Nutritional Characteristics, Microbial Loads, and Consumer Acceptability of Biscuits with Pumpkin, Red Bean, and Anchovy Flours as Wheat Flour Substitutes

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Received: 23 February 2025 ; Published online: 15 June 2026

Abstract

Biscuits are snacks widely consumed by all societal groups. This study aimed to develop nutrient-dense biscuits using red bean, anchovy, and pumpkin flours as partial substitutes for wheat flour, while ensuring that they are free from microbial and heavy metal contamination and are acceptable to consumers. The study was an experimental design using a completely randomized design consisting of five treatments with three repetitions. The biscuit formulations varied in the proportions of wheat, red bean, pumpkin, and anchovy flours, respectively, as follows: F0 (100%: 0% :0% :0%), F1 (60%: 20%: 10%: 10%), F2 (60%: 10%: 20%: 10 %), F3 (60%: 10%: 10%: 20 %), and F4 (50%: 20%: 20%: 10 %). The biscuit formula F3 had the highest nutrient content, contained 447 kcal 100 g^{-1} of energy and a protein content of 14.74 ± 0.33%, calcium content of 758 mg and zinc content of 26.74 mg. The microbial and heavy metal contamination levels were within safe consumption limits across all formulations. The consumer acceptability ratings ranged from moderate to extreme liking for all biscuit variants. The substitution of wheat flour with pumpkin, kidney bean, and anchovy flours results in nutrient-dense biscuits that are safe for consumption, free from microbial and heavy metal contamination, and well accepted by consumers. These biscuits offer a potential nutritional solution to malnutrition in children.

Keywords: Biscuit; Pumpkin flour; Red bean flour; Anchovy flour; Sensory acceptability; Nutrient composition

1 Introduction

Food products aimed at improving public health have been extensively developed (Goubgou et al., 2021). At present, the world suffers the double burden of malnutrition, which includes undernutrition (wasting or stunting), micronutrient deficiencies, overweight, obesity, and diet-related noncommunicable diseases (Organization, 2026). Popular food items play a vital role in the incorporation of essential nutrients, thereby catering to the growing health-conscious market that focuses on managing these disorders. Among these products, biscuits have significant potential to address nutritional requirements and prevent diet-related diseases. Thus, owing to their versatility, biscuits present an opportunity to manage nutrition-related disorders (Goubgou et al., 2021).

Biscuits, popular snacks enjoyed by a wide range of people, undergo a transformation in response to changing consumer demand (Yang et al., 2022). The average annual consumption of dry

 10.7455/ijfs/15.01.2026.a3

Nomenclature

AAS	Atomic absorption spectrophotometry	TPC	Total plate count
SNI	Indonesian National Standard		

bread and biscuits in Indonesia in 2020 was 22.834 kilograms per capita and 21.215 kilograms per capita by 2023 (of Agriculture Republic of Indonesia, 2023). As consumers become more health-conscious, their shopping habits shift toward products with higher nutritional value and better quality (Norazmir et al., 2014). This trend presents an excellent opportunity for food industry professionals to develop nutrient-dense biscuit products that cater to an evolving market, making them integral to industrial growth.

Biscuits are popular because of their variety of flavors, long shelf life, and relatively low cost (Goubgou et al., 2021). The essential ingredients of simple biscuits include wheat flour, water, sugar, fat, and eggs. Sugar is crucial for the enhancement of flavor, contributing to the biscuit structure, providing sweetness, softening the texture, and producing an appealing golden-brown color (Galla et al., 2017; Kohli et al., 2023). However, many commercially available biscuits have an unbalanced nutritional profile, often containing high levels of carbohydrates and fats while requiring more protein, vitamins, and minerals. To improve the nutritional content of the biscuits, alternative local ingredients can be used as substitutes such as anchovies, red beans, and pumpkin, which are rich in animal- and plant-based proteins and carotenoids (Ivanišová et al., 2020; Ratnasari & Yunianta, 2015; Sidoretno et al., 2022). Currently, in Indonesia, no commercially available biscuits combine the benefits of pumpkins, red beans, and anchovies.

Anchovies fresh or processed into fishmeal and flour are nutritional powerhouses. The transformation of anchovies into fishmeal and flour further enhances their nutritional profiles. Anchovy flour is rich in nutrients and contains 277 kcal of energy, approximately 60 g of protein, and 2.3 g of fat per 100 g, thus making it an excellent substitute for wheat flour in biscuit production and offering a practical method to enhance the nutritional quality of everyday foods (Thalib et al., 2021). Kidney beans are an excellent source of plant-based protein, starch, soluble and insoluble fibers, and essential minerals such as zinc, magnesium, and manganese, and are low in fat (Noah & Adedeji, 2021). These beans contain protein levels in the range of 20-30%, with high amounts of essential amino acids. For example, lysine is found in concentrations of approximately 5%, a nutrient typically not present in cereal grains. Kidney beans also offer substantial health benefits because they are rich in folic acid, calcium, carbohydrates, and protein (Hayat et al., 2022).

Pumpkin is an abundant agricultural commodity in Indonesia and known for its richness in β-carotene, vitamins, and fiber. Efforts to utilize pumpkin have led to the development of pumpkin flour, which is commonly used as a substitute for wheat flour in baked goods like biscuits, cookies, muffins, brownies, and cakes. Pumpkin contains 1569 µg of carotene per 100 g, and its flour is considered high in dietary fiber, meeting the food fiber content requirement of at least 6 g per 100 g (Anurag et al., 2022; Mustika & Kartika, 2020).

Nurlita and Asyik (2017) demonstrated that biscuits comprising a unique composition of 60% and 10% red bean and pumpkin flours, respectively, which are not typically found in traditional cookies, contained 6.25% moisture, 2.5% ash, 5.52% fat, 16.42% protein, and 69.31% carbohydrates. In a recent study (Binalopa et al., 2023), cookies made of 60% red bean flour and 40% pumpkin flour were found to be the most favoured by consumers. Proximate analysis of these cookies showed that they contained 3.45%

moisture, 2.74% ash, 8.55% protein, 27.78% fat, and 57.5% carbohydrate. Our study differs from others in the use of red bean and pumpkin proportions between 10 % and 20 %, and the inclusion of anchovy flour.

Therefore, this study aimed to develop nutrient-dense cookies that are free from microbial and heavy metal contamination, and acceptable to consumers. These cookies were evaluated for proximate composition and calcium, iron, and zinc contents, as well as for microbial and metal contamination. Consumer acceptance was also assessed. The development of biscuits formulated with pumpkin and red beans represents a promising approach for improvement of the nutritional quality of snack foods. By incorporating these ingredients, the resulting biscuits can serve as nutrient-dense food products that contribute to the dietary intake of protein and dietary fiber (Binalopa et al., 2023). Furthermore, these biscuits hold the potential for commercialization, offering an opportunity to introduce healthier snack options into the market and support a shift toward more health-conscious consumer choices.

Table 1: Biscuit Dough Formulation with Wheat, Pumpkin, Kidney Bean, and Anchovy Flours

Material (g)	Formulas (g)				
	F0	F1	F2	F3	F4
Wheat flour	800	480	480	480	400
Pumpkin flour	0	160	80	80	160
Red bean flour	0	80	160	80	160
Anchovy flour	0	80	80	160	80
Butter	400	400	400	400	400
Refined sugar	320	320	320	320	320
Egg	220	220	220	220	220
Milk powder	100	100	100	100	100
Cornstarch	40	40	40	40	40
Vanilla	5	5	5	5	5
Peanut	400	400	400	400	400
Salt	5	5	5	5	5
Chocolate bar	500	500	500	500	500

Notes: Formulate biscuits with wheat, red bean, pumpkin, and anchovy flours.
F0 (100% : 0% : 0% : 0%); F1 (60% : 20% : 10% : 10%); F2 (60% : 10% : 20% : 10%); F3 (60% : 10% : 10% : 20%); F4 (50% : 20% : 20% : 10%)

2 Materials and Methods

2.1 Materials

The main ingredients used in this study were pumpkin (*Cucurbita moschata*), red beans (*Phaseolus vulgaris* L.) and anchovy (*Stolephorus sp*). Pumpkin and anchovies were purchased from traditional markets on Seram Island, Maluku, whereas red beans were obtained from traditional markets on Kisar Island, Maluku, Indonesia. The other ingredients for making the biscuit were wheat flour, butter, refined sugar, eggs, milk powder, corn starch, vanilla, peanuts, salt, and chocolate bars. Those ingredients were purchased at Mardika Market, Ambon, Maluku, Indonesia. The composition of ingredients for each treatment is presented in Table 1.

2.2 Methods

The Development of Pumpkin and Red Bean

Pumpkins were harvested from Seram Island, Maluku, Indonesia. Mature but not overly ripe pumpkins were selected, which were thoroughly washed with clean water to remove dirt from the skin and manually peeled. After peeling, the pumpkins were cut, and the seeds and internal pulp were removed. The pumpkin flesh was blanched for 5 min, chopped into slices with thickness of 0.1 - 0.3 cm. The pumpkins were dried using a tray dryer at 60 ^oC for 12 h. After drying, the pumpkins were ground using a milling machine and sieved to a fine consistency (80-mesh sieve). The resulting pumpkin flour was stored in sealed polyethylene plastic bags to maintain its quality (Binalopa et al., 2023).

The red beans were thoroughly cleaned and then soaked in clean water for 12 hours, with the water changed every 6 hours to prevent fermentation. Following this, the beans were blanched in hot water at 85 ^{o}C for 30 minutes. After blanching, the beans were dehydrated in an air draft dehydrator at 55 ^{o}C for 20 hours. Finally, the dried beans were milled, sieved through a 0.05 mm mesh, and packaged in high-density polyethylene (Forwoukeh et al., 2024).

Anchovy Processing for Fishmeal Production

Anchovies harvested from Seram Island, Maluku, Indonesia were used for fishmeal production. The processing began by washing the anchovies and soaking in a 0.8% sodium bicarbonate solution for 45 min. The anchovies were then boiled at 80 ^{o}C for 15 min, squeezed for 10 min, dried at 55 ^{o}C for five hours, and ground and sieved through an 80-mesh sieve (Litaay et al., 2023).

Preparation of Peanut butter

Peanut were selected based on their quality, washed, and drained. Peanuts were roasted in an oven for 8-10 min and cool to obtain a uniformly roasted product. Skin, low-weight kernels, discolored kernels, and other unwanted parts were removed. The cleaned groundnut kernels were then ground at low speed in a mixer for 1-2 min. The resulting peanut butter was stored in an airtight container and kept at a cool temperature of 14 ^{o}C (Timbabadiya et al., 2017).

Preparation of Biscuits

The biscuits were prepared using the formulations listed in Table 1. The experimental design is presented in this table, which involved using a completely randomized design featuring various formulations of wheat, pumpkin, red bean, and anchovy flours at different percentages. F0 (100%: 0%: 0%: 0%); F1 (60%: 20% :10% :10%); F2 (60% :10% :20% :10%); F3 (60%: 10% :10% :20%) and F4 (50% :20% :20% :10%). The biscuit batches were prepared in three replications for each formulation. These formulations were chosen based on their nutritional value and potential to enhance the sensory properties of the biscuits.

The flours were incorporated into an essential mixture that included butter, powdered sugar, eggs, milk powder, cornstarch, vanilla, peanut butter, salt, and chocolate bars in all treatments (Nurlita & Asyik, 2017). The control group, designated as Formula 0, contained 100 % wheat flour.

The meticulous preparation process commenced with the thorough mixing of wheat, pumpkin, red bean, and anchovy flours, cornstarch and milk powder. Butter and powdered sugar were mixed using a mixer for approximately 5 min, after which the eggs were added and mixed for 10 min. Peanut butter and salt were added while stirring, after which the previously prepared flour mixture was meticulously incorporated and thoroughly blended. The resulting dough was allowed to rest for approximately 10 min. Subsequently, it was rolled to a thickness of 0.7 cm and cut into round pieces with a diameter of 5 cm.

The biscuits were baked on a baking sheet at a precisely controlled temperature of 200 ± 5 ^{o}C for approximately 10 min (Karki, 2016). Each biscuit formulation was baked separately to ensure precision. This result is also supported by figure 1. After baking, the biscuits were dipped in melted chocolate and cooled until the chocolate hardened, as shown in Figure 2. The final products were packaged and tested for nutritional and heavy metal content, microbial contamination, and organoleptic properties.

Nutrient Content Analysis

The crude protein and carbohydrate contents of the formulated biscuits were determined using standard laboratory methods (AOAC International, 2010). The fat content was measured using a Soxhlet extractor (method 930.09). The protein content was assessed using the Kjeldahl method with a Kjeldahl analyzer (method 978.04), calculated as nitrogen (N) multiplied by 6.25. Ash content was determined gravimetrically in a muffle furnace at 550 ^{o}C (method 930.05). Carbohydrate content was estimated by difference. The energy content was calculated by multiplying the percentages of protein, fat, and carbohydrates by their respective Atwater values of 4, 9, and 4. All analyses were performed in triplicate (Arama et al., 2023).

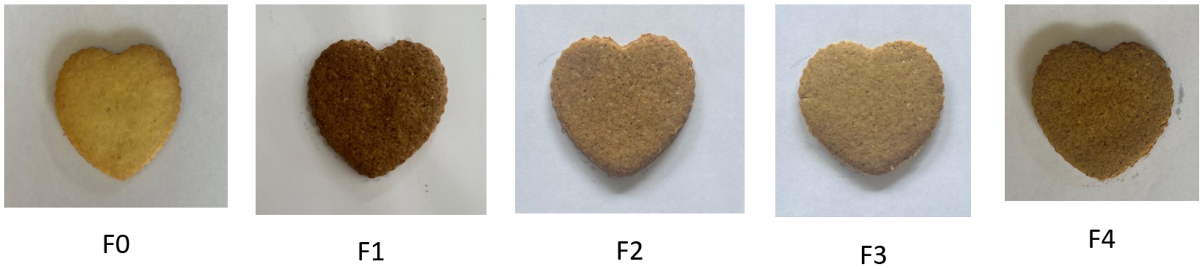

Figure 1: Biscuits formulated from different flours using a mixture design prior to being dipped in chocolate

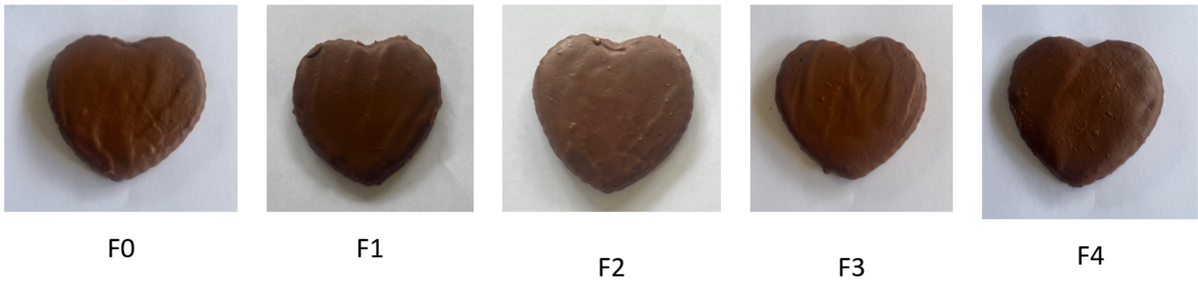

Figure 2: Biscuits formulated from different flours using a mixture design after being dipped in chocolate

Additionally, calcium, zinc, and iron contents were analyzed using atomic absorption spectrophotometry (AAS). Calcium, zinc and iron levels in the biscuit formula, and the absorbance was measured using a conditioned and regulated atomic absorption spectrophotometer, where the iron content was determined at a wavelength of 248.3 nm. In comparison, the calcium content was carried out at a wavelength of 422.7 nm with an air-acetylene flame. The zinc content was carried out at a wavelength of 213.90 nm (Putri et al., 2020).
Accuracy: The test is conducted using a sample that was spiked with a standard solution. The experimental spiking concentrations correspond to 80% (low concentration), 100% (medium), and 120% (high) of the sample's concentration. Spiking occurs at the beginning of the sample preparation, prior to microwave digestion. Accuracy is determined by calculating the percent recovery of the spiked analyte. Linearity: Instrument linearity is evaluated by measuring the absorbance of standard mineral solutions (Fe, Zn, and Cu) at various concentrations. The linearity tests for Fe and Cu were conducted using concentrations from 0 to 0.5 mg/L, while the Zn mineral concentration ranged from 0 to 1.0 mg/L. Each standard solution's absorbance was measured, and the measurements were repeated three times for accuracy. Limit of Detection (LOD) and Limit of Quantitation (LOQ): The values for the Limit of Detection Instrument (LDI) and Limit of Quantitation (LOQ) are derived from the linearity analysis data, specifically by determining the standard error of calibration (sy/x). The theoretical LDI is calculated using the formula [3(sy/x)]/b. Confirmation is conducted by performing seven repeated measurements of solutions at the theoretical LDI concentration for each of the three minerals (Fe, Zn, and Cu). The theoretical LOQ is determined using the formula [10(sy/x)]/b. Confirmation of the LOQ values is carried out by creating a solution with a concentration equal to the theoretical LOQ value and measuring it with seven replicates for each mineral. The Limit of Detection Method (LDM) is established by mea-

suring seven replications of the mineral concentration in the sample prepared at the theoretical LDI values (Faridah et al., 2020).

Microbial Analysis

For the microbial analysis, various indicators were measured throughout, for 12 days. Total aerobic plate count (TPC) was determined following ISO 4833-2:2013, in which 10 g or 10 mL of food was homogenized by sterile saline and diluted. One milliliter of diluted samples from the dilution series was spread onto plate count agar petri dishes and incubated at 30 ^{o}C for 48 h before counting colonies (Luu-Thi & Michiels, 2021).
To qualify coliform, a presumptive test for TC was performed using three tubes of lauryl sulphate tryptose (LST) broth. Nine mL in each tube were inoculated with one mL of the previously prepared 1:10, 1:100, and 1:1000 dilutions. Tubes containing inverted Durham's tubes were used for gas detection and were incubated aerobically at 37 ^{o}C for 24 - 48 h. All LST tubes showing both turbidity and gas within 48 h were recorded as positive presumptive tubes, and the Most Probable Number (MPN) was obtained from MPN food tables for the recorded three tube dilutions. Results were recorded as the presumptive MPN of coliform bacteria per g. A confirmed test was done for all positive presumptive tubes. Three loopfuls of presumptive positive tubes were inoculated into each Brilliant Green Lactose Bile Broth (BGLBB). All tubes were shaken on a vortex mixer and incubated at 35 - 37 ^{o}C for 24 - 48 h. Tubes showing turbidity and gas were recorded as positive confirmed tests for TC. Positive confirmed BGLBB tubes were streaked on eosin methylene blue (EMB) plates for 24 h at 37 ^{o}C. The typical nucleated (dark-centered) colonies with or without sheen were subjected to Gram staining. The typical microscopic appearance of gram-negative short non-spore-forming rods identifies the presence of TC (Ali et al., 2023).
E. coli was performed by ISO 16649-2:2001. Ten grams or 10 mL of food was homogenized and diluted, and 1-mL samples were placed into the center of petri dishes, mixed with 15 mL of Tryptone Bile X-glucuronide agar, and incubated at 44 ^{o}C for 24 h after solidification. After the incubation period, colonies of glucuronidase-positive *E. coli* were counted.
To qualify Salmonella spp., 25 g or 25 mL of food was pre-enriched in a nonselective liquid medium (peptone buffer) at 37 ^{o}C for 24 h. Next, 1 mL was incubated for another 24 h at 42 ^{o}C in 9 mL of selective Rappaport-Vassiliadis enrichment broth and streaked for single colonies onto xylose lysine deoxycholate agar and Salmonella-Shigella agar. Biochemical and serological tests confirmed presumptive Salmonella colonies by using triple sugar iron agar, urea agar, L-lysine decarboxylation medium, an indole test, and a Voges-Proskauer test (Luu-Thi & Michiels, 2021).

Heavy Metal Analysis

Heavy metal analysis of the samples was performed at the Center for Standardization and Industrial Services, Surabaya, Indonesia. The sample was weighed, with as much as 2 g to 10 g, using an analytical scale with an accuracy of 0.00001 g, then put into a 300 mL Erlenmeyer flask and placed in an acid chamber. The sample was combined with 5 mL of concentrated nitric acid (HNO3) and homogenized. Furthermore, the sample was placed on a hotplate at 100 ^{o}C for 15 minutes. The dissolved sample was added to 2 mL of distilled water and cooled for 10 minutes at room temperature. A sample solution was homogenized with 2 mL of perchloric acid (HClO4). Furthermore, the solution was heated again until white vapor of perchloric acid (HClO4) formed for 15 minutes on a hotplate. The unsaturated solution was still heated, and 10 mL of concentrated nitric acid (HNO3) was added until it underwent a complete digestion process. If a precipitate and white vapor had formed, the destruction stage was complete, and the sample solution was cooled for 15 minutes at room temperature.
The samples were analyzed using the Shimadzu *Atomic Absorption Spectrophotometer* (AAS) type AA-6880 to obtain the heavy metal content values of Pb and Cu. In contrast, the Perkin Elmer AAnalyst 100 was used to detect the heavy metal value of Hg. The wavelengths used to measure the concentration of heavy met-

als were 253.7 nm, 283.3 nm, and 324.7 nm respectively for Hg, Pb, and Cu. (Susilowati et al., 2022). Arsenic concentration was analyzed using an Atomic Absorption Spectrophotometer equipped with a Hydride Vapor Generator (HVG) unit (Agilent AA280FS/VGA, USA) at a wavelength of 193.7 nm (Cahyady et al., 2021; Helaluddin et al., 2016).

Sensory Acceptance of Biscuits

The sensory acceptance of the biscuits was evaluated using an organoleptic test based on a hedonic scale. The attributes assessed were color, aroma, taste, and texture. Sensory tests were performed by 50 semi-trained panelists (12 males and 38 females, age between 20-23 years). The panelists were nutrition students who had passed a sensory evaluation course. Before conducting the evaluation, panelists received refresher training on sensory evaluation based on the hedonic method. They were selected based on their status as regular biscuit consumers.

The evaluation was conducted in a sensory laboratory where individual panel rooms were used for testing. Each sample consisted of one biscuit from each formulation, which was presented in a disposable container labelled with a three-digit code. The attributes of color, aroma, taste, texture, and overall acceptability were evaluated using a structured 9-point hedonic scale with the following scoring system: 1 = dislike extremely - 9 = like extremely. The panelists were instructed to cleanse their palates with water between samples to ensure accurate taste perception (Mongi & Gomezulu, 2022).

Statistical Analysis

All experiments were performed in triplicate, and the results are presented as mean ± standard deviation. The collected data, namely, water content, protein content, fat, carbohydrates, calcium, zinc, and iron, were initially subjected to the Shapiro-Wilk test to assess normality, followed by Levene's test to evaluate the homogeneity of variances. Parametric one-way analysis of variance was applied if the data were normally distributed and homoscedastic. Statistical significance was set at a 95 % confidence level ($p < 0.05$). When significant differences were detected, a post-hoc analysis was conducted using the least significant difference test. For data that did not meet the assumptions of normality or homogeneity, the non-parametric Kruskal-Wallis test was used. When significant differences were identified in the Kruskal-Wallis test, pairwise comparisons were conducted using the Mann-Whitney U test. The results of the organoleptic evaluation, which represent ordinal data, were analyzed using the Friedman test. Any significant findings were further examined using the Wilcoxon signed-rank test to explore the pairwise differences between treatments.

The study was conducted after approval was obtained from the Health research ethics committee Poltekkes Kemenkes Maluku No. DP.04.03/6.2/1319/2024.

3 Results and Discussion

3.1 Results

Proximate and Mineral Composition of Biscuits

Proximate compositions of the biscuit formulations are listed in Table 2. The results indicate that treatment F3 exhibited the highest energy content (averaging 447.91 kcal), whereas treatment F1 had the lowest energy content (averaging 343.68 kcal). In addition, treatment F3 demonstrated the highest protein content (14.74%), whereas the lowest protein content was recorded for treatment F0 at 11.06%. The fat content was the highest in treatments, F4 and F0, with an average of 26.89%. The highest calcium content was also observed for treatment F3 (averaging 758 mg/kilograms), whereas treatment F1 had the highest zinc content (averaging 26.94 mg/kilograms). Mineral composition of biscuits are listed in Table 3. Statistical analysis revealed significant differences in energy, protein, carbohydrate, fat, calcium, iron, and zinc contents among the formulations ($p < 0.05$).

Microbiological Quality of Biscuits

The maximum permissible limits in biscuits were set for total plate count (TPC) as $< 10^5$ CFU/g,

Table 2: Proximate Composition of the Biscuits

Formulas	Nutrients			
	Energy (kcal)	Proteins (%)	Carbohydrates (%)	Fats (%)
F0	435.67 ± 13.16^{c}	11.06 ± 0.09^{a}	37.34 ± 0.22^{d}	26.89 ± 1.54^{c}
F1	343.68 ± 1.78^{a}	12.29 ± 0.31^{b}	30.73 ± 0.62^{c}	19.07 ± 0.29^{a}
F2	363.34 ± 22.89^{ab}	12.53 ± 0.46^{b}	30.76 ± 0.56^{c}	21.13 ± 2.17^{a}
F3	447.91 ± 22.26^{c}	14.74 ± 0.16^{d}	29.61 ± 0.22^{b}	24.65 ± 1.17^{b}
F4	382.72 ± 13.16^{b}	13.24 ± 0.05^{c}	26.97 ± 0.34^{a}	26.89 ± 1.54^{c}
p value	0.000*	0.000*	0.000*	0.000*

Note: * Significant at $p < 0.05$.
Note: Values bearing different superscript letters on the same column are significantly different ($p < 0.05$).

Table 3: Mineral Profile of Biscuit Formulations

Formulas	Nutrient			
	Water content (%)	Calcium (mg)	Iron (mg)	Zinc (mg)
F0	4.62 ± 0.08^{a}	466.89 ± 12.52^{a}	52.02 ± 0.35^{c}	24.91 ± 0.38^{a}
F1	6.65 ± 0.16^{c}	635.56 ± 25.31^{b}	41.22 ± 2.25^{b}	26.94 ± 1.01^{b}
F2	6.38 ± 0.06^{b}	622.42 ± 24.39^{b}	41.22 ± 2.25^{b}	24.73 ± 1.03^{a}
F3	6.35 ± 0.12^{b}	758.00 ± 29.03^{c}	37.83 ± 0.39^{a}	26.74 ± 0.87^{b}
F4	6.69 ± 0.09^{c}	723.16 ± 27.75^{c}	38.96 ± 0.62^{ab}	24.91 ± 0.38^{a}
p value	0.016*	0.000*	0.017*	0.003*

Note: * Significant at $p < 0.05$.
Note: Values bearing different superscript letters on the same column are significantly different ($p < 0.05$).
Biscuit formulation with wheat, red bean, pumpkin, and anchovy flours: F0 (100% : 0% : 0% : 0%), F1 (60% : 20% : 10% : 10%), F2 (60% : 10% : 20% : 10%), F3 (60% : 10% : 10% : 20%), F4 (50% : 20% : 20% : 10%).

for total coliform as 10 CFU/g and absence of *E. coli* and *Salmonella* (Institute of Food Science and Technology, 2020). The results of the microbial contamination tests indicated that none of the formulations exceeded the safety threshold. The microbial contamination test results measured on day 12 of storage are presented in Table 4.

Heavy Metal Content in Biscuits

The maximum permissible limits (MPL) for heavy metal contaminants in food, according to USDA, are lead 0.2 mg/kg, copper 0.01 mg/kg, mercury < 0.02 mg/kg, and arsenic < 0.5 mg/kg (USDA, 2018). The results of metal contamination tests, presented in Table 5, indicated that all formulations met the maximum limit requirements. Lead concentrations in the five formulations were below 0.0007 mg/kg, copper levels were less than 0.0075 mg/kg, mercury was detected at levels below 0.0004 mg/kg, and arsenic concentrations ranged from less than 0.0030 mg/kg to 0.03 mg/kg.

3.2 Sensory Evaluation of Biscuits

Organoleptic tests revealed that the average acceptance levels of the color, aroma, taste, and texture of the biscuit formulas ranged from “like moderately (score 7)” to “like extremely (score

Table 4: Microbiological Quality of Biscuits

Microbial	Formulas					Recommended Reference Value*
	F0	F1	F2	F3	F4	
Total plate number (colonies/g (mL))	2.63×10^3	2.67×10^3	2.67×10^3	2.83×10^3	2.31×10^3	Maximum 5×10^4 Colony/g (mL)
Most Probable Number (MPN) Coliform (colonies/g (mL))	0.24	0.086	0.086	0.1	0.093	Maximum 1×10^2 Colony/g (mL)
Escherichia coli	(–)	(–)	(–)	(–)	(–)	Negative
Salmonella	(–)	(–)	(–)	(–)	(–)	Negative

*(Unicef, 2022)
Biscuit formulation with wheat, red bean, pumpkin, and anchovy flours: F0 (100% : 0% : 0% : 0%), F1 (60% : 20% : 10% : 10%), F2 (60% : 10% : 20% : 10%), F3 (60% : 10% : 10% : 20%), F4 (50% : 20% : 20% : 10%)

Table 5: Heavy Metal Content in Biscuits

Metal	Formulas					Maximum permissible limits (MPL)*
	F0	F1	F2	F3	F4	
Lead (mg/kilograms)	< 0.0007	< 0.0007	< 0.0007	< 0.0007	< 0.0007	Maximum 0.2
Copper (mg/kilograms)	< 0.0075	< 0.0075	< 0.0075	< 0.0075	< 0.0075	Maximum 0.01
Mercury (mg/kilograms)	< 0.0004	< 0.0004	< 0.0004	< 0.0004	< 0.0004	Maximum 0.02
Arsenic (mg/kilograms)	< 0.0030	0.003	0.003	0.0067	< 0.0030	Maximum 0.5

*(USDA, 2018)
Biscuit formulation with wheat, red bean, pumpkin, and anchovy flours: F0 (100% : 0% : 0% : 0%), F1 (60% : 20% : 10% : 10%), F2 (60% : 10% : 20% : 10%), F3 (60% : 10% : 10% : 20%), F4 (50% : 20% : 20% : 10%)

9)." The average acceptance levels of panelists for the biscuit formulas are presented in Table 6. Notably, the partial substitution of wheat flour with pumpkin, red bean, and anchovy flours significantly influenced the sensory perception of these attributes. The highest total acceptance scores for color, taste, aroma, and texture were recorded for group F0 (30.42), followed closely by groups F4 (30.28), F2 (27.24), and F1 (27.20). The lowest score was observed for group F3.
Statistical analyses indicated significant differences ($p < 0.05$) in consumer acceptances regarding taste, aroma, and texture of the biscuit formulas. However, no significant difference ($p > 0.05$) was observed in the acceptance for biscuit color among the various formulas.

3.3 Discussion

Biscuits are convenient, ready-to-eat snack foods that are affordable and enjoyed by individuals of all age groups across many countries (Milkesa, 2020). They have the potential to serve as better food options for fulfilling nutritional requirements and preventing diet-related diseases. Often consumed as snacks or as accompaniments to other foods, biscuits are available in various shapes and flavors. Their long shelf life and convenience contribute to increasing production and consumption on a global scale (Goubgou et al., 2021).
The average energy content of the formulations ranged from 343.68 to 447.91 kcal/100 g, aligning

Table 6: Average Acceptance Levels for Biscuit Formulas by Panelists

Formulas	Color	Flavor	Aroma	Texture	Total
F0	7.38 ± 1.21	7.42 ± 1.29^{c}	7.64 ± 1.13^{b}	7.98 ± 0.82^{c}	30.42 ± 2.23^{c}
F1	7.16 ± 0.89	6.08 ± 1.57^{b}	6.92 ± 1.10^{a}	7.28 ± 1.37^{b}	27.20 ± 3.56^{b}
F2	7.18 ± 1.31	6.30 ± 1.61^{b}	6.90 ± 1.31^{a}	7.14 ± 1.39^{b}	27.24 ± 4.06^{b}
F3	7.18 ± 1.17	5.50 ± 1.84^{a}	6.74 ± 1.64^{a}	6.02 ± 1.88^{a}	25.00 ± 5.1^{a}
F4	7.32 ± 1.26	7.40 ± 1.11^{c}	7.32 ± 1.20^{b}	8.24 ± 0.91^{c}	30.28 ± 3.19^{c}
p value	0.741	0.003*	0.003*	0.000*	0.000*

Note: * Significant at $p < 0.05$.
Note: Values bearing different superscript letters on the same column are significantly different ($p < 0.05$).
Biscuit formulation with wheat, red bean, pumpkin, and anchovy flours: F0 (100% : 0% : 0% : 0%), F1 (60% : 20% : 10% : 10%), F2 (60% : 10% : 20% : 10%), F3 (60% : 10% : 10% : 20%), F4 (50% : 20% : 20% : 10%)

with the local supplementary food requirements for toddlers (300-450 kcal/day). Formulation F3 exhibited the highest energy content of 436.71 kcal/100 g. The energy content of a food product is influenced by its protein, fat, and carbohydrate compositions. The protein levels in all five formulations met the requirements for local supplementary foods for toddlers, with the protein content increasing with the addition of anchovy and red bean flour. Proteins serve as vital energy sources, supporting growth and tissue maintenance; their deficiency can lead to growth retardation in children (Manik et al., 2020).

Anchovies are rich in proteins and contain a balanced composition of essential and nonessential amino acids, polyunsaturated fatty acids, and phospholipids. Incorporating anchovies into snack products enhances their nutritional value, providing an excellent source of proteins, omega-3 fatty acids, vitamins, and minerals (Kari et al., 2022; Savitri et al., 2021). Animal-based proteins sourced from fish are typically highly digestible, meaning that a significant proportion of their amino acids are effectively absorbed by the body. In contrast, plant-based proteins often contain anti-nutritional factors and have complex structures that can hinder their digestibility. Additionally, the methods of cooking, processing, and preparation can impact how well proteins are digested. The structure of proteins plays a crucial role in how they are broken down and digested in the gastrointestinal tract (Ajomiwe et al., 2024).

The calcium contents of the formulations were enhanced by the inclusion of bean flour. Minerals, such as calcium, are found in food as both inorganic and organic compounds, often dissolved in water. This leads to variations in the release and absorption of calcium. Bioavailability, or biological availability, refers to the extent to which a nutrient is converted by the human body into a form that can be absorbed and utilized in metabolic processes or stored for future use (Shkembi & Huppertz, 2021).

Biscuits made by substituting wheat flour with pumpkin, red bean, and anchovy flours can enrich nutritional content because of their high protein and calcium levels, and at an affordable price. These biscuits provide 28% of the energy and 53% of proteins that are required to meet the nutritional needs of toddlers. Consequently, these biscuits are a valuable nutritional source for malnourished children, pregnant women, elderly individuals, and individuals with insufficient protein intake.

The International Commission on Microbiological Specifications for Foods indicates that cooked ready-to-eat foods with microbial counts ranging from 0 to 10^3 CFU/g are considered acceptable, those between 10^4 and 10^5 CFU/g are tolerable, and counts exceeding 10^6 CFU/g are deemed unacceptable (Peters et al., 2017). Although achieving a 100 % absence of microorganisms in food production is not feasible, effective control measures can significantly reduce their presence. The total plate count is a commonly used method for evaluating the hygienic quality of food (Ramashia et al., 2024). The researchers found that the total plate count (TPC) in all biscuit formulations ranged from 2.31 to 2.83 × 10^3 CFU/g, while coliform levels ranged from 0.086 to 0.24 CFU/g, both of which were below the recommended maximum limits. *Escherichia coli* and *Salmonella* were not detected in any of the biscuit samples, indicating that proper hygienic conditions were maintained during the products' preparation, processing, and storage. High temperatures of the baking process may also have contributed to these results. Therefore, the biscuit prepared with substituted pumpkin, red bean, and anchovy flours is microbiologically safe for human consumption. These findings are consistent with those reported by Noah Abimbola and Banjo Olabisi (2020), who observed lower total aerobic counts in cookies fortified with red kidney beans (*Phaseolus vulgaris* L.) and moringa seeds (*Moringa oleifera*), with no contamination by *Staphylococcus* spp. or *Salmonella* spp. in any of the samples. The absence of these pathogens reflects high hygiene standards adopted during food preparation. Similarly, Abraha et al. (2018) reported that microbial analysis (TPC) of biscuits supplemented with sturgeon fillet powder remained below the permissible limits, with no significant differences observed among the experimental samples. The evaluation of total bacterial count is widely used to measure the microbial quality of fish and fish-derived products. Factors contributing to microbial contamination of food include endogenous microorganisms and cross-contamination, with inadequate and unhygienic handling of equipment and ingredients being significant contributors. Contamination can also arise from improper handling of wrapping and packaging materials (Oladipo & Oluwaseun, 2019). Therefore, proper preparation and processing are essential to prevent the microbial contamination of processed foods. Ensuring microbial quality and safeguarding products against pathogenic microorganisms are crucial for maintaining consumer confidence and promoting products (Chaudhary et al., 2014).

The results of the heavy metal contamination tests, as presented in Table 5, indicated that all formulations complied with the established maximum permissible limits. The lead concentrations in the five biscuit formulations were below 0.0007 mg/kg. Lead is a known carcinogenic metal that can adversely affect the human brain and kidneys. Chowdhury et al. (2025) reported trace amounts of lead in cookies (0.2 mg/kg) and dry cake (0.4 mg/kg), while it was undetectable in toast and salted biscuits. Copper contamination in the biscuit formulations was below 0.0175 mg/kg. Copper poisoning in humans may lead to symptoms such as diarrhea, nausea, liver and kidney damage. Copper levels in biscuits have been previously reported, such as Onwordi et al. (2024) documenting the highest concentration of 1.37 ± 0.28 mg/kg in crackers commonly consumed in the southwestern Nigerian cities of Lagos and Ibadan. Mercury contamination was recorded at levels below 0.0004 mg/kg, and arsenic contamination ranged from below 0.0030 mg/kg to 0.03 mg/kg, all remaining within the acceptable safety limits.

Heavy metals can be disseminated through air, water, and soil. Other sources of heavy metal contamination include the use of chemicals such as pesticides and fertilizers, wastewater used in agricultural practices, food additives, machinery, and equipment, and contamination from packaging materials (Basaran, 2022).

Sensory evaluation is a crucial determinant of consumer acceptance of the organoleptic properties of food, including color, taste, aroma, texture, appearance, and overall appeal (Hussein et al., 2021). Organoleptic testing involves evaluating food products using the five senses to assess attributes, such as color, taste, aroma, and texture, to determine acceptance of the product by each panelist. The quality parameters evaluated included color, taste, aroma, and texture, using a 9-point hedonic scale (Farzana et al., 2022).

In baked foods, structural interactions have a significant impact on sensory properties, including texture, flavor, and the overall eating experience. These interactions, occurring between different ingredients and within the food matrix, influence how the food feels in the mouth, releases flavors, and retains its structure (Anandharamakrishnan et al., 2023). Thermally treated food may stimulate a cascade of reactions commonly known as the Maillard reactions (MR), also referred to as nonenzymatic browning. These reactions are particularly important for the formation of flavor and color in heated food (Starowicz & Zieliński, 2019).

Color is a vital attribute because it can stimulate appetite and serves as the first quality criterion for consumers evaluating food products (Wronkowska et al., 2018). Market acceptance and final product price of a food item are influenced by its appearance and color (Kumari et al., 2023; Laganà et al., 2022). This attribute is one of the parameters used to monitor the baking and roasting processes, as brown pigments develop during browning and caramelization reactions (Adeola & Ohizua, 2018; Tomsone et al., 2020). Based on the Friedman test, no significant differences in color were observed among the five formulations. This was attributed to the uniform brown color of the biscuits across all formulations, which resulted from their chocolate coating. The color and appearance of the biscuits in all formulations were deemed acceptable to consumers.

The flavor of biscuits can be influenced by several factors, with various volatile compounds generated during baking due to the Maillard reaction. Higher protein levels may indirectly affect the Maillard reaction rate, either through deamination of bound amino acids or hydrolysis. In addition, elevated protein levels during roasting promote the formation of pyrazines, contributing to the roasted flavor profile. Furthermore, the thermal degradation of lipids during baking produces different volatile compounds that can affect the flavor of biscuits (Hayat et al., 2022). Significant differences in taste were observed among the five formulations. The amount of ingredients used as substitutes influencede the taste of the biscuit formulation. The panelists' assessment of formulation F4 was comparable to the control formula. The substitution of 20% pumpkin flour, 20% red bean flour, and 10% anchovy flour was preferred, as it produced a well-balanced savory and sweet taste.

Aroma is crucial in determining the palatability of food as it is closely related to the olfactory perception of taste. A distinctive and appealing aroma enhances food desirability among consumers, making it an essential consideration in food processing (Khasanah & Mumpuni, 2021). The highest aroma-liking score in the control group was 7.64, followed by group F4 with a score of 7.32. Panelists who preferred the formulation without substituting red bean, pumpkin, and anchovy flour tended to favor the biscuits' original, distinctive, and pleasant aroma. Red beans are known to exhibit a beany flavor, which is attributed to the presence of the lipoxygenase enzyme that can generate off-flavors. However, in this study, the beany flavor and the aroma of anchovy were effectively masked by other ingredients such as butter, eggs, and chocolate, making the formulation acceptable and even preferred by some panelists.

The texture of biscuits is influenced by flour-particle interactions with proteins and starch, resulting in varying degrees of hardness. During the cooking process, water absorption leads to starch gelatinization and protein denaturation, further affecting texture. Moreover, the presence of sugar in the biscuit dough formulation influences the texture (Tadju et al., 2021). A significant difference was noted in the level of texture acceptance among the formulations, with group F4 receiving the highest liking score, categorized as "like very much." The level of liking for all sensory attributes was comparable between the control and F4 groups. According to study by Diana et al. (2020), a biscuit formulation incorporating 20 g of bilih fish flour into one chocolate formula dough was found to be the most preferred.

The biscuit developed in this study, based on local ingredients such as pumpkin, red bean, and anchovy, had high levels of protein and calcium. In addition to its nutritional benefits, the product met food safety standards, indicating its feasibility as a supplementary food for malnourished toddlers. The use of these accessible and affordable ingredients further supports their application in community nutrition programs. The limitations of this study include the lack of digestibility evaluation, sensory data limited to young adult participants, and the absence of industrial-scale validation.

4 Conclusion

The results of our study demonstrated that wheat flour substitution with pumpkin, red bean, and anchovy flours improved the nutritional content of the biscuits, particularly in terms of protein and calcium levels. The levels of microbial and heavy metal contamination were within safe consumption limits, and the biscuits were well accepted by consumers. Biscuits formulated with pumpkin, red bean, and anchovy flours as wheat flour substitutes have the potential to improve nutrient intake in malnourished children.

Acknowledgements

The authors thank Politeknik Kesehatan Kemenkes Maluku for funding this research, all participants, and the research assistants. We would like to thank Editage (www.editage.com) for English language editing.

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