Germination alters the microstructure, in vitro protein digestibility, α-glucosidase and dipeptidyl peptidase-IV inhibitory activities of bioaccessible fraction of pigeon pea (Cajanus cajan) seeds

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Abstract

Germination was investigated as a bioprocess for enhancing the digestibility and bioactivity of pigeon pea (Cajanus cajan). Pigeon pea seeds were germinated for 0, 4, 24 and 48 h. Electrophoresis revealed that the 7S vicilin subunits were the most abundant proteins in control and germinated seeds. FTIR showed a loss of β-sheet and a gain of α-helix contents, and microscopy showed cell wall degradation in germinated seeds. Germination decreased the seed protein yield due to partial hydrolysis of proteins. After in vitro digestion, 48-h germination increased the protein digestibility-corrected amino acid score of the pigeon pea flour and isolated protein. Germination also enhanced the inhibitory activity of the seed digesta against α-glucosidase but not dipeptidyl peptidase IV. Taken together, germination could be used to enhance the nutritional quality and bioactivity of pigeon pea towards improving its future utilization as novel healthy food for mitigating food insecurity.

KEYWORDS
amino acid composition, Cajanus cajan germination, dipeptidyl peptidase-IV, PDCAAS, pigeon pea, protein quality, α-Glucosidase

1 INTRODUCTION

Food insecurity is a global menace mostly caused by a limited availability of safe and nutritionally balanced food in a population. Thus, there is a need for diversification of food sources to include neglected and underutilized plants. Pigeon pea (Cajanus cajan) is an underutilized legume with a potential to contribute in achieving food security. Pigeon pea seeds contain up to 24% proteins on a dry weight basis (Singh, 2016), and the proteins are rich in lysine (Boachie et al., 2019; Krishnan et al., 2017), the limiting amino acids of cereals. However, as with other legume proteins, the digestibility and bioaccessibility of pigeon pea seed proteins are impeded by the complex microstructure and presence of interfering compounds in the seed matrix. Some inhibitors of legume protein digestibility, such as phytate, tannins, and protease inhibitors (Becker & Yu, 2013), can be reduced or removed by food processing, thus improving protein accessibility. For instance, the in vitro protein digestibility increased in legume seeds subjected to microwave treatments (Drulyte &
Orlien, 2019; Sun et al., 2020), fermentation (Çabuk et al., 2018; Di Stefano et al., 2019) and germination (Ohanenye et al., 2020; Sangronis & Machado, 2007). The bioprocesses, fermentation and germination, are inexpensive and effective at improving the quality of legume proteins (Ohanenye et al., 2020; Varnosfaderani et al., 2019). Fermentation depends on extrinsic factors, such as bacteria, to initiate changes whereas germination is an innate phenomenon in seed development.

Germination involves a cascade of biochemical activities causing changes in protein content by activating the transcriptional machinery of dry seeds (Ohanenye et al., 2020; Weitbrecht et al., 2011). Carbohydrate remobilization and metabolism have been central to many seed germination studies because it serves as the primary source of energy. Remobilization of seed proteins provides the amino acids needed for protein synthesis, although it can also occur in response to energy demands of germinating seeds (Angelovici et al., 2011). Prior to germination, seeds imbibe water (i.e., soaking), which triggers the biochemical reactions associated with germination (Ohanenye et al., 2020; Wolny et al., 2018). Soaking for up to 6 h was reported to increase the soluble protein and amino acid contents of mung bean seeds (Widjajaseputra et al., 2019), whereas in pigeon pea seed flour protein solubility was not influenced by soaking (Sun et al., 2020). After soaking and the associated biochemical processes, germination occurs when the radicle protrudes through the seed testa.

Germination of various legume seeds, such as chickpea (Cicer arетinium L.), lentil (Lens culinaris Merr.) and yellow pea (Pisum sativum L.), over a 6-day period increased their protein contents (Xu et al., 2019). However, in pigeon pea (Cajanus cajan), germination was reported to decrease the crude protein content (Oloyo, 2004), whereas prolonged germination increased protein solubility (Sharma, Singh, Singh, 2019b). These findings present germination as a bioprocessing method with a potential to influence the protein content of legume seeds. Equally important is the protein quality, which is defined by many factors such as the absorption, the post-digestion utilization of specific amino acids, the rates of obligatory oxidation and the indispensable amino acid composition (Friedman, 1996). Germination was reported to selectively increase the methionine content of germinated soybean seeds (Mora-Escobedo et al., 2014) and the valine, leucine, isoleucine and methionine contents of germinated lupin seeds (Chilomer et al., 2010; Martinez-Villaluenga et al., 2010). It is unclear how the germination-induced amino acid changes affect the protein secondary structure and protein chemistry of the legume seeds. Germination also increased the protein bioaccessibility and bioactivity profile of legume seeds after in vitro digestion (Di Stefano et al., 2019; Sangronis & Machado, 2007), thus making it suitable as a tool for enhancing the nutritional and health-promoting properties of legumes such as pigeon pea seeds.

Diabetes is a public health concern with a global morbidity estimated to rise to 10.4% by 2040 (WHO, 2016). Type 2 diabetes mellitus, representing ~90% of total diagnosed diabetes cases, is linked to dietary and environmental factors (Evans et al., 2000). People with type 2 diabetes have glucose intolerance and difficulty with blood glucose homeostasis (Page & Reisman, 2013). Glucose metabolism has been targeted in the development of many anti-diabetic drugs and natural products (Kerr et al., 2018; Salehi et al., 2019).

In humans, glucose metabolism is regulated by the activities of α-amylase, α-glucosidase and dipeptidyl peptidase (DPP)-IV. α-Amylase and α-glucosidase are directly involved in the catabolism of dietary carbohydrates and oligosaccharides, respectively, to release glucose, whereas DPP-IV degrades the incretin hormones, glucose-dependent insulinotropic peptide and glucagon-like peptide, thereby inhibiting glucose metabolism (Di Stefano et al., 2018; Lacroix & Li-Chan, 2016). Therefore, α-glucosidase and DPP-IV inhibitors can be used to lower the blood glucose level of people with type 2 diabetes (Di Stefano et al., 2018; Lacroix & Li-Chan, 2016). A previous study reported that germination of common legume seeds altered the seed microstructure and enhanced the α-glucosidase and DPP-IV inhibitory activities of the digested samples (Di Stefano et al., 2019). Moreover, germination was reported to influence the in vitro antioxidant capacity (Sharma, Singh, Singh, 2019a) and functional properties of pigeon pea seed flours (Acevedo et al., 2017). There is a dearth of information on germination-induced changes in the seed protein properties, in vitro protein digestibility and bioaccessibility and bioactivities related to glucose metabolism.

Therefore, it was hypothesized that germination will alter pigeon pea seed microstructure, secondary protein structure and amino acid composition, thus increasing the in vitro protein digestibility (protein digestibility-corrected amino acid score, PDCAAS) and inhibitory activities of the digesta against glucose-metabolizing enzymes (α-glucosidase and DPP-IV). These effects hold potential benefits towards improving the utilization of pigeon pea seeds and the production of novel healthy food products.

### 2 | MATERIALS AND METHODS

#### 2.1 | Materials and chemicals

Pigeon pea seeds (Cajanus cajan) were purchased from a vendor at the New Relief Market (Enugu, Nigeria). Precision Plus Protein dual colour standards were purchased from BioRad (Mississauga, ON, Canada), GelCode Blue Safe Protein Stain was purchased from Fisher Scientific (Toronto, ON, Canada). Pepsin (from porcine gastric mucosa, ≥250 units mg⁻¹ solid), α-amylase from porcine pancreas, pancreatin (from porcine pancreas, 8xUSP specification), calcium chloride, di-Na-tetraborate decahydrate, sodium dodecyl sulphate (SDS), DL-dithiothreitol (DTT), o-phthalaldehyde (OPA), L-serine, hydrochloric acid (37%), ethylenediaminetetraacetic acid (EDTA), glycine, ura solution BioUltra (8 M in water), β-mercaptoethanol, bile extract, bromophenol blue, α-glucosidase, DPP-IV, diprotin A and acarbose were purchased from Millipore Sigma (Burlington, MA, USA). Milli-Q water was prepared using the Milli-Q water supply system (Millipore Corporation, Billerica, MA, USA).
2.2 | Germination of pigeon pea and sampling

Pigeon pea seeds (10 g) were sterilized for 10 min with 70% (v/v) ethanol and then rinsed three times with sterile distilled water. The seeds were then placed in a sterile petri dish and ~3 ml of distilled water was added to each of the petri dishes, enough to cover the seeds. The petri dishes were covered and placed in an incubator protected from light with temperature set at 25°C. The seeds were rinsed with distilled water every 24 h for the duration of the experiment, and ~2 ml of distilled water was added to the petri dishes, enough to cover half of the swollen seeds size. Samples were collected at four different time points: (i) after washing (Time 0; control), (ii) 4 h after soaking, (iii) 24 h and (iv) 48 h in accordance with previous studies (Dukan et al., 1999, 2001). Seeds from the sampling points were frozen at −80°C, freeze-dried and pulverized for further analysis. The germination experiment was conducted in triplicate.

2.3 | Imaging of pigeon pea seeds and protein microstructures by scanning electron microscopy

To understand the impact of germination on the seed microstructure, pigeon pea seeds from the control, 4-h soaking and 24- and 48-h germination were cut in a transverse section with a scalpel into a thin sliced layer (~1 mm). The thin sliced layer was imaged by scanning electron microscopy (SEM) microscopy to view the seed microstructure using a Phenom ProX Desktop SEM system (Thermo Fisher Scientific, Einhoven, Netherlands). Images were obtained using a high sensitivity multi-mode backscatter electron (BSE) detector at 10 kV. Samples, which were not fixed or coated, were placed on a carbon disk stuck to an aluminium stub and placed on the ‘Charge Reduction Sample Holder’. SEM images were acquired at 820X magnification.

2.4 | In vitro digestion of pigeon pea samples for assessment of protein digestibility

In vitro simulated gastrointestinal digestion was conducted according to the consensus three-phase in vitro digestion model described (Minekus et al., 2014), with modifications. In the oral phase, 1 g of pulverized flour was mixed with 1 ml of the simulated salivary fluid and salivary α-amylase (300 U/ml), and the mixture was incubated for 2 min at pH 7.0. In the gastric phase, the resulting 2 ml bolus was mixed with 2 ml of the simulated gastric fluid and porcine pepsin (5,000 U/ml), and the mixture was incubated with continuous shaking (120 rpm) for 2 h, with the pH kept at 3.0. In the intestinal phase, an equal amount of the simulated intestinal fluid, pancreatin (100 trypsin U/ml) and bile extract (10 mM) was added to the resulting gastric chyme (4 ml), and the pH was raised to 7.0 before incubation for 2 h with continuous shaking (120 rpm). The digestion process was performed at 37°C. After digestion, enzymatic activities were halted by vortex mixing and immediate transfer of the samples into a freezer (~20°C). The samples were then freeze dried to obtain the digesta powder.

2.5 | Determination of the free amino nitrogen content and degree of hydrolysis

The germinated seed flour and their in vitro digesta were sequentially diluted to 1 mg/ml with Milli-Q water followed by centrifugation (Centrifuge 5,430 R, Eppendorf, Germany) at 10,000 g for 5 min at 23°C to collect the supernatant for free α-amino group determination (Nielsen et al., 2001). Resulting residues were discarded. Supernatants obtained from the digesta represent the bioaccessible fraction of the digested samples. OPA reagent consisted of di-Na-tetraborate decahydrate, SDS, OPA, ethanol, DTT and Milli-Q water (Nielsen et al., 2001). A 30-μl aliquot of the sample supernatant, standard (serine, 0.1 mg/ml) or blank (Milli-Q water) was mixed with 225 μl of OPA reagent in a 96-well microplate. The mixture was incubated for 2 min at 23°C with shaking and the absorbance (A) was read at 340 nm with a Spark multimode microplate reader (Tecan, Switzerland). Degree of hydrolysis (%) and free amino nitrogen content (milliequiv serine-NH₂/g sample) were calculated as previously reported (Nielsen et al., 2001).

2.6 | Amino acid composition analysis

The flour digests from the control and 48-h germinated seeds were centrifuged at 1,792 g for 5 min after which the supernatant was collected, frozen at ~80°C, freeze-dried and pulverized. Amino acid composition of the pulverized digesta and undigested proteins from the control and the 48-h germinated seeds was analysed at the SPARC BioCentre, The Hospital for Sick Children (Toronto, Canada) as previously reported (Mohan & Udenigwe, 2015) using the Pico-Tag method. Samples were first hydrolysed using the vapour phase of 6 M HCl/1% phenol at 110°C for 24 h under nitrogen. For analysis of cysteine residues, samples were dissolved in 88% formic acid and incubated at 55°C for 5–10 min, followed by addition of 100 μl of freshly prepared performic acid solution and heating at 55°C for 30–45 min. Samples were vacuum dried and then hydrolyzed as above. For analysis of tryptophan residues, samples were hydrolyzed with 4.2 N NaOH at 110°C for 24 h under nitrogen. For all analysis, sample hydrolysis was followed by pre-column derivatization with phenyl isothiocyanate and reverse-phase liquid chromatography using a Waters ACQUITY UPLC (Milford, MA, USA). The percentage content of each amino acid was recorded and used for in vitro PDCAAS calculations, where the degree of hydrolysis of the samples was multiplied by their amino acid scores (AAS) as previously reported (Nosworthy et al., 2017).

2.7 | Extraction and characterization of pigeon pea seed proteins

2.7.1 | Seed protein isolation

Pigeon pea proteins were isolated as reported (Adebiyi & Aluko, 2011), with minor modification. Briefly, pigeon pea flour (control and 48-h
germinated) was suspended in 0.25 M NaOH (10%, w/v) and stirred for 4 h to extract the proteins. The supernatant was recovered by centrifuging at 18,000 g for 45 min at 4 °C (Lynx 4,000, Thermal Fisher Scientific, Waltham, MA, USA), followed by adjusting the pH to 4 using 3 M HCl to precipitate the proteins. After centrifugation, the precipitate was collected, suspended in Milli-Q water and adjusted to pH 7 using 3 M NaOH. Finally, the isolated proteins were lyophilized and stored at −20 °C until further analysis. Protein assay was performed using the DC (detergent compatible) following the manufacturer’s instruction (Bio-Rad, CA, USA). The protein isolation yield (Yi) was calculated as

\[ Y_i(\%) = \frac{M_p}{M_0} \times 100. \]  

Here, \( M_p \) is the mass (g) of the isolated protein, and \( M_0 \) (g) is the mass of the pigeon pea flour. The protein yield (Yp) was calculated as

\[ Y_p(\%) = Y_i \times \% P. \]  

Here, \% P is the percent protein content of the isolated protein.

The isolated proteins were also subjected to in vitro digestion and their amino acid compositions determined as described previously.

### 2.7.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyse the profiles of the soluble proteins of pigeon pea seeds using stacking gel of 3% concentration (w/v, pH 6.8) and a gradient separating gel at 18%, 15%, 12%, 9% and 6% (w/v, pH 8.8). The pigeon pea seed proteins samples (at 20 mg/ml) were mixed with a sample buffer at 1:1 (v/v), which composed of 3%, 2.5%, 2%, 1.5% and 1% SDS for the 18%, 15%, 12%, 9% and 6% gradient gels, respectively; 0.5% bromophenol blue; 62.5 mM Tris–HCl (pH 6.8); 20% glycerol and 5% β-mercaptoethanol. The protein samples were heated at 95 °C for 5 min after which the samples were cooled on ice prior to loading. A 10-μl aliquot of the standard molecular weight marker and protein samples (at 10 mg/ml) were loaded into the sample wells. The SDS-PAGE running voltage was set at 120 V on a BioRad Mini-PROTEAN Tetra Cell electrophoresis unit (Mississauga, ON, Canada). To view the protein bands, GelCode™ Blue Safe Protein Stain (Fisher Scientific, Toronto, ON, Canada) was used to stain the protein bands. Afterwards, the gel was de-stained by placing it in Milli-195 Q water overnight (~12 h), and bands were imaged using a ChemiDoc Imaging System (BioRad Inc., Quebec, Canada). The protein band intensity was quantified using an open source software ImageJ 1.x (NIH, USA).

### 2.7.3 Fourier transform infrared spectroscopic analysis

Fourier transform infrared (FTIR) spectrometer (Nicolet 6,700 FTIR, Thermo Fisher Scientific, Waltham, MA USA) was used to compare the structures of proteins isolated from the control and 48-h germinated seeds. The spectra of the samples were obtained at 4,000 to 500 cm⁻¹ after subtracting background in the absorbance mode. Afterwards, the overlapping bands were separated into multi-component peaks using the PeakFit v4.12 software (SeaSolve Software Inc., Framingham, MA, USA) to smoothen the baseline and for Gaussian deconvolution and second derivation of FTIR spectra in the amide I region (1,600–1,700 cm⁻¹; Sun et al., 2020). Then, the resulting peaks were resolved into their corresponding types of protein secondary structures as previously reported (Qian et al., 2016). Specific conformations were assigned in the following order: β sheet (1,610–1,640 cm⁻¹), random coil (1,641–1,648 cm⁻¹), α-helix (1,656–1,658 cm⁻¹), turn (1,665–1,670 cm⁻¹) and β antiparallel (1,680–1,692 cm⁻¹).

### 2.8 Glucose metabolizing enzyme inhibition assays

#### 2.8.1 α-Glucosidase inhibition assay

Inhibition of yeast α-glucosidase was evaluated using the method of (Lalegani et al., 2018), with modifications. Briefly, 150 μl of α-glucosidase (0.4 U/ml) in 0.1 M phosphate buffer (pH 6.9) was mixed with 250 μl of the bioaccessible part of the pigeon pea digesta and incubated at 37 °C for 5 min. After pre-incubation, 100 μl of 5 mM PNPG solution in 0.1 M phosphate buffer (pH 6.9) was added and the mixture incubated at 37 °C for 30 min. Absorbance of the solution was measured every 2 min at 405 nm. For the control assay, the phosphate buffer solution was used instead of the sample solution. Inhibition of α-glucosidase was calculated as

\[ \text{Enzyme inhibition} (\%) = \frac{Ac − As}{Ac} \times 100. \]  

As and Ac represent the slopes of the absorbance curves for the samples and control, respectively. A standard curve was prepared with 1–250 μg/ml acarbose (standard inhibitor) and the inhibitory activity of the samples reported as μg acarbose equiv. per mg of flour. Also, the half-maximal inhibitory concentration (IC50) of acarbose was determined.

#### 2.8.2 Dipeptidyl peptidase-IV inhibition assay

DPP-IV inhibition was determined following the procedure reported (Nongoniema & Fitzgerald, 2013). Briefly, 50 μl of sample solution was mixed with 50 μl of Gly-Pro-pNA (substrate; 0.2 mM) and incubated for 10 min at 37°C. The reaction was started by the addition of 50 μl of DPP-IV (final concentration 0.0025 units/ml) prepared in Tris–HCl buffer (100 mM, pH 8). The reaction continued for 1 h at 37°C, and absorbance of the released p-nitroaniline was measured at 405 nm. Enzyme inhibition (%) and the IC50 of diprotin A (standard inhibitor) were determined as shown in 2.8.1. A standard curve was
prepared with 1–50 μg/ml diprotin A and the inhibitory activity of the samples reported as μg diprotin A equiv. per mg of flour.

2.9 | Statistical analysis

Statistical analyses were conducted using Genstat for Windows 12th Edition (VSN International Ltd, Herts., UK). Analysis of variance (ANOVA) was performed to identify factors that significantly affected variance in the analysed properties. ANOVA was performed on the data specifying a nested treatment structure of a common baseline (non-germinated/control seeds before soaking and germination). Least significant difference (LSD) values were calculated from each analysis, for comparison of appropriate treatment means, using general analysis of variance. A significance threshold of $P < 0.05$ was adopted, for all analyses. SigmaPlot for Windows SPW11 (Systat Software, Inc., London, UK) was used to plot the graphs. Except for SEM and FTIR, which were conducted on pulled replicate samples, all the experiments were conducted in triplicate ($n = 3$).

3 | RESULTS AND DISCUSSION

3.1 | Germination changed the pigeon pea seed microstructure

SEM was used to evaluate the microstructures of the non-germinated (control), soaked (4-h) and germinated (24-h or 48-h) pigeon pea seeds. SEM image showed that the microstructure of control seed cotyledon was compact (Figure 1A). However, the seed microstructure started changing after 4 h of soaking, such that the membrane covering the cell bodies became more visible, but intact (Figure 1B), compared to the control seed. This feature is supported by Bewley (1997), who reported that imbibition caused structural changes, especially to the cell membranes. For the seeds germinated for 24 h (Figure 1C), the membrane became more transparent with the appearance of empty cell compartments. Unlike the soaked seeds, germination caused noticeable degradation of the cell compartments (Figure 1C,D), which became more pronounced in the seeds germinated for 48 h (Figure 1D).

SEM imaging of the control pigeon pea seed showed a compact structure of the starch granules and protein bodies, as previously reported in the SEM images of other legume seeds (Sun et al., 2020; Swanson et al., 1985). The disruption of the cell wall compartments from imbibition (4-h soaking) to germination, depicted by sprout emergence (48-h), could be because of a direct activity of seed hydrolases or a collapse from the degradation and emptying of the cell macromolecules through remobilization. Recently, Di Stefano et al. (2019) reported a similar result where germination partially degraded the microstructures of yellow pea and green lentil seeds. Germination for 4 and 24 h slightly decreased the net free amino nitrogen content (Table 1), but the value increased at 48 h of germination. This effect could be due to protein degradation in the germinated pigeon pea seeds. Seed germination occurs in three phases, namely, imbibition,
reactivation of metabolism and protrusion of radicle (Bewley et al., 2013). These germination phases are characterized by the reactivation of metabolism and the release of hydrolytic enzymes for remobilizing the seed reserve energy store (Ohaneny et al., 2020). This process may have facilitated the emptying of cell compartments in the germinated pigeon pea seeds. Cell wall degradation may have resulted from the metabolism of the reserve nutrients or occurred to grant access to the hydrolyses. Other studies have reported similar findings, especially the degradation of the protective membrane from the soaked seeds through to protrusion of the radicle (Gómez-Maqueo & Gamboa-deBuen, 2016; Swanson et al., 1985).

3.2 | In vitro protein digestibility of the pigeon pea seed flours

Simulated gastrointestinal in vitro digestion of flours from the control, 4-h soaked and 24- and 48-h germinated pigeon pea seeds was used to determine the effect of the seed matrix on protein digestibility. In vitro degree of hydrolysis measures protein digestibility. As shown in Table 1, germinating the pigeon pea seeds for 48 h caused a 32.4% increase in the flour protein digestibility compared to the control. No significant differences in digestibility were found between the other treatments and the control, which is in agreement with previous studies (Sangronis & Machado, 2007). Legume seed proteins have reduced digestibility when compared to animal proteins because of antinutritional factors present in the seed matrix (Gilani et al., 2012; Ohaneny et al., 2020). The antinutrients form insoluble complexes with the seed proteins, inhibit proteolytic enzymes or prevent protein uptake (Becker & Yu, 2013; Ohaneny et al., 2020). Food processing methods such as soaking, cooking and fermentation have been reported to improve digestibility of legume proteins (Di Stefano et al., 2019). Germination also reduces the activities of antinutritional factors (Chilomer et al., 2010; Sofi et al., 2019), thus removing the inhibitors of protein digestibility. Furthermore, the improved protein digestibility of the 48-h germinated pigeon pea seeds may be because of the effects of hydrolyses released during germination, which caused partial hydrolysis of the proteins prior to the in vitro digestion (Table 1).

To further evaluate the nutritional benefits of the germinated pigeon pea seed flours, the AAS and in vitro PDCAAS were used in the assessment of protein digestibility (FAO/WHO, 1991). Amino acid composition of the bioaccessible part of the in vitro digesta of pigeon pea seed flour is shown in Table 2. Germination increased the total bioaccessible amino acids by 18% compared to the control, with increases in the bioaccessible amounts of both the non-essential and essential amino acids. Chilomer et al. (2010) and Martínez-Villaluenga et al. (2010) reported increases in the essential amino acid content (valine, leucine, isoleucine and methionine) of two germinated lupin seeds, with concomitant decrease in non-essential and some other essential amino acid contents. As shown in Table 3, calculated AAS indicated that methionine + cysteine were the first limiting amino acids (Schwab, 2011) for both the control and germinated pigeon pea seeds whereas threonine and lysine were the second and third limiting amino acids, respectively.

Germination for 48 h caused a slight change in the AAS, with a small increase in the first limiting AAS. This change resulted in a 37.7% increase in the in vitro PDCAAS of the pigeon pea seed flour (Table 3). The sulphur-containing amino acids increased by 62.8% in the seeds germinated for 48 h compared to the control. Higher contents of the sulphur-containing amino acids have been reported for the embryos compared to the cotyledons of pigeon pea and chickpea seeds (Singh & Jambunathan, 1982). Thus, the embryo growth during germination may be responsible for this enrichment in the pigeon pea seeds. The increased in vitro PDCAAS could also be due to the increased degree of hydrolysis following partial digestion of the seed proteins during germination. Taken together, germination improved the protein quality of pigeon pea seed flours, but the in vitro PDCAAS of the bioprocessed legume is still considered low for nutritional purposes.

3.3 | Germination influence on protein content, yield and protein profile of pigeon pea seeds

Pigeon pea proteins were isolated to evaluate the effect of germination on the protein profile and properties. Pigeon pea seed proteins are made up of globulins (54–60%), albumins (10–15%), glutelins (10–15%) and prolamins (4–5%; Krishnan et al., 2017; Singh & Jambunathan, 1982). As shown in Figure 2, SDS-PAGE showed that the two major bands in all the samples were found at 64 and 47 kDa, which correspond to 7S vicilin subunits (Krishnan et al., 2017). Compared to the control, the total band intensity was decreased in all the germinated samples, with the lowest value (—11.8%) observed for the 4-h soaked seeds. Particularly, the seed germination caused 3.6–4.7% and 2.5–20.7% decreases in intensity of Band b (47 kDa) and band e (16 kDa). In contrast, there was a 3.3–23.7% increase in intensity of the 64 kDa band. Furthermore, the 32 and 18 kDa bands (c and d) increased by 17.1–19.8% and 8.7–10.5%, respectively, in the proteins isolated from germinated seeds. These bands correspond to the cysteine-rich subunits of basic 7S globulin known as γ-protein, which is synthesized in developing seeds (Krishnan et al., 2017). This protein

### Table 1

| Sample | FAN content | In vitro DH (%) |
|--------|-------------|-----------------|
| Control | 202.8 ± 6.4<sup>b</sup> | 41.1 ± 4.9<sup>b</sup> |
| 4 h     | 185.7 ± 6.6<sup>c</sup> | 45.6 ± 2.9<sup>b</sup> |
| 24 h    | 181.2 ± 3.7<sup>c</sup> | 49.5 ± 2.2<sup>b</sup> |
| 48 h    | 213.7 ± 2.5<sup>a</sup> | 54.4 ± 2.3<sup>a</sup> |

Note. Control, non-germinated seeds; 4 h, seeds soaked for 4 h; 24 h, seeds germinated for 24 h; 48 h, seeds germinated for 48 h; FAN, free amino nitrogen content (milliequiv Ser-NH<sub>2</sub>/g protein); DH, degree of hydrolysis. Different letters in each column indicate significantly different mean values (P < 0.05).
may have contributed to higher amount of bioaccessible cysteine and corresponding AAS of the 48-h germinated seed flour (Tables 2 and 3). Protein isolation yield of the pigeon pea seeds decreased continuously from soaking to germination for 48 h (Table 4). The endogenous contents of germinating seeds leak into the surrounding medium as a result of rapid water uptake (Bewley, 1997; Bewley et al., 2013), and this may be responsible for the progressive reduction in the protein content recovered from the soaked to the germinated seeds. Oloyo (2004) also reported a decrease in the crude protein content in germinated pigeon pea seeds. Protein content of the seeds did not change after the 48-h germination. However, the protein yield decreased by 21% ($P < 0.05$) after germination (Table 4). This may be due to the germination-related degradation of seed storage proteins (Bewley, 1997; Ohanenye et al., 2020). With no roots and leaves for nutrients uptake and production, the energy required for germination must come from within the seed (Bewley, 1997) and released by the hydrolases, which are suppressed in dormant seeds (Ohanenye et al., 2020).

### TABLE 2

| Amino acid | Control protein (μg/mg sample) | % | 48 h germinated seeds protein (μg/mg sample) | % |
|------------|---------------------------------|---|----------------------------------------|---|
| Non-essential amino acids (NAA) | | | | |
| Asx#       | 23.92                           | 10.78 | 28.39 | 10.83 |
| Glx##      | 46.9                             | 21.13 | 53.47 | 20.39 |
| Ser        | 11.36                            | 5.12  | 13.79 | 5.26  |
| Gly        | 7.26                             | 3.27  | 8.43  | 3.22  |
| Arg        | 14.6                             | 6.58  | 17.24 | 6.57  |
| Ala        | 8.86                             | 3.99  | 10.59 | 4.04  |
| Pro        | 10.2                             | 4.60  | 12.27 | 4.68  |
| Tyr        | 6.94                             | 3.13  | 8.23  | 3.14  |
| Cys        | 0.88                             | 0.40  | 1.18  | 0.45  |
| Essential amino acids (EAA) | | | | |
| His        | 8.11                             | 3.66  | 9.27  | 3.54  |
| Thr        | 6.72                             | 3.03  | 8.05  | 3.07  |
| Val        | 9.83                             | 4.43  | 11.98 | 4.57  |
| Met        | 2.78                             | 1.25  | 3.32  | 1.26  |
| Ile        | 8.76                             | 3.95  | 10.88 | 4.15  |
| Leu        | 17.22                            | 7.76  | 21.68 | 8.27  |
| Phe        | 21.65                            | 9.75  | 24.26 | 9.25  |
| Lys        | 12.38                            | 5.58  | 15.99 | 6.10  |
| Trp        | 3.54                             | 1.60  | 3.21  | 1.23  |
| NAA        | 130.92                           | 58.99 | 153.59 | 58.56 |
| EAA        | 90.99                            | 41.01 | 108.64 | 41.44 |

Note. Asx# = (Asp + Asn); Glx## = (Glu + Gln).

### TABLE 3

| Amino acid score (AAS) | Flour | 48-h germinated |
|------------------------|-------|-----------------|
|                        | Thr   | Met+Cys | Val | Ile | Leu | Phe + Tyr | His | Lys | Trp | In vitro PDCAAS |
| Control                | 0.89  | 0.66    | 1.27 | 1.41 | 1.18 | 2.04     | 1.93 | 0.96 | 1.45 | 26.5 ± 2.3 c |
| 48-h germinated        | 0.90  | 0.68    | 1.31 | 1.48 | 1.25 | 1.97     | 1.86 | 1.05 | 1.12 | 36.5 ± 1.5h   |
| Isolated protein       | 1.30  | 0.80    | 1.25 | 1.06 | 0.78 | 1.30     | 2.09 | 0.46 | 1.07 | 30.4 ± 0.3c  |
| 48 h germinated        | 1.05  | 1.69    | 1.29 | 1.36 | 1.03 | 2.00     | 1.69 | 1.06 | 1.52 | 70.0 ± 0.8a  |

In vitro PDCAAS, protein digestion-corrected amino acid score (PDCAAS) = AAS × degree of hydrolysis (%). AAS = FAO reference of first limiting amino acid (mg/g) / measured amount of same amino acid (mg/g protein). FAO reference (mg/g protein): Thr, 3.4; Met+Cys, 2.5; Val, 3.5; Ile, 2.8; Leu, 6.6; Phe + Tyr, 6.3; His, 1.9; Lys, 5.8; Trp, 1.1. Bolded = first limiting amino acids.
3.4 Germination influence on amino acid composition, secondary structure and digestibility of the isolated proteins

FTIR spectra for the proteins isolated from control and 48-h germinated pigeon pea seeds showed the presence of seven peaks between 1,613 to 1,685 cm\(^{-1}\) (Figure S1), and these were resolved to their corresponding secondary structural properties (Qian et al., 2016). After quantification, a loss of 4% \(\beta\)-sheet and a gain of 5% \(\alpha\)-helix contents were found in the isolated proteins from the 48-h germinated seeds compared to proteins from control seeds. No differences were found for the contents of \(\beta\)-antiparallel, random coil and turn structures in the two samples. Amino acid composition differed between the proteins; for instance, cysteine increased by 2.2 folds and lysine increased by 1.7 folds after 48-h germination (Table 5). Such changes have important structural implications especially for cysteine, which may be involved in the folding and stability of the protein structure through the formation of disulphide bonds.

Loss in \(\beta\) sheet and a gain in \(\alpha\)-helix contents in the 48-h germinated seed proteins meant a change in the protein structures because of degradation or protein composition due to de novo synthesis during germination (Rajjou et al., 2008). The \(\alpha\) helix and \(\beta\) sheets play different roles in protein–protein interactions, protein quaternary structure and peptide/protein aggregation (Boyle, 2018). The increase in the \(\alpha\)-helix content may be related to the influence of environmental changes to the germinating seed proteome (Bartlett & Whipple, 2013; Penfield & MacGregor, 2017). Conversely, the lower \(\beta\)-sheet contents may have resulted from the transition of storage proteins to non-storage proteins through remobilization associated with germination (Bewley & Black, 1994; Kesari & Rangan, 2011).

\(\beta\) sheets are structurally more stable than the \(\alpha\) helices; thus, their changes due to germination may also affect the behaviour of the proteins during thermal food processing. The capacity of \(\beta\) sheets to inhibit the in vitro digestibility of maize proteins was demonstrated (Martínez-Velasco et al., 2018). Also, less folded proteins are more digestible than highly folded ones (Joye, 2019). Unlike the flour proteins (Table 1), the isolated proteins did not differ substantially in their in vitro degree of hydrolysis (Table 4). Conversely, the changes in amino acid composition resulted in a major shift in the AAS such that the first limiting amino acids were lysine for the isolated control seed protein and leucine for the isolated 48-h germinated seed protein (Table 3). The second and third limiting amino acids also differed between the isolated control and 48-h germinated seed proteins. Notably, a combination of germination and protein isolation increased the AAS of cysteine + methionine by 2.5 folds and in vitro PDCAAS of the pigeon pea samples by 2.6 folds. Although the combination of legumes with cereals has been suggested to improve the limiting amino acid contents (Ohanenye et al., 2020), this study showed that pigeon pea flour can be combined with its protein isolate to achieve a balanced amino acid profile for nutritional purposes.

3.5 Inhibition of glucose metabolizing enzymes

Soaking for 4 h and germination for 24 h reduced (\(P < 0.05\)) the \(\alpha\)-glucosidase inhibitory activity (acarbose equiv.) of the bioaccessible

| Sample | \(Y_i\) (%) | Protein content (%) | \(Y_p\) (%) | In vitro DH |
|--------|-------------|---------------------|-------------|------------|
| Control | 31.2 ± 0.08\(^a\) | 64.9 ± 1.86\(^b\) | 20.24 | 65.5 ± 1.1\(^{bc}\) |
| 4-h     | 29.0 ± 0.17\(^b\) | 53.8 ± 1.07\(^c\) | 15.60 | 63.2 ± 1.6\(^d\) |
| 24-h    | 27.0 ± 0.57\(^c\) | 65.5 ± 2.12\(^{ab}\) | 17.69 | 65.8 ± 0.9\(^b\) |
| 48-h    | 22.9 ± 0.10\(^d\) | 69.8 ± 2.34\(^a\) | 15.98 | 68.2 ± 0.8\(^a\) |

Note. \(Y_i\), isolation yield (%); \(Y_p\), protein yield (%); DH, degree of hydrolysis; Control, non-germinated seeds; 4 h, seeds soaked for 4 h; 24 h, seeds germinated for 24 h; 48 h, seeds germinated for 48 h. Different letters in each column indicate significantly different mean values (\(P < 0.05\)).
pigeon pea seed digesta by 12.5% and 19.8%, respectively, compared to the control (Table 6). In contrast, germinating the seeds for 48 h increased \( (P < 0.05) \) the enzyme inhibition by 17.3%. For DPP-IV, soaking for 4 h led to a 9.5% decrease in the enzyme inhibitory activity (diprotin A equiv.), whereas germinating the pigeon pea seeds for 24 and 48 h did not have any effect on DPP-IV. Furthermore, there were no significant differences in DPP-IV inhibitory activity among the germinated seed samples. The enhancement of the \( \alpha \)-glucosidase inhibition is consistent with the higher degree of hydrolysis obtained for the 48-h germinated sample, which may have released more small active peptides. Thus, the bioprocess could be adopted for improving health-related benefits of the underutilized pigeon pea seeds. Similarly, Ha et al. (2016) reported an increase in \( \alpha \)-glucosidase inhibition after 48-h germination of barley seeds, whereas extending the germination time to 67 h decreased the bioactivity. Germination-related increase in the \( \alpha \)-glucosidase inhibitory activity of chickpea and yellow pea seed digesta was also suggested to be mediated by peptides and polyphenols released during germination or in vitro digestion of the legume seeds (Di Stefano et al., 2019).

### TABLE 5
Amino acid composition of the proteins isolated from non-germinated (control) and germinated (48 h) pigeon pea seeds

| Amino acid | Control seed protein (µg/mg sample) | % | 48 h germinated seeds protein (µg/mg sample) | % |
|------------|-----------------------------------|---|------------------------------------------|---|
| Non-essential amino acids (NAA) | | | | |
| Asx\# | 88.83 | 13.26 | 52.75 | 10.25 |
| Glx## | 158.85 | 23.72 | 104.01 | 20.21 |
| Ser | 42.44 | 6.34 | 25.78 | 5.01 |
| Gly | 32.22 | 4.96 | 19.68 | 3.82 |
| Arg | 47.81 | 7.14 | 31.24 | 6.07 |
| Ala | 31.65 | 4.73 | 20.51 | 3.98 |
| Pro | 32.44 | 4.84 | 21.34 | 4.15 |
| Tyr | 21.11 | 3.15 | 17.46 | 3.39 |
| Cys | 7.23 | 1.08 | 15.82 | 3.07 |
| Essential amino acids (EAA) | | | | |
| His | 26.59 | 3.97 | 16.59 | 3.22 |
| Thr | 29.7 | 4.43 | 18.42 | 3.58 |
| Val | 29.28 | 4.37 | 23.17 | 4.50 |
| Met | 6.15 | 0.92 | 5.96 | 1.16 |
| Ile | 19.92 | 2.97 | 19.64 | 3.82 |
| Leu | 34.62 | 5.17 | 34.84 | 6.77 |
| Phe | 33.93 | 5.07 | 47.24 | 9.18 |
| Lys | 18.01 | 2.69 | 31.61 | 6.14 |
| Trp | 7.90 | 1.18 | 8.62 | 1.67 |
| NAA | 463.58 | 69.23 | 308.59 | 60.02 |
| EAA | 206.10 | 30.77 | 206.09 | 39.98 |

Asx\# = (Asp + Asn); Glx## = (Glu + Gln).

### TABLE 6
Inhibition of the glucose metabolizing enzymes (\( \alpha \)-glucosidase and dipeptidyl peptidase-IV) by in vitro digesta of the non-germinated (control) and germinated pigeon seed flours

| Sample | \( \alpha \)-Glucosidase inhibition | Dipeptidyl peptidase-IV inhibition |
|--------|-----------------------------------|----------------------------------|
|        | µg Acarbose equiv/mg flour | µg Diprotin equiv/mg flour |
| Control | 3.98 ± 0.07\( ^a \) | 1.05 ± 0.01\( ^a \) |
| 4 h | 3.48 ± 0.07\( ^c \) | 0.95 ± 0.04\( ^b \) |
| 24 h | 3.19 ± 0.19\( ^c \) | 1.02 ± 0.01\( ^a,b \) |
| 48 h | 4.67 ± 0.24\( ^a \) | 1.07 ± 0.00\( ^a \) |

Note. Control, non-germinated seeds; 4 h, seeds soaked for 4 h; 24 h, seeds germinated for 24 h; 48 h, seeds germinated for 48 h.

\( ^a\)Acarbose for \( \alpha \)-glucosidase and diprotin A for dipeptidyl peptidase-IV. Different letters in each column indicate significantly different mean values (\( p < 0.05 \)).

4 | CONCLUSIONS

In this study, the effect of germination of pigeon pea seed on its microstructure, protein secondary structure, in vitro protein digestibility and inhibitory activity against glucose metabolizing enzymes were investigated. The results showed that the hypothesis held true as the seed microstructure was altered by germination, which reduced the integrity of the cell compartments. The cell structure collapse may
have been triggered by the nutrient remobilization by the hydrolases associated with germination. Protein digestibility, measured as the in vitro PDCAAS of the pigeon pea seed proteins, was slightly increased by germination, with more pronounced increase observed after protein isolation. These changes were attributed to the changes in the AAS and partial hydrolysis of the pigeon pea proteins by proteases released during germination. Furthermore, the reduction of the β-sheets content of the pigeon pea seed proteins by germination may have contributed to the improved in vitro protein digestibility. Taken together, the findings showed that germination improved the nutritional quality and bioactivity of pigeon pea seeds, which enhance the prospects of the legume in functional food product development. Future studies are needed to evaluate the nutritional and health benefits of the germinated pigeon pea seeds in humans.

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CONFLICT OF 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.

AUTHOR CONTRIBUTION
Ikenna Ohanenye conceived the project and conducted the investigation, formal analysis, validation, visualization, writing—original draft and writing—review and editing. Xiaohong Sun conducted the investigation, validation and writing—review and editing. Roghayeh Amini Sarteshniz had conducted the investigation, validation and writing—review and editing. Chibuike Udenigwe conceived the project and conducted the formal analysis, visualization, funding acquisition, resources, supervision and writing—review and editing.

ETHICAL STATEMENT
No human participants or animals were used in this study.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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