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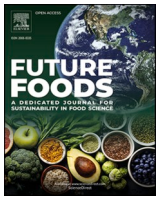

# Fungal-mediated solid-state fermentation ameliorates antinutritional factors but does not improve *in vitro* digestibility of marama (*Tylosema esculentum*) beans

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ABSTRACT

Beans from the orphan legume *Tylosema esculentum* (marama plant) have the potential to partially or completely replace soybeans in human and animal diets. However, their high levels of antinutritional factors and low levels of certain essential amino acids may limit this utility. This study aimed to enhance the food/feed value of marama beans through fungal-mediated solid-state fermentation (SSF) using *Aspergillus oryzae, Aspergillus sojae*, and their co-culture, focusing on the beans' functional and nutritional properties. Contrasts revealed that fermented beans had lower ($p < 0.05$) acid detergent fiber (ADF), acid detergent lignin (ADL), cellulose, and hemicellulose levels compared to non-fermented beans, while crude fat content was unchanged ($p > 0.05$). Beans fermented with *A. sojae* showed higher crude protein (CP) content ($p < 0.05$) than non-fermented beans. Fermented beans exhibited lower ($p < 0.05$) phytic acid and trypsin inhibitor activity, but significantly higher ($p < 0.05$) total phenolic content compared to non-fermented beans. Non-fermented beans had higher ($p < 0.05$) *in vitro* enzyme protein digestibility (IVPD) than single-strain-fermented beans. Fungal fermentation increased ($p < 0.05$) dispersibility and reduced water and oil absorption capacities but did not affect ($p > 0.05$) pH and bulk density. In conclusion, fungal-mediated SSF reduced some fiber fractions, phytic acid, and trypsin inhibitor activity in marama beans, though this did not result in enhanced simulated protein digestibility.

## 1. Introduction

Soybean cultivation in sub-Saharan Africa requires significant inputs such as fertilizers, pesticides, herbicides, and irrigation water. This makes soybeans an economically, environmentally, and socially unsustainable source of dietary protein in regions where climatic conditions are generally unfavorable for their cultivation. Consequently, identifying and evaluating alternative legumes adapted to local climatic conditions is essential. One such alternative is marama bean (*Tylosema esculentum*), a leguminous tuberous plant native to the Kalahari Desert regions of Namibia, Botswana, and parts of South Africa. The yield of marama beans ranges from 0.5 to 2 tons per hectare, whereas soybean yields typically range between 3 and 4 tons per hectare (Omotayo and Aremu, 2021). At current productivity levels, marama cultivation requires nearly twice the amount of land to achieve yields comparable to soybeans. However, ongoing domestication studies aim to enhance the agronomic performance of marama plants and establish large-scale production systems (Chimwamurombe, 2016) thus positioning marama beans as a commercially viable alternative to soybeans. Traditional preparation methods include roasting the seeds in hot sand, boiling fresh beans with water or milk, and grinding them into flour for porridge (Jackson et al., 2022). Additionally, marama beans are processed into

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oil, butter, and a milk-like beverage like soymilk (Cullis et al., 2023) making them a versatile food source. Recent research has also explored the potential of marama beans as a soybean substitute in animal feed formulations (Alabi, 2023; Alabi et al., 2024; Vilakazi et al., 2025).

While marama beans rival soybeans in nutrient composition (Amonsou et al., 2012) and contain higher levels of tyrosine and proline (Holse et al., 2011), they lack essential amino acids such as methionine and cysteine, which are crucial for animal growth (Amonsou et al., 2012). These sulfur-containing amino acids contribute to protein synthesis and glutathione production, which play a critical role in tissue development and protection of cells against oxidative stress (Liu et al., 2024). Furthermore, Alabi et al. (2022) reported that marama beans contain approximately four times the trypsin inhibitor activity (measured as trypsin unit inhibitors, TUI) of soybeans, with values of 245.45 TUI/mg DM and 66.98 TUI/mg DM, respectively. High trypsin inhibitor activity impairs protein digestion by inhibiting trypsin enzyme activity (Savage and Morrison, 2003). In a feeding trial, Alabi et al. (2024) demonstrated that diets containing up to 6.5 % raw full-fat marama bean meal negatively affected broiler chickens, leading to reduced growth performance, lower carcass weights, and increased visceral organ weights. These adverse effects were attributed to high levels of trypsin inhibitors and phytic acid in the beans. Additionally, marama beans are rich in dietary fiber (19–27 % DM) (Holse et al., 2011; Jackson et al., 2010), which reduces nutrient digestibility. Other antinutritional factors, including tannins, elastase inhibitors, and cyanogenic glycosides, may further limit nutrient bioavailability (Alabi et al., 2022; Alabi, 2023). To maximize the utility of marama beans as a dietary protein source, strategies are needed to reduce their antinutritional factors while enhancing their digestibility and functional properties to achieve nutritional parity with soybeans. Solid-state fermentation (SSF) using filamentous fungi is a promising technique for this purpose. Studies have shown that fungal-mediated SSF can reduce antinutritional activity, improve digestibility, and enhance the nutritional and functional properties of food/feed substrates. Indeed, *A. sojae, A. ficuum*, and their co-culture have been successfully used to valorize canola meal (Olukomaiya et al., 2020c) and lupin flour (Olukomaiya et al., 2020a), while *A. oryzae* has proven effective for faba meal (Chen et al., 2013).

*Aspergillus sojae* and *A. oryzae* were selected for this study due to their well-documented ability to produce a diverse range of hydrolytic enzymes and their established use in food and feed applications, supported by their GRAS (Generally Recognized as Safe) status (El-Gendi et al., 2021). *Aspergillus oryzae* is known to produce key enzymes such as cellobiohydrolases, β-glucosidases, endoxylanases, proteases, amylases, glutaminases, and metallopeptidases (Irajie et al., 2016; Naz et al., 2020; Yang et al., 2023), while *A. sojae* primarily produces glutaminases, proteases, endo-polygalacturonases, and exo-polygalacturonases (Irajie et al., 2016; Yang et al., 2023). These enzymes are particularly effective in degrading antinutritional compounds such as phytic acid, trypsin inhibitors, and phenolics, as well as breaking down carbohydrates, polypeptides, and nucleic acids (Hu et al., 2011), ultimately improving food and feed digestibility and nutritional quality. To date, no studies have explored the influence of SSF on the nutritional and functional properties of marama beans. This study aimed to evaluate the effects of fermenting marama beans with *A. sojae* and *A. oryzae* on their chemical composition, functional properties, and *in vitro* multi-enzyme protein digestibility. It was hypothesized that SSF with *A. oryzae* and *A. sojae* would reduce antinutritional factors while improving the nutritional value, functional characteristics, and protein digestibility of marama beans.

## 2. Materials and methods

### 2.1. Marama beans and fungal strains

Hard-shelled marama nuts were sourced from Malwelwe village, Botswana (23.94282° S; 25.1999° E), an area that receives 350 – 450 mm of rainfall annually and experiences temperatures ranging from 34 – 36 °C in summer to 0 – 5 °C in winter (Alabi et al., 2022). The soil type in this area is Kalahari sands. The nuts were cracked by hand to remove their hard seed coat. Kernels were removed from shells manually, milled through a 1 mm sieve, and autoclaved prior to SSF for 8 days. *Aspergillus sojae* (ATCC 20,235) and *A. oryzae* (ATCC 9362) were obtained as live cultures from the National Collection of Fungi at the ARC Plant Protection Research Institute, Pretoria, South Africa. Fungi were maintained on potato dextrose agar (PDA) plates at 30 °C for 4 days, after which they were stored at 4 °C until further use. To obtain spore suspensions, mycelial plugs (1 cm × 1 cm) of *A. oryzae* and *A. sojae* were individually transferred aseptically from stock cultures to fresh PDA plates and incubated at 30 °C for 7 days or until sufficient conidial formation was observed. Plates were flooded with sterile 0.1 % Tween 80 to submerge the conidia, and spores were gently dislodged using a sterile glass rod. Spore suspensions were quantified using a haemocytometer.

### 2.2. Solid-state fermentation of marama beans

Solid-state fermentation was carried out in 500 mL Erlenmeyer flasks (150 g autoclaved bean sample per flask) in independent quadruplicate flasks for each treatment. The moisture content was adjusted to 45 % with distilled water after autoclaving at 121 °C for 15 min. After cooling to room temperature, the moistened substrates were inoculated with the respective spore seed to obtain a spore count of $1 \times 10^7$ spores for *A. sojae,* and *A. oryzae* separately. For co-culture preparations, equal volumes of each spore suspension were combined to yield a final spore count of $1 \times 10^7$ per flask prior to substrate inoculation. Inoculation was performed simultaneously for all treatments, including the co-culture, and substrates were thoroughly mixed under aseptic conditions to ensure homogeneous distribution of the spores.

The control flasks contained autoclaved substrates with no fungal inocula. All flasks were incubated at 30 °C for 8 days, during which the moisture content was not monitored or maintained. After incubation, the bean substrates were dried at 60 °C until a constant weight was achieved, which took approximately 48 h, following the protocol of Van Soest et al. (1991), to avoid heat-induced chemical changes. While AOAC recommends drying grains at 105 °C, the lower temperature was chosen to preserve the chemical integrity of the fermented marama beans for subsequent analyses of phenolics, trypsin inhibitors, fiber, and protein. The dried samples were then milled through a 0.5 mm sieve and stored at −20 °C until further analysis. Fermented substrates were then analysed for chemical composition, *in vitro* enzymatic digestion of dry matter and protein, and functional properties.

### 2.3. Proximate composition

Proximate components were quantified in unfermented and fermented marama beans and substrates according to AOAC (2016). The components of interest were fibre (method 962.09), crude ash (method 923.03), crude fat (method 960.39), and crude protein (method 990.03). Moisture was measured as the loss in weight when samples were dried in an oven at 60 °C until they reached a constant weight. Crude ash was determined as the residue after complete incineration of samples in a muffle furnace at 550 °C. Crude fat content was determined as the loss in weight of samples upon oil extraction using petroleum ether solvent in an ANKOM X15 extractor (ANKOM Technology, Macedon, New York, USA). Total nitrogen content was determined using the Dumas total combustion method with a Carlo Erba Elemental Analyser 2100 (Elemental Microanalysis Ltd., Okehampton, UK) and was converted to crude protein (CP) by multiplying percentage N content by a factor of 6.25. Fibre was determined as neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL), cellulose, and hemicellulose content of the marama bean samples using the

ANKOM200 Fibre Analyzer (ANKOM Technology, Macedon, New York, USA). Marama bean samples were serially refluxed with neutral and acid detergent solutions to determine NDF and ADF fractions, respectively. The NDF was determined without the use of sodium sulphite or heat-stable amylase and expressed inclusive of residual ash as described by Van Soest et al. (1991). Acid detergent lignin was determined by difference after dissolving the cellulose fraction in ADF residue using 72 % $H_2SO_4$ (Van Soest et al., 1991). The difference between NDF and ADF was recorded as hemicellulose content, the weight loss of ADF after $H_2SO_4$ treatment was measured as cellulose.

### 2.4. Antinutritional factors

#### 2.4.1. Phytic acid

Phytic acid content was determined using a modified colorimetric procedure described by Olukomaiya et al. (2020c). To start, 1 g of the marama bean substrate was combined with 20 mL of 2.4 % HCl and shaken on an orbital shaker for 16 h at room temperature. Afterward, the mixture was centrifuged at 4000 × *g* for 10 min at 10 °C. The resulting supernatant was mixed with 2 mL of sodium chloride, shaken for 20 min, and allowed to stand in a test tube at 4 °C for one hour. The tube was then centrifuged again at 4000 × *g* for 20 min at 10 °C. Absorbance of the supernatant was measured at a wavelength of 500 nm using a spectrophotometer, with reverse osmosis water serving as the blank. Phytic acid content was calculated based on a standard curve prepared with the dodecasodium salt of phytic acid and expressed as mg phytic acid per gram of substrate (Olukomaiya et al., 2020c).

#### 2.4.2. Trypsin inhibitors

The trypsin inhibitor was assessed following the method outlined by Mbata et al. (2009). In summary, the BAPA solution was prepared by dissolving 40 mg of benzoyl-DL-arginine-p-nitroanilide (BAPA) hydrochloride in 1 mL of dimethyl sulfoxide and then diluting it with 100 mL of 0.05 M tris-buffer (pH = 8.2) preheated to 37 °C. The trypsin solution was made by dissolving 4 mg of trypsin (2 × crystallized, salt-free; Worthington Biochemicals Corp., Freehold, N.J.) in 200 mL of 0.001 M HCl. Marama bean extracts were prepared by mixing 1 g of sample with 50 mL of 0.01 N NaOH. Raw samples were extracted for 1 hour, while fermented samples were extracted for 3 h, maintaining a pH range of 8.4–10.0. In the case of the fermented samples, longer extraction times were necessary to extract the maximum amount of trypsin inhibitors, as these samples had been exposed to an additional 48 h of heat treatment compared to the raw samples (Mbata et al., 2009). The reaction was stopped by adding 1 mL of 30 % acetic acid, and the absorbance of the filtered solution was measured at 410 nm using a reagent blank. The blank consisted of 1 mL of 30 % acetic acid, trypsin, 2 mL of water, and 5 mL of the BAPA solution. Trypsin inhibitor activity was expressed as trypsin units inhibited (TUI), where one TUI represents a 0.01 increase in absorbance at 410 nm per 10 mL of the reaction mixture.

#### 2.4.3. Total phenolics

The total phenolic content was measured using the Folin-Ciocalteu method as described by Prior et al. (2005). Extraction was performed twice by mixing 0.5 g of the sample with 10 mL of 80 % methanol and allowing it to stand for 15 min. A 25 μL aliquot of the extract was added to 125 μL of 0.7 M sodium carbonate ($Na_2CO_3$) and 125 μL of 10 % Folin-Ciocalteu reagent. The reaction mixture was incubated in the dark for 30 min. Absorbance was then recorded at 625 nm using a GENESYS™ 180 UV–Vis Spectrophotometer (ThermoFisher Scientific), with gallic acid serving as the standard. Results were expressed as milligrams of gallic acid equivalents (GAE) per gram of the sample.

#### 2.4.4. In vitro multi-enzyme digestibility

Marama bean substrates (1 g) were combined with 5 mL of distilled water and incubated at 40 °C for 30 min. After incubation, the pH was adjusted to 2.7 – 2.9 by adding 0.35 mL of 1 M HCl, followed by the addition of 1 mL of pepsin solution (10 mg). The mixture was incubated at 40 °C for 1 hour. Subsequently, 0.3 mL of 1 M NaOH was used to adjust the pH to 5.9–6.1, after which 1 mL of 5 % pancreatin solution was added, and the mixture was incubated for 2 h at 40 °C. Next, 1 mL of 40 % 5-sulfosalicylic acid dihydrate was added, vortexed, and left to react for 30 min. The mixture was centrifuged at 2500 × *g* for 20 min, and the supernatant was removed. The residue was treated with 40 % 5-sulfosalicylic acid dihydrate and 10 mL of 95 % ethanol, vortexed, and centrifuged again at 2500 × *g* for 20 min, repeating this process once more. Finally, the residue was mixed with 10 mL of acetone, centrifuged twice for 20 min at 2500 × *g*, dried at 100 °C for 1 hour, weighed, and analyzed for total nitrogen and crude protein content. The *in vitro* enzyme protein digestibility (IVPD) was calculated as:

$$IVPD\ (\%) = (P - A)/P \times 100,$$

Where **P** is the amount of protein in the sample before digestion, and **A** is the amount of protein after digestion (Olukomaiya et al., 2020c).

### 2.5. Functional properties

#### 2.5.1. pH and bulk density

The pH of both fresh and dried bean substrates was measured using a BASIC 20+ CRISON pH meter. Bulk density (BD) for fermented and unfermented bean substrates was evaluated following the procedure outlined by Oladele and Aina (2007). Two grams of the bean substrate were placed in a 10 mL graduated cylinder, which was gently tapped on a solid surface until a constant volume was achieved. Bulk density (g/mL) was calculated as the ratio of the weight (g) to the volume (mL) of the milled bean substrate.

#### 2.5.2. Water absorption capacity

Water absorption capacity (WAC) was measured at 25 °C following the method described by Traynham et al. (2007). Approximately 1 g of the bean substrate was mixed with 10 mL of reverse osmosis water and centrifuged at 3000 × *g* for 30 min. After centrifugation, the supernatant was carefully decanted, and the final weight of the sample was recorded. WAC was calculated as the percentage increase in the sample's weight after centrifugation.

#### 2.5.3. Oil absorption capacity

Oil absorption capacity (OAC) was determined using the method outlined by AOAC (2016). Approximately 0.5 g of bean substrate was combined with 3 mL of canola oil and centrifuged at 3000 × *g* for 30 min. After centrifugation, the excess oil was carefully decanted, and the sample was weighed. OAC was expressed as the percentage increase in the sample's weight after oil absorption.

#### 2.5.4. Dispersibility

The dispersibility of fermented and unfermented bean substrates was assessed using the method outlined by Elkhalifa et al. (2017). One gram of the bean substrate was placed in a 20 mL measuring cylinder and mixed with reverse osmosis water to reach a total volume of 10 mL. The mixture was vigorously shaken and allowed to settle for 3 h, after which the final volume was measured and recorded as the sample's dispersibility.

#### 2.5.5. Statistical analysis

Each SSF treatment was replicated four times using independent fermentation chambers as experimental units. All the data were tested for normality and homogeneity of variances before running the general linear models (GLM) procedure (SAS, 2010). To compare the physicochemical, functional, and nutritional properties, a one-way statistical analysis was carried out on 8-day incubation data from control, *A. oryzae, A. sojae*, and their co-culture treatments according to the following

model:

$$Yij = \mu + Si + Ejj$$

Where $Y_{ij}$ = physicochemical, functional, and nutritional properties, μ = population mean, $S_i$ = the effect of fungal strains, and $E_{ij}$ = random error. The effects of control and three SSF treatments (*A. oryzae, A. sojae*, and their co-culture) on response variables were analyzed using pre-planned mutually orthogonal contrasts. The specific contrasts tested were: **Contrast 1**, comparing the average response of SSF treatments to the control; **Contrast 2**, comparing the average response of *A. oryzae* and *A. sojae* SSF treatments to the co-culture treatment; and **Contrast 3**, comparing *A. oryzae* SSF treatment to *A. sojae* SSF treatment. The probability of difference (PDIFF) option in the lsmeans statement was used to compare treatment means.

## 3. Results and discussion

### 3.1. Chemical composition and pH

Contrast analysis revealed that fungal-mediated SSF did not affect ($p > 0.05$) crude fat content (Table 1) and pH (dried and fresh samples) (Table 3) of marama beans. Crude fat content in marama beans ranged between 335.3 – 358.9 g/kg DM, while pH ranged from 5.66 – 5.80. Our crude fat results are consistent with the findings of Olukomaiya et al. (2020c) regarding the effects of SSF with *A. sojae* and *A. ficuum* on canola meal. However, they differ from those reported for lupin flour fermented with *A. sojae* and *A. ficuum*, where fermentation increased crude fat content compared to the non-fermented flour (Olukomaiya et al., 2020a). The discrepancy in results between the two previous studies, despite using the same fungal strains, indicates that substrate type plays a critical role. This suggests that the effect of SSF on fat content is strongly influenced by the substrate used. Contrary to our expectations, the pH of marama bean was not affected by SSF in the current study. The expectation of lower pH in the fermented substrate is based on an increase in the production of organic acids from microbial degradation of carbohydrates (Olukomaiya et al., 2020a, 2020c). Indeed, Heidari et al. (2022) demonstrated that SSF reduced the carbohydrate levels of mechanically fractioned canola meal. This result contradicts several reports that found the pH of fermented substrates to be lower than in non-fermented substrates such as canola meal (Olukomaiya et al. (2020c), lupin flour (Olukomaiya et al., 2020a) and camelina meal (Olukomaiya et al., 2020b). It is important to consider the differences in substrate types and fungal strains used between the current study and the cited ones, as these factors may explain the observed discrepancies in the pH of fermented substrates. For instance, marama beans have a largely insoluble carbohydrate content of approximately 19 – 27 % DM, which is lower than that of many other legumes (Holse et al., 2011) such as canola meal and lupin flour. However, the pH of dried marama beans (5.66 – 5.8) is favorable for food/feed production as it could help inhibit the growth of foodborne pathogens, thereby improving product stability.

Of all the fermented substrates, only *A. sojae*-fermented beans had a higher CP content ($p < 0.05$) than the control, while the others were statistically similar to the control (Table 1). The planned contrast between individual fungal strains and the co-culture showed that the co-culture-fermented marama beans had a lower CP content. The high CP content of fermented beans could be due to the ability of *A. sojae* to utilize the carbon and energy content of the marama bean to produce more fungal protein (Olukomaiya et al., 2020a; Terefe et al., 2021). These findings demonstrate that fermenting marama beans with *A. sojae* could increase the protein content of the substrate, a desirable nutritional outcome. Our results corroborate the findings by Olukomaiya et al. (2020a), who reported that the CP content of lupin flour fermented with *A. sojae, A. ficuum*, and co-culture was significantly higher compared to the non-fermented flour. Likewise, Kumitch et al. (2020) found that pea protein-enriched flour fermented with *A. oryzae* had higher CP content compared to non-fermented flour. Analysis of the amino acid profile of fermented substrates would be useful for non-ruminants and humans because marama beans have low levels of methionine and cysteine (Amonsou et al., 2012).

### 3.2. Fiber fractions

Fibre reduces digestibility by limiting enzyme access to nutrients (Ng et al., 2020) making high-fibre food/feedstuffs unsuitable for simple non-ruminants and humans. Contrast analysis confirmed that SSF reduced ($p < 0.05$) the fibre content of the fermented marama beans (Table 1) in support of our hypothesis. *Aspergillus oryzae*-fermented beans had similar ($p > 0.05$) ADF content (152.3 g/kg DM) as non-fermented beans (157.1 g/kg DM), while beans fermented with the co-culture had the lowest ADF content (98.8 g/kg DM). Furthermore, co-culture-fermented beans had lower ADL content (44.3 g/kg DM) compared to non-fermented (66.4 g/kg DM) beans, however, all fermented beans had statistically similar ($p > 0.05$) ADL content. For the co-culture, this might suggest some synergism between the two fungal strains when used in composite form. However, fermentation with *A. oryzae* (40.7 g/kg DM), *A. sojae* (61.3 g/kg DM), and their co-culture (91.1 g/kg DM) produced substrates whose hemicellulose content did not differ ($p > 0.05$), in contrast with the findings made using lupin flour (Olukomaiya et al., 2020a), camelina meal (Olukomaiya et al., 2020b) and canola meal (Olukomaiya et al., 2020c). However, the average hemicellulose content of the fungal fermented beans was significantly lower ($p < 0.05$) than that of the non-fermented beans (Table 1). Surprisingly, the cellulose content of non-fermented beans and beans fermented with *A. oryzae* and *A. sojae* did not differ ($p > 0.05$). However, SSF using the co-culture significantly ($p < 0.05$) reduced cellulose content when compared to non-fermented beans. This corroborates the findings of Olukomaiya et al. (2020a) and Olukomaiya et al. (2020c), who reported that SSF reduced the cellulose content of lupin flour and

**Table 1**
Chemical composition (g/kg DM, unless otherwise stated) of marama beans subjected to *Aspergillus*-mediated solid-state fermentation.

| | | | | | | p values[2] | | | |
|---|---|---|---|---|---|---|---|---|---|
| Parameters | Control | *A. oryzae* | *A. sojae* | Co-culture | SEM[1] | GLM | C1 | C2 | C3 |
| Crude protein | 343.7$^{b}$ | 398.6$^{ab}$ | 425.1$^{a}$ | 396.2$^{ab}$ | 13.93 | 0.0098 | 0.5357 | 0.0020 | 0.1687 |
| Crude fat | 340.1$^{a}$ | 358.9$^{a}$ | 343.9$^{a}$ | 335.3$^{a}$ | 18.89 | 0.8303 | 0.3965 | 0.9824 | 0.7540 |
| Neutral detergent fiber | 311.5$^{a}$ | 192.0$^{b}$ | 190.3$^{b}$ | 189.9$^{b}$ | 19.79 | <0.0019 | 0.1263 | 0.0003 | 0.9870 |
| Acid detergent fiber | 157.1$^{a}$ | 152.3$^{a}$ | 129.0$^{b}$ | 98.8$^{c}$ | 3.99 | <0.0001 | 0.0002 | <0.0001 | 0.0002 |
| Acid detergent lignin | 66.4$^{a}$ | 56.9$^{ab}$ | 47.7$^{ab}$ | 44.3$^{b}$ | 4.56 | 0.0205 | 0.4418 | 0.0032 | 0.6120 |
| Hemicellulose | 154.3$^{a}$ | 40.7$^{b}$ | 61.3$^{b}$ | 91.1$^{b}$ | 18.14 | 0.0045 | 0.0125 | 0.0043 | 0.2686 |
| Cellulose | 90.7$^{a}$ | 95.3$^{a}$ | 81.4$^{a}$ | 54.5$^{b}$ | 5.11 | 0.0050 | 0.0057 | 0.0034 | 0.0029 |

[a,b]Means with common superscripts do not statistically differ ($P > 0.05$).
[1] SEM = standard error of the mean.
[2] p values: GLM = General linear model; C1 = contrasting the average response to SSF treatments *versus* the control; C2 = contrasting the average response to *A. oryzae* and *A. sojae* SSF treatments *versus* the co-culture treatment; and C3 = contrasting *A. oryzae* and *A. sojae* SSF treatment means.

camelina meal.

As expected, results from the present study showed a decrease in fibre content of marama beans upon fermentation. These results confirm reports by Olukomaiya et al. (2020c) who found that fermenting canola meal significantly reduced NDF content compared to non-fermented canola meal. Similarly, Olukomaiya et al. (2020a) found that SSF reduced the ADF content of lupin flour. The decrease in the ADF, hemicellulose, and cellulose fibre fractions could be attributed to the ability of fungi to degrade fibre during SSF. However, the effectiveness of fungal strains varied with the co-culture exhibiting higher potency than individual strains deployed separately. Indeed, marama beans fermented with the co-culture had the lowest ADF and cellulose compared to those fermented with individual strains (Table 1). This outcome suggests a synergistic interaction between the enzymes produced by the two strains, resulting in enhanced fibrolytic activity. Indeed, *A. oryzae* and *A. sojae* produce ligninolytic and cellulolytic enzymes such as cellobiohydrolases and laccases, which hydrolyse cellulose and lignin, respectively (Asemoloye et al., 2020; Irajie et al., 2016; Naz et al., 2020). These findings demonstrate that using the co-culture to ferment marama beans can produce a low-fibre substrate that can be used as a functional ingredient in food and feed formulations.

### 3.3. Antinutritional factors and in vitro digestibility

Preplanned contrast analysis showed that SSF significantly lowered ($p < 0.05$) phytic acid and trypsin inhibitors compared to the non-fermented beans (Table 2). The reduction in phytic acid content upon fermentation of substrate could be due to the ability of *A. oryzae* and *A. sojae* to synthesize the enzyme phytase (Olukomaiya et al., 2020a, 2020c). Phytic acid forms complexes with carbohydrates, proteins, and minerals, thus limiting their bioavailability and accessibility by digestive enzymes (Sharma and Singh, 2023). Hence, a reduction in the phytic acid content has the potential to increase the bioavailability of minerals in legumes such as marama beans. This is especially important given that simple non-ruminants and humans do not secrete the enzyme phytase. Sharma and Singh (2023) also reported reduced phytic acid content in cereal flours fermented with *A. oryzae*.

The beans fermented with *A. oryzae* had lower ($p < 0.05$) trypsin inhibitors compared to beans fermented with the co-culture. The potency of this strain against trypsin inhibitors has been reported by Chen et al. (2013), who observed trypsin inhibitor activity being reduced to nondetectable levels in soy meal upon SSF. However, it appears no studies have demonstrated the effectiveness of *A. sojae* in reducing trypsin inhibitors in legume grains. Trypsin inhibitors are naturally occurring proteins that block the activity of trypsin, a key digestive enzyme in animals and humans, thereby reducing protein digestibility (Chen et al., 2013) when legumes like marama beans are consumed raw. The decrease in trypsin inhibitors in fermented foodstuffs is due to microbial degradation during SSF (Terefe et al., 2021). However, no specific enzymes for the breakdown of trypsin inhibitors have been identified, though fungal proteolytic enzymes produced during SSF may be responsible. The trypsin inhibitor activity in marama beans remained relatively high (compared to raw soybeans) even after fungal fermentation. To address this, additional mitigation strategies could be used to enhance the nutritional value of this orphan legume. These could include extending SSF incubation times, use of proteolytic and composite enzymatic treatments, sprouting of whole marama seeds, and using chemical treatments (alkali or acid) to denature trypsin inhibitors. Additionally, long-term solutions such as selective breeding of low-trypsin inhibitor marama bean varieties and genetic modification through targeted gene editing could be explored. A combination of these strategies may provide synergistic effects for more effective trypsin inhibitor reduction.

Non-fermented (16.6 mg GAE/g DM) and co-culture-fermented (18.1 mg GAE/g DM) beans had lower total phenolic content compared to beans fermented with *A. oryzae* (28.1 mg GAE/g DM) and *A. sojae* (34.1 mg GAE/g DM) separately. The total phenolic content of marama beans fermented with individual fungal strains did not differ ($p > 0.05$). The finding that total phenolic content in beans fermented with either *A. oryzae* or *A. sojae* was significantly higher than in the non-fermented beans (Table 2) is consistent with a report by Liu et al. (2022), who observed an increase in total phenolics in soybean, chickpea, black bean, and mung bean after SSF with *Cordyceps militaris*. Other studies have also demonstrated that fermentation with *A. oryzae* increases the total phenolic content of pea protein isolates (Khorsandi et al., 2024; Kumitch et al., 2020); *Moringa oleifera* seed flour (Puspitasari et al., 2024), and chickpea, faba bean, and lentil protein isolates (Stone et al., 2024). The observed increase in phenolic content in fermented marama beans can be attributed both to the *de novo* synthesis of phenolics by the fungi and to the enzymatic liberation of fibre-bound phenolics from the substrate. Indeed, fungi have been reported to synthesize a variety of simple and complex phenolic compounds, which serve critical functions such as microbial defense, pigmentation, and protection against ultraviolet radiation (Liu et al., 2022). Phenolic acids in plant-based substrates are present in free and soluble conjugated forms and insoluble, fibre-bound compounds. During SSF, these bound phenolics can be released through enzymatic action, particularly by fibrolytic enzymes produced by fungi, thereby increasing the pool of solvent-extractable phenolics (Dey et al., 2016). However, marama beans fermented using the co-culture exhibited a reduced phenolic content relative to those fermented with individual fungal strains. This observation may be attributed to antagonistic interactions between the co-inoculated fungi (Sanneboyina and Audipudi, 2024), potentially mediated through competitive nutrient utilization or the synthesis of antifungal secondary metabolites. Nonetheless, current literature does not substantiate antagonistic interactions specifically between *A. oryzae* and *A. sojae*.

Contrary to our hypothesis, contrast analysis revealed that dry matter digestibility (DMD) and *in vitro* enzyme protein digestibility (IVPD) values were higher ($p < 0.05$) in non-fermented compared to fermented beans (Table 2). A comparison of fermented marama beans

**Table 2**
Phytic acid, trypsin inhibitor, and phenolics content and *in vitro* digestibility of marama beans subjected to *Aspergillus*-mediated solid-state fermentation.

| | | | | | | p values[3] | | | |
|---|---|---|---|---|---|---|---|---|---|
| Parameters[1] | Control | *A. oryzae* | *A. sojae* | Co-culture | SEM[2] | GLM | C1 | C2 | C3 |
| Phytic acid (mg/g DM) | 33.54$^{a}$ | 0.14$^{b}$ | 0.16$^{b}$ | 0.26$^{b}$ | 0.73 | <0.0001 | 0.0002 | <0.0001 | 0.9351 |
| Trypsin inhibitors (TUI/g DM) | 233.8$^{a}$ | 73.1$^{c}$ | 86.0$^{bc}$ | 100.1$^{b}$ | 4.44 | <0.0001 | 0.0002 | <0.0001 | 0.0895 |
| Total phenolics (mg GAE/g DM) | 16.6$^{b}$ | 28.1$^{a}$ | 34.1$^{a}$ | 18.1$^{b}$ | 2.30 | 0.0004 | 0.0746 | 0.0055 | 0.0004 |
| Dry matter digestibility (g/kg) | 696.6$^{a}$ | 607.5$^{c}$ | 630.9$^{bc}$ | 668.5$^{ab}$ | 9.65 | 0.0099 | 0.0065 | 0.0166 | 0.0512 |
| Protein digestibility (g/kg DM) | 641.8$^{a}$ | 460.7$^{b}$ | 536.1$^{b}$ | 631.6$^{a}$ | 13.45 | 0.0019 | 0.0008 | 0.0244 | 0.0074 |

$^{a,b}$Means with common superscripts do not statistically differ ($P > 0.05$).
[1] Parameters: GAE = gallic acid equivalent; TUI = trypsin unit inhibitors.
[2] SEM = Standard error mean.
[3] p values: GLM = General linear model; C1 = contrasting the average response to SSF treatments *versus* the control; C2 = contrasting the average response to *A. oryzae* and *A. sojae* SSF treatments *versus* the co-culture treatment; and C3 = contrasting *A. oryzae* and *A. sojae* SSF treatment means.

showed that *A. sojae* and *A. oryzae* produced beans with lower ($p < 0.05$) DMD and IVPD compared to co-culture-fermented beans. Non-fermented beans and co-culture-fermented beans had statistically similar DMD and IVPD values. The findings of the present study are similar to those reported by Olukomaiya et al. (2020a), who found that non-fermented lupin flour had higher ($p < 0.05$) protein digestibility compared to the fermented lupin flour. The reason for the lower digestibility of substrates fermented with individual fungal strains is unknown.

Antinutritional factors in foodstuffs reduce palatability and bioaccessibility of nutrients by increasing the gut viscosity and binding to nutrients, mucosal proteins, and digestive enzymes (Erdaw et al., 2016). Therefore, a reduction in some fibre fractions, phytic acid, and trypsin inhibitor activity suggest that SSF can improve the bioavailability of nutrients when animals or humans consume fermented marama beans. However, our results did not support this hypothesis, as contrast analysis revealed that *in vitro* enzyme protein digestibility of non-fermented (control) beans was higher than in fermented beans (Table 2). A possible explanation is that fermentation led to a higher retention of indigestible residual proteins, as fungal proteolytic enzymes hydrolyzed the more digestible protein fractions. Comparing fermented marama beans, those fermented with *A. sojae* or *A. oryzae* had lower protein digestibility than those with their co-culture, further confirming the co-culture's superior hydrolytic activity, as previously observed with ADF and cellulose content. We acknowledge that this study could have benefited from complementary analyses such as SDS-PAGE or a determination of the degree of hydrolysis to further elucidate the mechanisms underlying protein modifications during fermentation. However, IVPD is still the most pertinent metric that reflects the net effect of both proteolysis and any inhibitory factors that may limit the digestibility of marama bean, thereby providing a holistic assessment of protein utilization. The lack of expected correlations between phytic acid and trypsin inhibitor content and simulated protein digestibility of fermented marama beans requires further research. The higher levels of phenolics recorded in single-strain fermented marama beans could benefit animals and humans, as some of these compounds have putative antioxidant activity (Shahidi and Ambigaipalan, 2015). However, a study by Nyembwe et al. (2015) demonstrated that tannin phenolics in marama beans may negatively affect foodstuffs' sensory properties, palatability through astringency, as well as nutrient digestibility. Indeed, tannin phenolics may reduce the activity and effectiveness of secreted digestive enzymes by forming starch/protein-polyphenol complexes and V-amylose complexes (Han et al., 2020; Kan et al., 2022; Ngo et al., 2022; Romero Hernández et al., 2022). This could provide a plausible explanation for the lower protein digestibility observed in the single-strain fermented substrates that had relatively higher total phenolic content in the current study.

### 3.4. Functional properties

Marama beans did not vary ($p > 0.05$) in terms of bulk density across all groups (Table 3). These findings are contrary to those of Olukomaiya et al. (2020c), who found that bulk density was higher in fermented than in non-fermented canola meal. Furthermore, contrast analysis revealed no significant difference ($p > 0.05$) in oil absorption capacity (OAC) between the fermented and control beans, but co-culture fermentation resulted in lower OAC compared to individual strains. These findings are also contrary to those of Olukomaiya et al. (2020c), who found that OAC in fermented canola meal was higher than in non-fermented canola meal. The discrepancies between the current and previous studies could be attributed to the different substrates used across the two studies.

Contrast analysis revealed that SSF reduced ($p < 0.05$) the WAC of marama beans regardless of the fungal strain used. Beans fermented with the co-culture had the least WAC (16.6 %) while control beans had the highest value (73.9 %). These findings are similar to those made by Olukomaiya et al. (2020c) on the effects of SSF on the WAC of canola meal. The reduction in WAC upon fermentation points to an SSF-induced reduction in hydrophilic groups (Olukomaiya et al., 2020c) in marama beans, which could pose a challenge when the substrate is used in diets that require moisture treatment prior to pelleting. The drastic reduction in WAC in co-culture-fermented marama beans could be due to a significant reduction in their ADF and cellulose content, as already reported. The WAC of a substrate is often positively correlated with its fiber content. This relationship arises because dietary fibers, particularly insoluble fibers like cellulose, have a strong ability to bind and retain water. These fibers also have hydrophilic functional groups, which can form hydrogen bonds with water, enhancing absorption (Yamazaki et al., 2005). The dispersibility of non-fermented beans (18.3 %) was significantly ($p < 0.05$) lower than that in beans fermented with the co-culture (48.3 %). The mean dispersibility of beans fermented with individual strains, *A. sojae* and *A. oryzae*, was lower ($p < 0.05$) than in beans fermented with the co-culture. Dispersibility of *A. sojae*- and *A. oryzae*-fermented beans did not differ ($p > 0.05$) from that of non-fermented beans. These results contradict Olukomaiya et al. (2020b) and Olukomaiya et al. (2020c), who found that there were no SSF-induced variations in the dispersibility values of camelina meal and canola meal. The higher dispersibility in marama beans fermented with co-culture *versus* those fermented with single strains suggests a synergistic interaction between the two fungal strains, as observed for fibre and protein digestibility in the current study. Higher dispersibility could be due to SSF-induced disruption of the crystalline and double helical structure of starch granules, resulting in reorganization or modification (Adebiyi et al., 2016). Higher dispersibility is desirable as it promotes uniform dough formation during food or feed preparation and enhances protein digestibility by improving protein solubility (Awuchi et al., 2019). Accordingly, a positive association between dispersibility and protein digestibility of fermented substrate has been reported in canola meal (Olukomaiya et al., 2020a), but not in the present study.

## 4. Conclusions

This study demonstrated that co-culture fermentation with *A. sojae*

**Table 3**
pH and functional properties of marama beans fermented with *Aspergillus* strains.

| | | | | | | p values[2] | | | |
|---|---|---|---|---|---|---|---|---|---|
| Parameters | Control | *A. oryzae* | *A. sojae* | Co-culture | SEM[1] | GLM | C1 | C2 | C3 |
| pH (dried) | 5.66[a] | 5.74[a] | 5.70[a] | 5.80[a] | 0.05 | 0.3268 | 0.7052 | 0.1899 | 0.2108 |
| pH (fresh) | 6.52[a] | 6.21[a] | 6.12[a] | 6.40[a] | 1.00 | 0.0562 | 0.2489 | 0.0549 | 0.0683 |
| Water absorption capacity ( %) | 73.9[a] | 48.9[b] | 49.7[b] | 16.6[c] | 4.62 | <0.0001 | <0.0001 | <0.0001 | 0.8990 |
| Bulk density (g/mL) | 0.50[a] | 0.52[a] | 0.50[a] | 0.52[a] | 0.007 | 0.1038 | 0.1356 | 0.2800 | 0.0737 |
| Oil absorption capacity ( %) | 54.7[a] | 45.6[ab] | 51.6[ab] | 33.4[b] | 4.73 | 0.0355 | 0.0637 | 0.0220 | 0.3878 |
| Dispersibility ( %) | 18.3[b] | 43.3[ab] | 38.3[ab] | 48.3[a] | 6.14 | 0.0233 | 0.2629 | 0.0061 | 0.2721 |

[a,b]Means with common superscripts do not statistically differ ($P > 0.05$).

[1] SEM = Standard error mean.

[2] p values: GLM = General linear model; C1 = contrasting the average response to SSF treatments *versus* the control; C2 = contrasting the average response to *A. oryzae* and *A. sojae* SSF treatments *versus* the co-culture treatment; and C3 = contrasting *A. oryzae* and *A. sojae* SSF treatment means.

and *A. oryzae* was more effective than single-strain fermentation in reducing fiber fractions, increasing total protein content, and improving the dispersibility of marama beans. However, both the co-culture and single strains had similar effects on the beans' proximate composition and some antinutritional factors (phytic acid and trypsin inhibitors). Co-culture fermentation had no significant impact on IVPD, whereas single-strain fermentation significantly reduced it. Despite reductions in anti-nutritional factors (including fibre fractions) and improved dispersibility, these changes did not translate into higher IVPD for the fermented marama beans.

## Declaration of generative AI in scientific writing

During the preparation of this work, the authors used generative AI to improve readability and language use in some parts of the paper. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

## Ethics statement

This study did not use human or animal subjects.

## CRediT authorship contribution statement

**Joel Gbenle:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Marlin Mert:** Writing – review & editing, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Nkateko N. Phasha:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Formal analysis. **Molatelo Junior Madibana:** Writing – review & editing, Supervision, Resources, Investigation, Funding acquisition. **Freddy Manyeula:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Oluwaseun Peter Bamidele:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Rashieda Toefy:** Writing – review & editing, Resources, Methodology, Funding acquisition. **Siphosethu Richard Dibakoane:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis. **Victor Mlambo:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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