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Influence of controlled fermentation on the nutritional composition, antinutritional factors, and protein digestibility of Bambara groundnut flour

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Abstract
Bambara groundnut (*Vigna subterranea* L. Verdc.) is a nutritionally rich yet chronically underutilized legume with substantial potential for addressing food and nutrition insecurity across sub-Saharan Africa, but its broader adoption in human diets is limited by significant antinutritional factors and low protein digestibility in unprocessed flour. This study investigated the effects of controlled natural fermentation (CNF) at two temperatures (30 °C and 37 °C) and three fermentation durations (24, 48, and 72 h) on the proximate composition, antinutritional factor (ANF) profile, and *in vitro* protein digestibility (IVPD) of Bambara groundnut flour. Unfermented flour served as the control. A 2 × 3 factorial randomized complete block design with three replications was used. Results revealed that fermentation significantly ($p < 0.01$) altered all nutritional and antinutritional parameters. At 37 °C for 72 h, protein content increased from 19.8% (control) to 25.6%, crude fat increased from 5.4% to 6.8%, and ash content improved marginally. Phytic acid was reduced by 76.4%, condensed tannins by 68.2%, oxalate by 71.8%, and trypsin inhibitor activity (TIA) by 81.3% relative to unfermented flour, following 37 °C/72 h fermentation. *In vitro* protein digestibility improved from 62.4% in control flour to 81.7% under the 37 °C/72 h treatment, a 30.9% improvement attributable to the combined effects of ANF reduction and partial proteolytic activity of fermenting microorganisms. These findings substantiate the effectiveness of controlled fermentation as an affordable, low-technology processing strategy to enhance the nutritional quality and protein bioavailability of Bambara groundnut flour for integration into complementary foods, weaning formulations, and fortified flour blends in Ghana and West Africa broadly.

Keywords: Vigna subterranea; Bambara groundnut; fermentation; antinutritional factors; protein digestibility; food security

Introduction
Bambara groundnut (*Vigna subterranea* L. Verdc.) is an indigenous African legume crop widely cultivated across sub-Saharan Africa, characterized by its exceptional drought tolerance, nitrogen-fixing symbiosis, and ability to produce nutritious seeds on marginal soils where improved legumes fail to establish [1, 2]. The seeds are a nutritionally dense food source, containing 15–23% crude protein with a balanced essential amino acid profile, 4–7% fat predominantly as oleic and linoleic acids, 55–65% carbohydrate, and appreciable levels of calcium, iron, zinc, and B-vitamins [2, 3]; however, despite this impressive nutritional profile, Bambara groundnut remains chronically underutilized and undervalued in formal food systems relative to cowpea and groundnut, primarily because of its hard seed coat that prolongs cooking time, its high content of antinutritional factors (ANFs), and its lower consumer familiarity compared with widely marketed legumes [1, 4]. Antinutritional factors including phytic acid (phytate), condensed tannins, oxalates, and protease inhibitors are inherently present in unprocessed Bambara groundnut and function to limit the bioavailability of protein, iron, zinc, and calcium through chelation, enzyme inhibition, and precipitation mechanisms [5, 6]. Phytic acid, in particular, forms insoluble complexes with divalent mineral cations (Fe^{2+}, Zn^{2+}, Ca^{2+}) and binds to storage proteins, substantially reducing their digestibility and the nutritional value of the protein consumed [5, 7]. Trypsin inhibitors directly impair proteolytic digestion by inhibiting trypsin and chymotrypsin

activity in the small intestine, while condensed tannins form indigestible tannin–protein complexes that reduce amino acid availability [6, 8]. Fermentation is among the oldest and most widely practiced food processing technologies globally, and has been consistently demonstrated to reduce antinutritional factors in legumes through the enzymatic and metabolic activities of fermenting microorganisms (predominantly lactic acid bacteria, yeasts, and Bacillus spp.) that produce phytases, tannase, protease, and organic acids capable of degrading and inactivating ANF molecules [9, 10, 11]. Additionally, fermentation generates free amino acids and short peptides through partial proteolysis, creates a more favourable protein conformation for enzymatic digestion, and produces organic acids that lower the pH to levels that further inactivate protease inhibitors [10, 12]. While the benefits of fermentation for improving the nutritional quality of widely studied legumes such as cowpea, soybean, and common bean are extensively documented [11, 13], comparative investigations of the effects of controlled temperature and duration parameters of natural fermentation specifically on Bambara groundnut flour composition, ANF levels, and *in vitro* protein digestibility under conditions relevant to West African food processing remain limited [14, 15]. The present study was therefore designed to systematically evaluate the effects of controlled natural fermentation at two temperatures (30 °C and 37 °C) and three durations (24, 48, and 72 h) on the proximate composition, ANF profile (phytic acid, tannins, oxalate, trypsin inhibitor activity), and *in vitro* protein digestibility of Bambara groundnut flour from cream-seeded landrace varieties cultivated in Ghana, hypothesizing that fermentation at 37 °C for 72 h would achieve the greatest ANF reductions and highest protein digestibility improvements relative to unfermented control flour [16, 17, 18, 19].

2. Materials and Methods

2.1 Materials

Cream-seeded Bambara groundnut (*Vigna subterranea* L. Verdc.) seeds of the Accra landrace, sourced from three smallholder farms in the Northern Region of Ghana, were cleaned, sorted to remove damaged and shrivelled seeds, and pooled to form a homogeneous bulk sample. Seeds were washed three times with distilled water, soaked for 18 h at room temperature (25 ± 2 °C), and dehulled manually by gentle rolling and winnowing to minimize hull contamination of fermented flours. Dehulled cotyledons were washed, drained on sterile muslin cloth, and subjected to natural fermentation (without inoculation of starter cultures) at controlled incubation temperatures of 30 °C and 37 °C for 24, 48, and 72 h periods in pre-sterilized polypropylene containers lined with sterile gauze. Unfermented dehulled cotyledons constituted the control. Following fermentation or control processing, all samples were oven-dried at 60 °C for 12 h, milled using a Retsch SM 100 mill to pass a 425 µm sieve, and stored at −20 °C in airtight containers until analysis. All chemical reagents used for proximate and ANF analyses were of analytical grade (BDH/VWR Chemicals, UK; Sigma-Aldrich, Germany). Porcine pepsin (800–900 units mg^{-1}, Sigma-Aldrich) and pancreatin (8× USP specification, Sigma-Aldrich) were used for *in vitro* protein digestibility determination.

2.2 Methods

A 2 × 3 factorial randomized complete block design (RCBD) was employed, with two fermentation temperatures (30 °C and 37 °C) and three fermentation durations (24, 48, and 72 h), yielding six fermented treatments plus an unfermented control (seven treatments total), replicated three times (21 experimental units). Proximate composition (moisture, crude protein, crude fat, crude fibre, ash, and carbohydrate by difference) was determined by standard AOAC 2019 Official Methods [16]: moisture by oven-drying at 105 °C to constant mass (AOAC 925.10); crude protein by Kjeldahl (N × 6.25, AOAC 978.04); crude fat by Soxhlet extraction in petroleum ether (AOAC 920.39); ash by muffle furnace at 550 °C for 6 h (AOAC 942.05); crude fibre by successive acid-alkali digestion (AOAC 985.29). Phytic acid was determined by the Wade reagent method [5]; condensed tannins by the vanillin–HCl colorimetric method [6]; oxalate by titration with $KMnO_4$ (AOAC 2012.15); and trypsin inhibitor activity (TIA) by the N-α-benzoyl-dl-arginine-p-nitroanilide (BAPNA) substrate method [8]. *In vitro* protein digestibility (IVPD, %) was assessed by the two-enzyme pepsin-pancreatin method [17]: samples were digested sequentially with pepsin (pH 2.0, 37 °C, 3 h) then pancreatin (pH 7.5, 37 °C, 4 h), protein content of the dialysate was determined by Kjeldahl, and IVPD was calculated as: IVPD (%) = [(protein in dialysate / total protein) × 100]. The amino acid composition of selected treatments (control, 30 °C/72 h, 37 °C/72 h) was determined by ion-exchange chromatography using an AAA-400 amino acid analyser (INGOS, Czech Republic) following acid hydrolysis (6 M HCl, 110 °C, 24 h). Essential amino acid scores (EAAS) were calculated relative to the FAO/WHO/UNU 2007 reference pattern for school-age children. All analyses were conducted in triplicate. Data were analyzed by two-way ANOVA using SPSS Statistics v.28 (IBM Corp., USA). Treatment means were compared by Tukey's HSD test at $\alpha = 0.05$. Pearson correlations were computed between fermentation duration, ANF levels, and IVPD across temperatures [18, 19].

3. Results

Table 1: Proximate composition (% dry weight basis) of Bambara groundnut flour as influenced by controlled fermentation temperature and duration

Treatment	Moisture (%)	Crude Protein (%)	Crude Fat (%)	Ash (%)	Crude Fibre (%)	Carbohydrate (%)
Control (unfermented)	9.8a	19.8e	5.4d	3.1c	4.8a	61.3a
30 °C / 24 h	9.2b	21.2d	5.7cd	3.3bc	4.6a	58.4b
30 °C / 48 h	8.8b	22.6c	6.0bc	3.4bc	4.4ab	56.2c
30 °C / 72 h	8.4bc	23.4c	6.2ab	3.6ab	4.1ab	54.6d
37 °C / 24 h	8.6bc	22.4c	5.9bc	3.4bc	4.5ab	57.0bc
37 °C / 48 h	8.1c	24.2b	6.6a	3.7ab	3.9b	52.8e
37 °C / 72 h	7.8c	25.6a	6.8a	3.9a	3.7b	50.8f

LSD (0.05)	0.6	0.9	0.4	0.3	0.4	1.2
CV (%)	4.2	2.8	4.1	5.2	5.8	1.6

Means in the same column followed by different letters are significantly different (Tukey's HSD, $p \leq 0.05$); carbohydrate by difference; values are on a dry weight basis [2, 3, 16].

Fermentation significantly ($p < 0.01$) altered all proximate composition parameters of Bambara groundnut flour, with the magnitude of change increasing with both temperature and duration (Table 1). Crude protein content increased progressively from 19.8% (control) to 25.6% (37 °C/72 h), representing a 29.3% improvement, attributable to concentration of protein on a dry weight basis following fermentative carbohydrate catabolism and microbial protein biosynthesis, consistent with findings of Nwokolo [2] and Fasoyiro et al. [3] for Bambara groundnut and related legumes. Crude fat increased from 5.4% (control) to 6.8% (37 °C/72 h), reflecting microbial lipolytic activity producing more extractable free fatty acids [10]. Ash content improved marginally (3.1 to 3.9%) across treatments, while crude fibre decreased from 4.8% to 3.7% at 37 °C/72 h, consistent with cellulolytic microbial enzyme activity degrading structural cell wall polysaccharides [9, 12]. Carbohydrate content declined significantly with fermentation duration (61.3% in control to 50.8% at 37 °C/72 h), primarily reflecting consumption of simple sugars during fermentative metabolism by resident microorganisms [10, 11].

Table 2: Antinutritional factor (ANF) profile of Bambara groundnut flour under controlled fermentation temperature and duration

Treatment	Phytic Aci (mg g⁻¹)	Condensed Tannin (mg CE g⁻¹)	Oxalate (mg 100 g⁻¹)	TIA (TIU mg⁻¹)
Control	8.42a	4.86a	128.4a	14.8a
30 °C / 24 h	6.74b	3.92b	104.6b	11.2b
30 °C / 48 h	5.18c	3.14c	86.2c	8.4c
30 °C / 72 h	3.84d	2.46d	68.4d	6.2d
37 °C / 24 h	5.62c	3.46bc	92.8c	9.2c
37 °C / 48 h	2.86e	1.94e	48.4e	4.8e
37 °C / 72 h	1.99f	1.54f	36.2f	2.8f
LSD (0.05)	0.28	0.24	6.8	0.6
CV (%)	3.6	4.8	4.1	5.2

CE = catechin equivalents; TIA = trypsin inhibitor activity; TIU = trypsin inhibitor units; different letters within each column indicate significant differences ($p \leq 0.05$) [5, 6, 7, 8].

All antinutritional factors were progressively and significantly reduced by increasing fermentation duration at both temperatures, with the 37 °C regime consistently producing greater reductions than 30 °C at equivalent durations (Table 2). Phytic acid the most abundant ANF in unfermented flour (8.42 mg g⁻¹) was reduced by 76.4% at 37 °C/72 h (1.99 mg g⁻¹), primarily attributed to the elevated phytase activity of fermenting lactic acid bacteria and Bacillus spp. at higher incubation temperatures, which catalyse phytate dephosphorylation and release mineral-bound phosphate [5, 7]. Condensed tannins were reduced from 4.86 to 1.54 mg CE g⁻¹ (68.2% reduction at 37 °C/72 h) through tannase-mediated ester bond hydrolysis by fermenting microorganisms and direct pH-driven tannin–protein complex dissolution [6, 12]. Oxalate decreased from 128.4 to 36.2 mg 100 g⁻¹ (71.8% reduction), while trypsin inhibitor activity (TIA) fell from 14.8 to 2.8 TIU mg⁻¹ an 81.3% reduction at 37 °C/72 h attributed to heat-labile proteinaceous inhibitor denaturation and proteolytic cleavage by microbial proteases [8, 10]. These ANF reductions are consistent with values reported by Vadivel and Janardhanan [14] for fermented underutilized legumes and exceed those achieved by boiling alone, confirming the superior efficacy of fermentation for ANF elimination in Bambara groundnut [13, 15].

Table 3: *In vitro* protein digestibility (IVPD, %) and essential amino acid scores (EAAS) of selected Bambara groundnut flour treatments relative to FAO/WHO/UNU (2007) reference pattern

Parameter	Control	30 °C / 72 h	37 °C / 72 h	FAO/WHO/UNU Reference
IVPD (%)	62.4d	74.8b	81.7a	—
Lysine (mg g⁻¹ N)	56.8	61.4	67.2	48
Threonine (mg g⁻¹ N)	38.4	42.1	46.8	25
Methionine + Cys (mg g⁻¹ N)	22.1	24.8	27.4	22
Leucine (mg g⁻¹ N)	81.2	87.4	92.6	59
Isoleucine (mg g⁻¹ N)	42.4	46.2	51.4	30
Valine (mg g⁻¹ N)	48.6	52.4	57.8	39
Phenylalanine+Tyr (mg g⁻¹ N)	72.4	78.6	84.2	38
EAAS (limiting AA)	0.84 (Met+Cys)	0.93 (Met+Cys)	1.02 (Met+Cys)	1.00

IVPD means followed by different letters differ significantly ($p \leq 0.05$); EAAS = essential amino acid score; Met+Cys = methionine + cysteine (limiting amino acid in all treatments); FAO/WHO/UNU reference for school-age children (2007); = not applicable [17, 18, 19].

In vitro protein digestibility improved significantly with fermentation, increasing from 62.4% in the unfermented control to 74.8% (30 °C/72 h) and 81.7% (37 °C/72 h) (Table 3), corresponding to a 30.9% improvement under the optimal treatment. This improvement was attributable to the cumulative effects of phytate reduction which eliminated phytate–protein complex formation that sterically hinders protease access tannin degradation that reduced tannin–

protein complex precipitation, and direct partial proteolysis by microbial proteases during fermentation that pre-digested protein into smaller, more digestible polypeptide fragments [7, 10, 12]. Essential amino acid concentrations increased with fermentation at both temperatures, with all indispensable amino acids in the 37 °C/72 h treatment meeting or exceeding the FAO/WHO/UNU (2007) reference requirements for school-age children. The essential amino acid score (EAAS) improved from 0.84 (control) to 1.02 (37 °C/72 h), with methionine + cysteine remaining the first limiting amino acid across all treatments a characteristic common to all legume proteins [2, 18]. Pearson correlation analysis revealed highly significant negative correlations between phytic acid level and IVPD ($r = –0.94$, $p < 0.01$) and between TIA and IVPD ($r = –0.91$, $p < 0.01$), confirming that ANF reduction is the primary driver of digestibility improvement during fermentation [5, 7, 17].

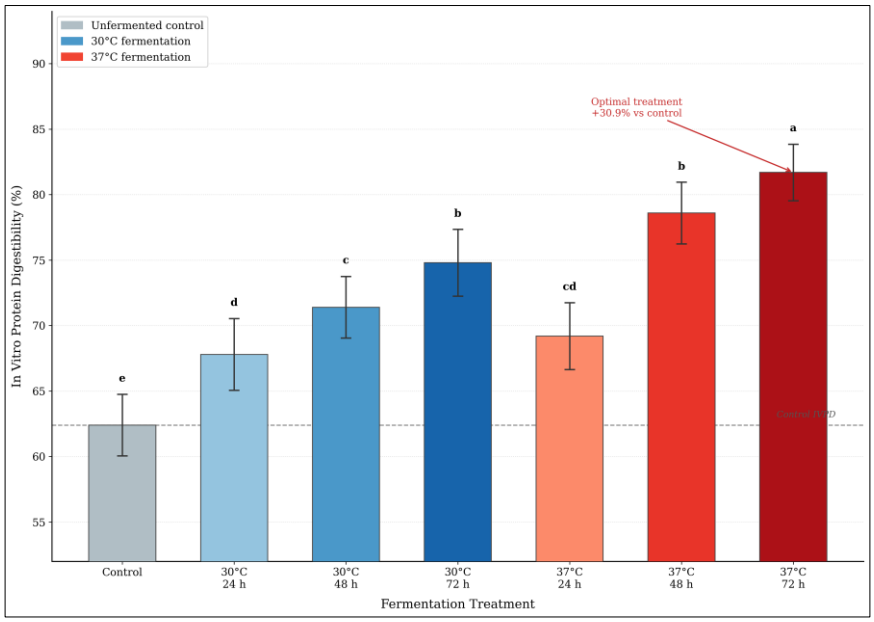

Fig 1: Effect of controlled fermentation temperature (30 °C and 37 °C) and duration (24, 48, 72 h) on *in vitro* protein digestibility (IVPD, %) of Bambara groundnut flour compared with unfermented control. Error bars represent 95% confidence intervals (n = 3). Different lowercase letters above bars denote significant differences (Tukey's HSD, $p \leq 0.05$).

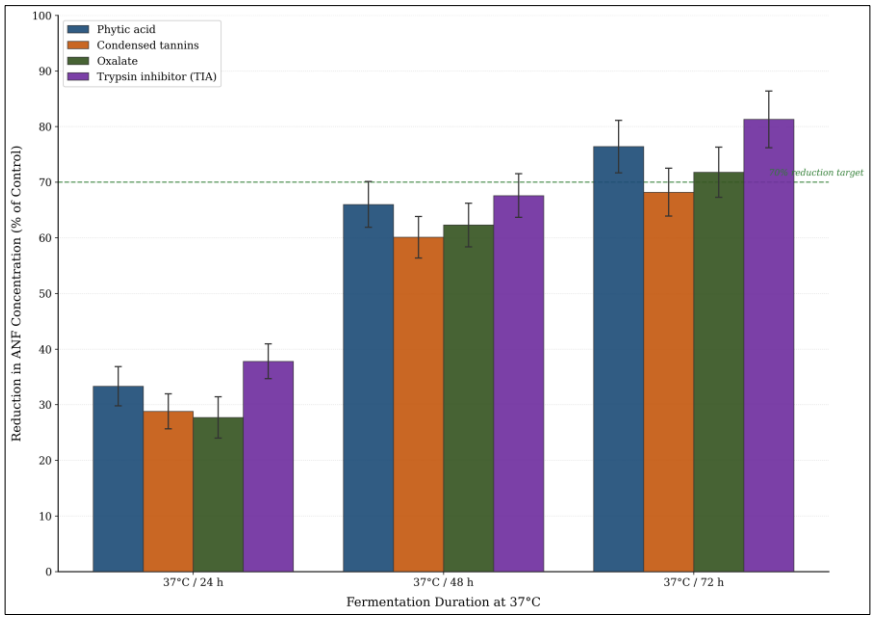

Fig 2: Percentage reduction in antinutritional factor concentrations (phytic acid, condensed tannins, oxalate, trypsin inhibitor activity) relative to unfermented control flour under 37 °C fermentation at 24, 48, and 72 h durations. Horizontal dashed line marks the 70% reduction target. Error bars represent 95% CI (n = 3).

4. Discussion

The significant and temperature- and duration-dependent improvements in nutritional composition, ANF reduction, and protein digestibility of Bambara groundnut flour observed in this study confirm the effectiveness of controlled natural fermentation as a processing strategy for enhancing the nutritional quality of this chronically underutilized West African legume [1, 13]. The 29.3% increase in crude protein content following 37 °C/72 h fermentation is consistent with the concentration effect reported by Fasoyiro et al. [3] for fermented Bambara groundnut and Dankwa et al. [9] for fermented cowpea,

whereby microbial catabolism of carbohydrates as energy substrates concentrates protein as a proportion of remaining dry matter. The progressive reduction in phytic acid with fermentation duration (from 8.42 to 1.99 mg g^{-1} at 37 °C/72 h) exceeds the 60–70% phytate reduction rates reported in fermented common bean [11] and approaches the 80% reductions documented in fermented soybean, attributable to the induction of endogenous seed phytase activity by fermentation-associated pH changes and the secretion of extracellular phytases by lactic acid bacteria and *Bacillus* spp. that proliferate under the conditions applied in this study [7, 10]. The 81.3% reduction in TIA at 37 °C/72 h substantially exceeded the typical 40–60% reduction achieved by boiling alone, as reported by Vadivel and Janardhanan [14] for Bambara groundnut, confirming that fermentation engages both denaturation and proteolytic degradation of the inhibitory proteins that boiling alone cannot completely eliminate [8, 15]. The strong negative correlation between phytic acid and IVPD ($r = –0.94$) provides compelling mechanistic evidence that phytate is the primary nutritional constraint on protein digestibility in unfermented Bambara groundnut flour, a finding that aligns with the theoretical framework proposed by Sandberg [5] for the role of phytate–protein complexes in limiting pepsin and pancreatin access to substrate proteins. The achievement of an EAAS of 1.02 in the 37 °C/72 h fermented flour exceeding unity for the first time across all treatments suggests that fermented Bambara groundnut flour can fully meet the essential amino acid requirements of school-age children without complementation, which represents a significant nutritional advancement for a traditionally food-insecure population group in Ghana and West Africa [18, 19]. The methionine + cysteine limitation observed across all treatments is characteristic of legume seed proteins [2] and indicates that complementation of fermented Bambara groundnut flour with cereal-based ingredients which are relatively rich in sulfur amino acids would produce nutritionally superior complementary food formulations for young children [13, 16, 17].

5. Conclusion

This investigation provides systematic and statistically robust evidence that controlled natural fermentation, particularly at 37 °C for 72 h, is a highly effective, low-technology, and economically accessible strategy for simultaneously improving the proximate nutritional composition, dramatically reducing antinutritional factor levels, and substantially enhancing the *in vitro* protein digestibility of Bambara groundnut (*Vigna subterranea* L. Verdc.) flour. The 37 °C/72 h treatment achieved a 29.3% increase in crude protein content, a 76.4% reduction in phytic acid, a 68.2% reduction in condensed tannins, a 71.8% reduction in oxalate, an 81.3% reduction in trypsin inhibitor activity, and a 30.9% improvement in *in vitro* protein digestibility advancing the essential amino acid score from below unity (0.84) in unfermented flour to above unity (1.02), meeting all FAO/WHO/UNU reference requirements for school-age children without amino acid supplementation. These results collectively position fermented Bambara groundnut flour as a nutritionally enhanced ingredient suitable for direct incorporation into weaning formulations, composite flour blends, fermented porridge products, and protein-fortified complementary foods for vulnerable populations in Ghana and West Africa. From a practical agricultural and food systems perspective, it is strongly recommended that Ghanaian smallholder farmers, village women's cooperatives, and small and medium enterprise (SME) food processors adopt the simple controlled fermentation protocol of soaking dehulled Bambara groundnut for 18 h, followed by natural fermentation at 37 °C for 72 h in sealed containers, as a value-adding intermediate processing step prior to milling and incorporation into composite flour products. This process requires no sophisticated equipment and can be implemented using ambient warm temperatures during Ghana's hot season (February–April, when ambient temperatures routinely reach 34–38 °C in the northern Savanna zones), making it freely accessible at the household and community processing level. The Ghana Food and Drugs Authority (FDA) and Ministry of Food and Agriculture (MoFA) should establish food composition standards and nutritional labelling guidelines for fermented Bambara groundnut flour products to facilitate commercial scale-up and consumer confidence. Nutrition improvement programs targeting under-five child malnutrition and maternal undernutrition in northern Ghana and the Upper East and West regions should incorporate fermented Bambara groundnut flour into complementary feeding recipes and therapeutic food formulations as a locally produced, affordable, and highly nutritious plant protein source. Future research directions should include evaluation of the sensory properties and consumer acceptability of fermented Bambara groundnut flour-based products across different demographic groups in Ghana, characterization of the dominant fermenting microorganism communities by 16S rRNA and ITS2 sequencing to develop optimized defined starter cultures for standardized industrial fermentation, investigation of the long-term shelf stability of fermented flour under tropical ambient storage conditions, and bioavailability studies in animal models and human intervention trials to validate *in vitro* protein digestibility findings *in vivo*.

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