulted in changes in moisture, ash, fat, and protein contents, as well as dietary fiber fractions (TDF, SDF, and IDF). The incorporation of RBRF also influenced WHC and increased the measured TAC, TFC, and TPC of the cookies.

Sensory evaluation showed that RBRF substitution affected the sensory properties of the cookies, including color, flavor, crispiness, and overall acceptability. Among the formulations evaluated, the 10% RBRF substitution showed the highest overall acceptability score, indicating that this formulation provided the most favorable sensory response among the tested treatments. Therefore, the 10% RBRF formulation can be considered the most acceptable formulation based primarily on the sensory evaluation results, while its physicochemical and functional characteristics also demonstrated measurable changes compared with the control. Further studies involving storage stability, starch digestibility, and resistant starch content are needed to better evaluate the functional potential of RBRF-substituted cookies.

5. ACKNOWLEDGMENTS

The authors gratefully acknowledge the Ministry of Higher Education, Science and Technology, Republic of Indonesia, for financial support through the Fundamental Research Grant program awarded to Dr. Nurhidajah [grant numbers 127/C3/DT.04.00/PL/2025:023/LL6/ PL/ AL.04/2025; 013/061026/PN/PRF/SP2H/2025].

6. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be authors as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVALS

The research procedure, including the human sensory evaluation, was conducted in accordance with the ethical standards and guidelines of the Code of Ethics issued by the University Muhammadiyah Semarang, Indonesia. Ethical approval was obtained under Approval No. 007/ KE/07/2025. All sensory panelists were informed about the purpose and procedures of the study, and informed consent was obtained from each participant prior to participation in the sensory evaluation.

9. DATA AVAILABILITY

All the data are available with the authors and shall be provided upon request.

10. PUBLISHER'S NOTE

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11. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors used ChatGPT (OpenAI, OpenAI OpCo, LLC, San Francisco, CA 94158, USA; https://chatgpt.com/) for language refinement, including grammatical correction and improvement of the clarity, readability, and phrasing of the manuscript. ChatGPT was not used to generate or modify research data, perform statistical or data analysis, interpret results, generate scientific conclusions, or create research findings. All AI-assisted output was critically reviewed, edited, and verified by the authors, who take full responsibility for the accuracy, originality, and integrity of the final manuscript.

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How to cite this article:
Nurhidajah N, Hersoelistyorini W, Ayuningtyas RA, Yonata D, Kathiresan S, Pranata B. Development and characterization of cookies partially substituted with retrograded black rice flour as a potential functional snack. J Appl Biol Biotech 2027. Article in Press. http://doi.org/10.7324/jabb.2027.303513