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Microbiological safety and anti-nutrient content of meat analogues from African yam beans, cowpeas, mucuna, and soybeans infused with *Hibiscus sabdariffa* (Zobo) calyces

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Abstract
This study evaluated the anti-nutrient composition and microbial quality of meat analogues developed from four leguminous seeds: African yam beans, cowpeas, mucuna, and soybeans. The legumes were dehulled, soaked, and blended with Roselle calyx extract (90:10 w/v) before being mixed with binder and oil, then cooked via extrusion. The final meat analogue formulation consisted of 70% legume protein, 20% binder, and 10% fat. Anti-nutrient analysis showed phytate (1.05–1.28 mg/100 g), tannin (1.21–2.16 mg/100 g), and trypsin inhibitor (0.18–1.52 IU/100 g) levels, all within acceptable limits for plant-based foods, indicating that processing significantly reduced inherent anti-nutritional factors. Microbial counts ranged from 1.07×10^5 to 1.83×10^5 CFU/g (bacteria) and 1.03×10^5 to 7.40×10^5 SFU/g (fungi), indicating hygienic production. In conclusion, the selected legume-based meat analogues demonstrated safe microbial levels and reduced anti-nutritional factors, supporting their potential as nutritious plant-based meat alternatives.

Keywords: Meat analogues, Leguminous seeds, Anti-nutritional factors, Microbial quality, Roselle (Hibiscus sabdariffa) extract

1. Introduction

Rising concerns about climate change, food insecurity, and diet-related diseases have spurred interest in sustainable food alternatives such as meat analogues, plant-based products designed to replicate the sensory and nutritional properties of meat (Kyriakopoulou *et al*., 2019 [1]; Kumar *et al*., 2016) [2]. Excessive red meat consumption has been linked to chronic illnesses including cardiovascular diseases and certain cancers (Wang & Beydoun, 2009) [3], while plant-based diets have shown benefits such as improved glycemic control and reduced chronic disease risk (Mohamed *et al*., 2017) [4].

Recent research has focused on optimizing meat analogues using soy, wheat gluten, and pea proteins (Sha & Xiong, 2020 [5]; Tso, Forde, & Lim, 2020) [6]. However, underutilized legumes like African yam bean, cowpea, and *Mucuna pruriens* remain largely unexplored despite their rich protein profiles and adaptability to sub-Saharan climates (Udensi *et al*., 2010 [7]; Aletor & Aladetimi, 1989 [8]; Antova *et al*., 2014 [9]). For instance, African yam bean contains 21–29% protein (Esan & Fasasi, 2013) [10], cowpea offers up to 31.7% protein (FAO, 2011) [11], and *Mucuna pruriens* provides 24–34% protein and bioactive compounds (Pathania *et al*., 2020 [12]; Attaugwu *et al*., 2022) [13].

Incorporating *Hibiscus sabdariffa* (zobo) calyces, known for their antioxidant and antimicrobial properties (Ali *et al*., 2005 [14]; Babalola *et al*., 2011) [15], further enhances the functional and sensory appeal of such products. Despite these advantages, research on integrating these ingredients into meat analogue formulations is sparse. This study addresses this gap by formulating and evaluating an extruded meat analogue from blends of African yam bean, cowpea, *Mucuna pruriens*, and soybeans infused with zobo calyces. It assesses anti-nutritional profiles and microbiological safety, contributing to the development of regionally adapted, health-promoting meat alternatives.

2. Materials and Methods

2.1 Material Procurement

African yam bean, Mucuna bean, cowpea, Soybeans, and Hibiscus sabdariffa calyces, along with other ingredients such as umami seasoning, vegetable oil, garlic powder, and black pepper, were obtained from a local market in Anyigba, Kogi State, Nigeria. The chemicals and reagents utilized in this work were all of analytical grade.

2.2 Experimental Design

This study was designed to examine the anti-nutritional and microbial properties of the meat analogue samples. Meat analogues were developed using African yam bean, mucuna bean, cowpea, and soybean with roselle calyces infusion to achieve the desired color and flavor. Four batches of meat analogue were produced as shown in Table 3.1 below. All the batches were formulated, thus 70 % *cowpea* /soybean/African yam bean/mucuna bean protein, 20 % Binder 10 % Fat, Chicken Sausage was served as control.

Table 1: Meat analogue formulations

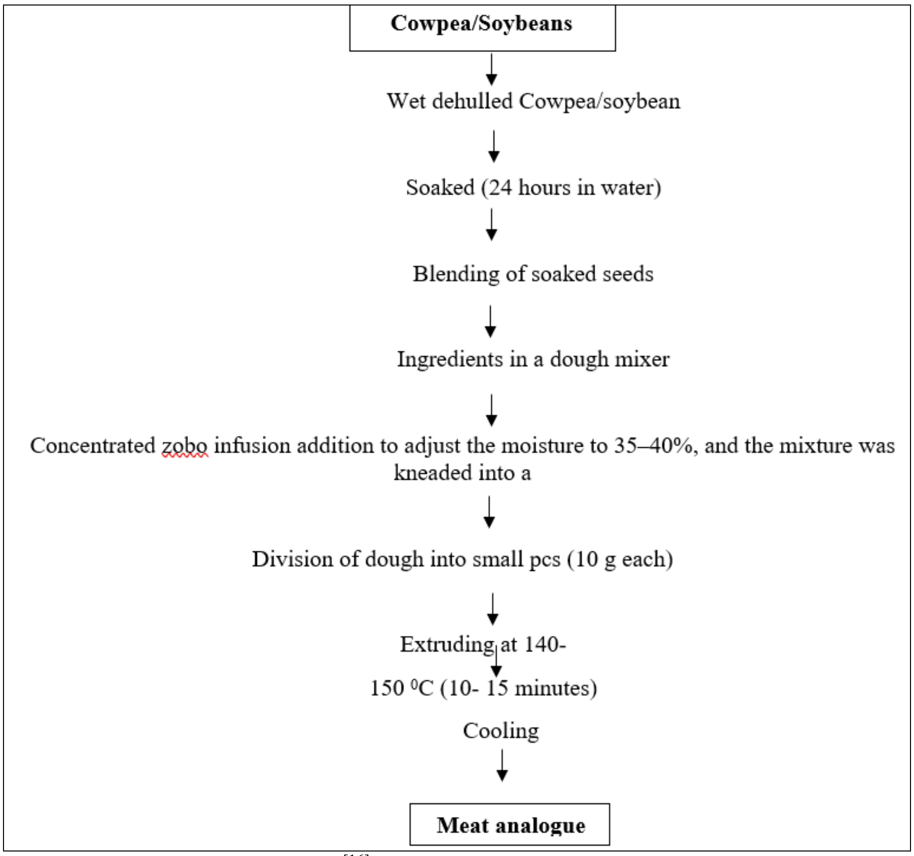
Ingredients	AAA	BBB	CCC	DDD
Protein source (%)	70	70	70	70
Binder (%)	20	20	20	20
Fat (%)	10	10	10	10

Key: AAA= Cowpea meat analogue, BBB = African yam bean meat analogue CCC = Mucuna meat analogue DDD = Soybean meat analogue EEE = Control.

2.3 Sample preparation and processing

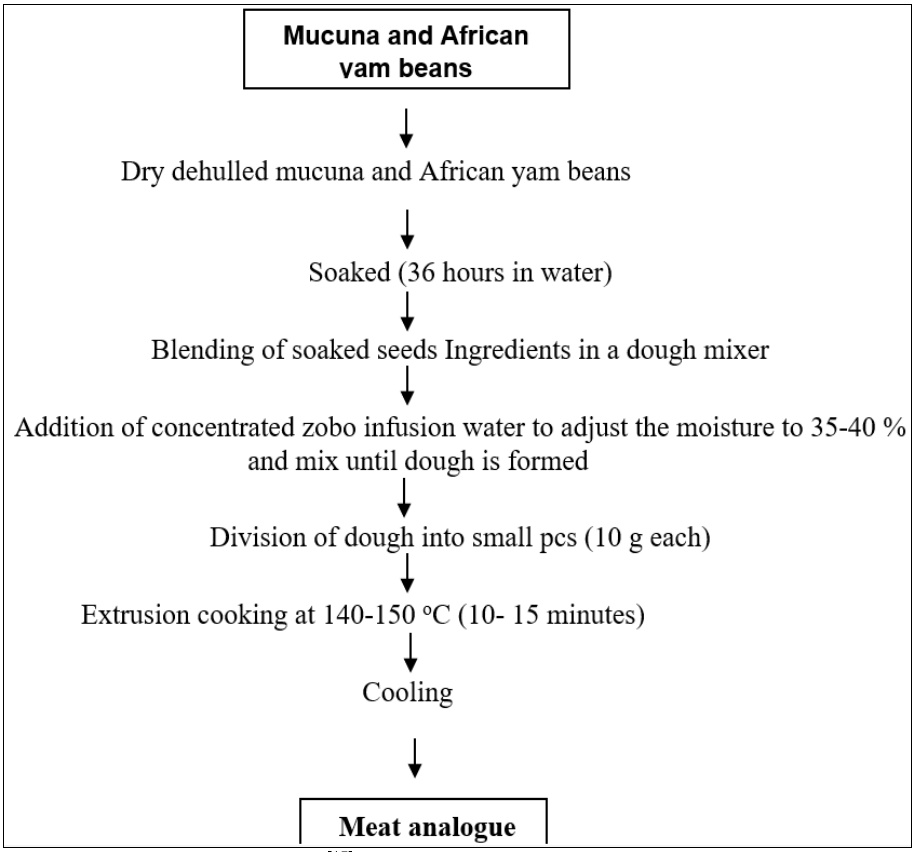
2.3.1 Seed and Bean preparation

Cowpea seeds and soybeans were cleaned to remove any dirt or impurities and soaked separately in water for 24 hours to soften them, while mucuna and African yam bean was dehulled mechanically before soaking for 32 hours. The zobo calyces were boiled in water to extract the color, flavor and nutrients. The boiled calyces were strained to obtain a concentrated infusion. The soaked seeds were drained and blended separately into a smooth paste. The Zobo calyces infusion and CMC were gradually added to the different pastes while kneading until a homogeneous mixture was obtained. The mixture was subjected to extrusion cooking using an extruder. The meat analogue was allowed to cool for 30 minutes before serving for sensory evaluation. The sample for analysis was stored in airtight containers in the freezer.



Source: Adetunji and Babalobi (2011) [16]

Fig 1: Production of Cowpea/ soybean meat analogue



Source: Uaboi-Egbenni *et al*. (2020) [17].

Fig 2: Production of Mucuna / African yam bean meat analogue

2.4 Method of Analysis

2.4.1 Trypsin Inhibitor Activity was determined using AOCS Method Ba 12a (2020) [18]. Dry samples of meat analogues were finely ground and extracted using 10 mM NaOH (50 mL) at room temperature for 3 hours with stirring. The extract was diluted to ensure 30–70% trypsin inhibition per 1.0 mL. Reaction mixtures were prepared in test tubes as follows:

- **Sample readings** (with inhibitor),
- **Sample blanks** (inhibitor + early acetic acid),
- **Reference readings** (no inhibitor),
- **Reference blanks** (no inhibitor + early acetic acid).

Reagents were added sequentially: water/extract, prewarmed BAPA solution, trypsin solution (start timing), and acetic acid after exactly 10 minutes. Mixtures were vortexed and centrifuged at 1500 × g for 5 minutes.
Absorbance of the supernatant was measured at 410 nm within 1 hour.

Calculation

One trypsin unit (TU) = 0.02 absorbance increase at 410 nm.
TIA was expressed as trypsin units inhibited (TUI) per gram of sample.

2.4.2 Estimation of Condensed Tannin Content

A slightly modified procedure based on Asres *et al*. (2018) [18] was employed. About 20 g of ground sample was weighed into a conical flask and treated with 100 mL of petroleum ether. The flask was sealed and left undisturbed for 24 hours to defat the sample. After this period, the mixture was filtered, and the remaining residue was air-dried for approximately 15 minutes to ensure complete evaporation of the solvent. The residue was then extracted with 100 mL of 10% acetic acid in ethanol and allowed to stand for 4 hours. After filtration, the resulting filtrate was collected, and 25 mL of ammonium hydroxide (NH_4OH) was added to precipitate alkaloids. The solution was gently heated on a hot plate to eliminate excess NH_4OH. A 5 mL aliquot was drawn from the remaining mixture, combined with 20 mL of ethanol, and titrated using 0.1 M sodium hydroxide (NaOH) with two drops of phenolphthalein as an indicator until a faint pink endpoint appeared. The percentage of tannic acid in the sample was then calculated using the formula:

$$\% \text{ Tannic acid} = (C_1 \times 100) / \text{weight of sample analyzed} \quad (1)$$

Where $\mathbf{C_1}$ = concentration of tannic acid.

2.4.3 Estimation of Phytate Content

Phytate determination followed a modified approach adapted from Asres *et al*. (2018) [18]. Precisely 0.2 g of the sample was soaked in 100 mL of 2% hydrochloric acid for 3 hours. The suspension was then filtered, and 50 mL of the filtrate was diluted with 100 mL of distilled water. Subsequently, 10 mL of 0.3% ammonium thiocyanate was added as an indicator. This solution was titrated against standard ferric chloride ($FeCl_3$) solution, with each milliliter containing 0.00195 g of iron. The concentration of phytic acid was calculated using the formula:

$$\text{Phytic acid (mg/100g)} = (\text{Titre value} \times 0.00195 \times 1.19 \times 100) / \text{weight of sample} \quad (2)$$

2.4.4 Microbial analysis
2.4.4.1. Determination of total plate count/total fungal count
The pour plate method, as described by Harrigan and McCance (2016) [19] was used to determine the total plate count. One gram of the sample was mixed with 9 ml of Ringer's solution from which a further tenfold serial dilution was made to obtain the 10^{-2} dilution. One millilitre solution from each of the dilutions was seeded into petri dishes (in duplicate), and 15 ml of sterile nutrient agar (total plate count) and potato dextrose agar (total fungal count) poured and mixed thoroughly with the inocula by gently rocking the plates. The plates were incubated at 37 ºC for 24 h, after which the colonies formed were counted and expressed as colony-forming units per gram of sample (cfu/g) using the formula:

$$\text{Bacteria cells (cfu/g)} = \frac{\text{Number of colonies} \times \text{original dilution}}{\text{Dilution factor} \times \text{volume of innoculum}}$$

$$\text{Total fungal count (sfu/g)} = \frac{\text{Number of colonies} \times \text{original dilution}}{\text{Dilution factor} \times \text{volume of innoculum}}$$

2.5 Statistical analysis
Analysis of Variance (ANOVA) was carried out on the parameters to compare the values obtained for the various meat analogue and Soybean meat analogue (control). This was done by using Statistical Package for Social Sciences (SPSS) Version 17.0 for Windows (SPSS Inc., Illinois, USA). The mean separation was carried out using the Least Significant Difference (LSD). Statistical significance was accepted at a 0.05 level of probability ($p \leq 0.05$).

3. Results and Discussions
3.1 Anti-nutritional Composition
The anti-nutritional properties of the meat analogue samples revealed relatively low levels of phytates, tannins, trypsin inhibitors, phenols, and flavonoids, indicating that the products are safe and potentially beneficial for consumption. The phytate content (1.05–1.28 mg/100g) observed across the samples was within acceptable limits and comparable to the findings of Oboh and Oomotosho (2015) [20], who reported similar values in wheat-African breadfruit-based meat analogues. These values were notably lower than the 2.10–3.61 mg/100g range reported by Edima-Nyah *et al.* (2019a) [21] in breadfruit-based snack bars, suggesting that the processing methods used in this study, such as dehulling and extrusion, may have contributed to phytate reduction. This is significant because phytates, while mildly beneficial as antioxidants, are known to chelate essential minerals like iron and zinc at higher concentrations, thereby impairing their bioavailability (Onimawo & Akubor, 2012) [22].

Tannin content ranged from 1.21 to 2.20 mg/100g, and although significant differences existed among the samples, all values remained well below the established safe limit of 90 mg/100g (Maseta *et al.*, 2016) [23]. This is consistent with results by Oguntause *et al.* (2019) [24], who recorded similar tannin levels in Bambara groundnut-based meat analogues. Tannins, while sometimes responsible for astringency and dark coloration in foods (Ogunwolu *et al.*, 2015) [25], can also exert antimicrobial and antioxidant effects when present in safe quantities, thereby contributing to the product's shelf stability and health benefits.

Trypsin inhibitor activity (TIA) levels were low, ranging from 0.18 to 1.52 TIU/100g. These levels are significantly lower than the 11.15 to 34.80 TIU/100g reported in wheat-breadfruit bread bars (Effiong & Edima-Nyah, 2023) [26], indicating effective reduction of these inhibitors, likely due to the heat from extrusion processing. Since trypsin inhibitors can interfere with protein digestion, their significant reduction enhances protein bioavailability. All samples were well below the toxic threshold of 200 TIU/100g (Inuwa *et al.*, 2011) [27], confirming their safety for human consumption.

The total phenolic content (0.13–2.21 mg GAE/g) varied significantly, with soybean-based samples recording the lowest levels. This aligns with the findings of Polidori *et al.* (2011) [28], who noted that phenolic compounds are not only natural antioxidants but also act as effective antimicrobial agents, making them vital for preserving food and improving health outcomes. The higher phenolic content observed in some legume-based analogues suggests enhanced antioxidant potential, which could help in reducing oxidative stress.

Similarly, the total flavonoid content (1.13–1.80 mg GAE/g) showed significant variation, with soybean-based samples exhibiting relatively higher values. Flavonoids are well-documented for their strong antioxidant properties and health-promoting effects, including anti-inflammatory, cardioprotective, and anticancer activities (Okwu, 2015) [29]. Their presence in these meat analogues enhances their functional food potential, especially in mitigating the risks associated with chronic diseases linked to oxidative damage.

In summary, the levels of anti-nutritional factors in the meat analogues were within safe limits, with some contributing positively to the health-promoting properties of the products. The relatively low concentrations of phytates, tannins, and trypsin inhibitors enhance nutrient bioavailability, while the presence of phenolic and flavonoid compounds suggests antioxidant potential, supporting the suitability of these analogues as nutritious and functional meat alternatives.

Table 2: Anti-nutrient composition of meat analogue

Parameters	Phytate (mg/100g)	Tannin (mg/100g)	Trypsin Inhibitor TIU/100g
AAA	$1.05^c \pm 0.029$	$1.93^b \pm 0.016$	$1.52^a \pm 0.002$
BBB	$1.28^a \pm 0.000$	$2.16^a \pm 0.042$	$0.30^c \pm 0.000$
CCC	$1.09^b \pm 0.042$	$2.20^a \pm 0.007$	$0.87^b \pm 0.000$
DDD	$1.15^b \pm 0.000$	$1.21^d \pm 0.028$	$0.18^e \pm 0.021$
EEE	$1.15^b \pm 0.084$	$1.41^c \pm 0.049$	$0.22^d \pm 0.007$

Values are averages of triplicate readings (mean ±standard deviation). Means within a row followed by different superscripts letter(s) are significantly differences ($p \leq 0.05$). Keys: AAA = Soyabeans meat analogue, BBB = Mucuna meat analogue, CCC = African yam beans meat analogue. DDD = Cowpea meat analogue, EEE = Sausage (control).

3.2 Microbiological Assessment
The microbiological assessment of the meat analogue samples revealed acceptable safety levels, as both bacterial and fungal counts remained within the permissible limits set by the International Commission on Microbiological Specifications for Foods (ICMSF, 2019) [30], which considers counts $\leq 10^5$ CFU/g safe for consumption. The relatively low bacterial counts indicate that the production process was conducted under hygienic conditions, minimizing microbial contamination. This is crucial, as

microbial quality not only reflects food safety but also influences shelf-life and consumer acceptability.

The higher bacterial load observed in the control sausage sample compared to the plant-based analogues may be attributed to its higher animal protein content, which is more prone to microbial proliferation. Animal-based products generally support faster microbial growth due to their rich nutrient composition, especially when compared to processed plant-based alternatives (Uzeh *et al*., 2016) [31]. The findings align with Onwuka (2014) [32], who emphasized that the microbial status of a food product often reflects the nature of its ingredients and the processing methods used. The lower microbial counts in the plant-based analogues could also be linked to the thermal effects of extrusion and the possible antimicrobial activity of bioactive compounds, such as phenolics and flavonoids, present in legumes and zobo calyces, which have been shown to inhibit microbial growth (Polidori *et al*., 2011) [28].

Fungal counts, although varying across samples, also remained well below critical thresholds, further affirming the microbiological stability of the products. The slightly elevated fungal count in the control sample may be due to post-processing contamination or higher moisture retention, which supports fungal growth. The values observed were significantly lower than those reported by Uzeh *et al*. (2016) [31] for dairy-based products, reinforcing the idea that plant-based matrices, when properly processed, can offer safer alternatives with extended shelf-life.

In essence, the microbiological safety of the legume-based meat analogues demonstrates the effectiveness of the processing methods used and underscores the potential of these formulations as safe, shelf-stable meat substitutes. This not only enhances consumer confidence but also supports broader adoption of plant-based alternatives in food systems where hygiene and shelf-life are critical concerns.

Table 3: Microbiological quality of meat analogue produced from selected legume seeds

Samples	Bacteria count (CFU/g)	Fungi Count (CFU/g)
AAA	1.16×10^5	1.03×10^5
BBB	1.69×10^5	7.00×10^5
CCC	3.0×10^5	4.10×10^5
DDD	1.07×10^5	2.90×10^5
EEE	1.83×10^5	7.40×10^5

Key: AAA = Soyabeans meat analogue, BBB = Mucuna meat analogue, CCC = African yam beans meat analogue; DDD = Cowpea meat analogue: EEE = Sausage (Control)

4. Conclusion

This study demonstrated that meat analogues produced from African yam beans, cowpeas, mucuna, and soybeans infused with Roselle calyx extract possess acceptable anti-nutritional and microbiological profiles. The levels of phytates, tannins, and trypsin inhibitors were all within safe limits, indicating that the processing methods effectively reduced these compounds. Microbial counts were also below the safety threshold, reflecting good hygienic practices during production. These findings highlight the potential of underutilized legumes as sustainable, nutritious, and safe alternatives for meat analogue production.

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