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Physicochemical properties and metabolic effects of cowpea protein isolate☆

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ABSTRACT

This study aimed to characterize the physicochemical properties, protein quality, and metabolic and intestinal parameters associated with the consumption of cowpea protein isolate (CPI). CPI was obtained by alkaline extraction, followed by isoelectric precipitation and freeze-drying. Its proximate composition, bioactive compounds content, and thermal, morphological, optical, and color properties were evaluated, along with amino acid profile, chemical score, and true digestibility. Male Wistar rats were fed with experimental diets containing 9% protein for 30 days. CPI showed high protein content (86.46%), total phenolics (1.79 mg GAE·g^{-1}), and 0.36 IU·mg^{-1} of trypsin inhibition. Electrophoresis revealed predominant bands between 50 and 75 kDa and an endothermic peak at 83 °C. Tryptophan, methionine, and cysteine were identified as limiting amino acids, supplying approximately 70% of nutritional requirements. In vivo analyses showed that CPI presented protein efficiency ratio higher than 2.75, net protein ratio of 3.55, True Digestibility of 89%, decreased LDL-c, triglycerides, and Castelli Index II. Regarding intestinal health, CPI preserved acetic and propionic acid production and reduced butyric acid, aminopeptidase, sodium–glucose cotransporter 1, and sucrase-isomaltase gene expression while maintaining Peptide transporter 1 levels. Further, CPI decreased superoxide dismutase activity and malondialdehyde content, increased claudin and zonulin gene expression, and decreased occludin gene expression. Overall, CPI demonstrated good nutritional properties, supported efficient protein utilization, and improved lipid and oxidative parameters. However, some intestinal effects suggest potential limitations related to bioavailability and epithelial function under the conditions tested.

1. Introduction

The increase in global consumption of plant-based proteins can be due to environmental impacts, concerns regarding animal welfare, health benefits associated with plant-based diets, and the growing demand for more sustainable foods. Plant-derived proteins, once regarded merely as complementary alternatives, have gained prominence and are now widely incorporated into human diets (Karabulut et al., 2024).

In this context, cowpea (*Vigna unguiculata* (L.) Walp.), also known as black-eyed pea, stands out as a legume of great nutritional, agronomic, and socioeconomic relevance. Commonly cultivated in tropical and semi-arid regions of Latin America, Africa, and South Asia, cowpea is an annual crop well adapted to adverse climatic conditions, which makes it an important component of food security in developing countries(Abebe & Alemayehu, 2022; Boukar et al., 2019). It is estimated that global cowpea market exceed US$ 6.3 billion, with projections reaching US$ 10.5 billion by 2030 (Transparency Market Research, 2020).

Nutritionally, cowpea is recognized for its content of phenolic compounds, flavonoids, and anthocyanins, which confer antioxidant and anti-inflammatory properties to the grain (López-Morales et al.,

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2020), but also for its high protein content and the presence of bioactive peptides with antihypertensive, antioxidant, and hypocholesterolemic potential (Vélez et al., 2023).

Besides, these compounds have been associated with improvements in intestinal morphology, enhanced brush border integrity, modulation of the microbiota, and reduction of inflammatory processes in rodent models (Gomes et al., 2022).

Despite these nutritional and functional qualities, the consumption of cowpea still faces certain sensory and technological limitations. Among the main challenges associated with cowpea are its characteristic flavor and the high concentrations of compounds such as tannins and phytic acid which, although exhibiting antioxidant properties, can reduce the bioavailability of nutrients (Peyrano et al., 2016).

The production of protein isolates from cowpea (CPI) has proven to be an effective approach to overcoming these limitations, as it concentrates the protein fraction and may reduce the concentration or chemically modify compounds that can interact with nutrients and hinder their bioavailability. This isolated protein fraction represents a valuable nutritional source, with protein content ranging from 85 to 92 g·100 g^{-1}, while preserving key nutritional properties and expanding its potential application in food formulations (Peyrano et al., 2016; Shevkani et al., 2015).

Previous studies have demonstrated the efficiency of CPI in terms of solubility under alkaline pH and emulsifying capacity, ranging from 106.8 to 146.9 g of oil·g^{-1}, in samples containing approximately 90% of protein (Horax et al., 2004). Recently, Zakki and Aryanti (2025) observed that pH adjustments in cowpea isolates not only increase protein content (63.54% in cowpea isolates compared to 21.5% in cowpea flour) but also improve the stability of oil-in-water emulsions, reinforcing the potential of CPI as a natural emulsifier and functional ingredient in food formulations. Rangel et al. (2004) conducted a 16-day biological assay with rats fed a diet containing 10% of protein to evaluate the protein quality of CPI. The results showed an average weight gain of 3.45 g, a Net Protein Ratio (NPR) of 0.7, and true digestibility of 86.9%, indicating adequate metabolic utilization of the protein. In addition, the protein isolate showed no trypsin inhibitory activity, demonstrating that it did not exert toxic effects on the animals and suggesting the reduction of relevant complexing factors after isolate processing.

Despite several studies evaluating the effects of CPI on technological and in vivo properties (Horaxet al. 2004; Peyrano et al., 2016; Rangel et al., 2004; Zakki & Aryanti, 2025), the available literature remains fragmented and limited to evaluate in vivo studies relating protein quality, lipid profile, oxidative stress, and intestinal health following CPI consumption. This limitation restricts a comprehensive understanding of the physiological and nutritional implications of CPI when used as a food protein source. Therefore, this study aimed to characterize cowpea protein isolate in terms of its physicochemical properties and to evaluate its protein quality, biochemical profile, oxidative status, and intestinal health in an animal model.

2. Materials and methods

2.1. Preparation of cowpea protein isolate

Cowpea protein isolate (CPI) was prepared using raw cowpea beans obtained from a grain distributor (Cajuri, Minas Gerais, Brazil) following the protocol described by Shevkani et al. (2015), with adaptations. Initially, cowpea grains were milled using a fixed-hammer rotor mill equipped with a frequency inverter and a thermostatic chamber (model MA-090CFT, Marconi®), employing a 1-mm stainless steel sieve to obtain the flour.

A 50 g portion of the flour was dispersed in 700 mL of deionized water under continuous agitation, and the pH of the suspension was adjusted to 9.0 using 1 mol·L^{-1} NaOH. The mixture was homogenized for 1 h at room temperature (24 °C), with continuous pH monitoring and readjustment to pH 9.0 whenever necessary. After alkaline extraction, the suspension was centrifuged at 10,000 ×*g* for 20 min at 4 °C using a Beckman J2-MC centrifuge (Beckman Coulter, USA). The pH of the resulting supernatant was adjusted to 4.5 with 1 mol·L^{-1} HCl to induce isoelectric precipitation of proteins and immediately subjected to a second centrifugation under the same conditions (10,000 ×g, 20 min, 4 °C).

The protein precipitate was collected, frozen at −70 °C for 24 h, and subsequently freeze-dried using a CoolSafe Basic freeze dryer (LaboGene®, Denmark) for 72 h at −55 °C, yielding the cowpea protein isolate.

2.2. Chemical and bioactive composition

The cowpea protein isolate (CPI) was characterized in relation to chemical composition and bioactive compounds and compared to raw cowpea bean flour (CBF).

Proximate Composition.

The proximate composition was determined using the methodologies proposed by AOAC International (2019). Moisture content was measured using the gravimetric method by calculating the difference between the wet and dry sample, using a forced-air oven (Nova Ética®, model 400 / 6ND, São Paulo, Brazil) (24 h at 105 °C; AOAC 934.01). Lipid content was measured by Soxhlet extraction (Tecnal, model TE-0364, Piracicaba/SP) (AOAC 945.16) for 8 h under reflux. Total ash was quantified after incineration (550 °C, 6 h) in a muffle furnace (Quimis, model Q320 M, Brazil) (AOAC 923.03). Protein concentration was determined using the semimicro Kjeldahl method (protein conversion factor 6.25; AOAC 2001.11). Carbohydrate content was calculated using the following equation: 100 − (%moisture + %lipids + %proteins + %ash).

Finally, caloric value was estimated considering 4 kcal/g for protein or carbohydrate and 9 kcal/g for lipids (Merrill and Watt, 1955).

Phytic Acid.

Phytic acid content was determined using the commercial K-PHYT Phytic Acid (Phytate) / Total Phosphorus kit (Megazyme, Wicklow, Ireland), following the manufacturer's protocol. Absorbance was measured at 500 nm using a spectrophotometer (Varioskan Lux, Thermo Scientific, USA), and the results were expressed as g of phytic acid per 100 g of sample.

Oxalic Acid.

The quantification of oxalic acid was performed in triplicate based on the method of Iwuoha and Kalu (1995), with modifications CPI (0.25 g) was hydrolyzed with 6 M HCl, followed by neutralization, precipitation at pH 4.4–4.6, and complexation with $CaCl_2$. After centrifugation, the precipitate was solubilized in H_2SO_4, and the tryptophan content was quantified by titration with 0.1 N $KMnO_4$ at 70 °C. Oxalic acid content was calculated using the formula: % Oxalic Acid = ($V_{KMNO4\ em\ mL}$ xN_{KMNO4} x 0.04502 × 100) / *m*(g), where *V* is the $KMnO_4$ volume (mL), *N* is its normality, and *m* is the mass of the sample (g).

Preparation of Extracts for Phenolic and Antioxidant Analyses.

A 1 g portion of each sample (raw cowpea bean flour and cowpea protein isolate) was weighed and extracted using a solution composed of 50% methanol, 70% acetone, and distilled water, as described by Rufino et al. (2017). Samples were shaken for 16 h and subsequently centrifuged at 3000 ×*g* for 10 min. The resulting supernatants were collected and stored under refrigeration until analysis (Awika et al., 2003). The same extract was used for the determination of condensed tannins, total phenolic content and Trolox equivalent antioxidant capacity (TEAC).

Condensed Tannins.

The determination of condensed tannins was performed using the vanillin/HCl reaction, according to the methodology proposed by Price et al. (1978). Absorbance was measured at 500 nm using a spectrophotometer (Varioskan Lux, Thermo Scientific, USA). Quantification was expressed as milligrams of catechin per g of sample, based on a calibration curve constructed from catechin standard solutions.

Total Phenolics.

Total phenolic content was determined using the Folin–Ciocalteu method, as described by Singleton et al. (1999). Aliquots of 500 μL of the extract were mixed with 500 μL of Folin–Ciocalteu reagent (20%) and 500 μL of sodium carbonate solution (7.5%). After vortex agitation and incubation for 30 min at room temperature (25 °C), absorbance was measured at 765 nm using a spectrophotometer (Varioskan Lux, ThermoFisher Scientific, USA). Quantification was performed based on a calibration curve constructed with gallic acid, described by the equation $y = 0.0049\times + 0.1397$, with a coefficient of determination $R^2 = 0.9918$. Results were expressed as milligrams of gallic acid equivalents per gram of sample (mg GAE·g^{-1}).

Trolox Equivalent Antioxidant Capacity (TEAC) Assay.

Antioxidant capacity (TEAC) was determined using the ABTS radical cation method, as described by Leite-Legatti et al. (2012) and Pavan et al. (2014). The $ABTS^{+}\bullet$ radical was generated by reacting ABTS with potassium persulfate and incubating the mixture in the dark for 12 h. The solution was diluted in ethanol to an absorbance of 0.70 ± 0.02 at 734 nm (Varioskan Lux, ThermoFisher Scientific, USA). For the assay, 50 μL of the extract were added to 250 μL of the ABTS solution, and the absorbance was measured after 6 min of reaction in the dark. Results were expressed as μmol Trolox·g^{-1}.

Trypsin Inhibitor Activity.

Trypsin inhibitor activity was determined according to Frota et al. (2018) and Kakade (1969) using benzoyl-DL-arginine-p-nitroanilide (BAPNA) as the substrate. CPI samples were extracted in Tris-HCl buffer (pH 8.2) containing $CaCl_2$. The supernatant was used for enzymatic assays, and absorbance was measured at 410 nm (SpectraMax® ABS Plus, Molecular Devices, USA). Inhibitory activity was initially calculated based on the reduction in absorbance relative to a control and subsequently converted to trypsin inhibitor units per milligram of sample (IU·mg^{-1}), providing a quantitative measure of inhibitory capacity.

Protein Characterization and Digestibility.

Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) of the CPI was performed according to Laemmli (1970), using a 4% stacking gel and a 12% resolving gel. Gels were prepared with 30% of acrylamide/bisacrylamide and Tris-HCl buffer (0.5 M, pH 6.8 for stacking and 1.5 M, pH 8.8 for resolving). Protein samples were prepared to load specific protein amounts—100 ng, 200 ng, and 350 ng per well—with volumes adjusted according to previously determined protein concentration. Samples were mixed at a 3:1 ratio with sample buffer (0.5 M Tris-HCl pH 6.8, 10% SDS, 1% bromophenol blue, β-mercaptoethanol, and 10% glycerol) and brought to a final volume of 30 μL per well with distilled water. Samples were denatured at 98 °C for 10 min prior to loading. Electrophoresis was performed for two hours. A standard molecular weight marker (Bio-Rad), containing proteins ranging from 6.5 to 200 kDa, was used to estimate the molecular weight of the bands. Gels were stained with Coomassie Blue and destained using an acetic acid and methanol solution.

The profile of indispensable and dispensable amino acids in the CPI was determined according to AOAC method 994.12/2000 (Association of Oficial Analytical Chemist 2000) and the methodology described by Liu et al. (1995). The obtained values were compared with the amino acid composition of casein, used as the reference protein in the biological assay (Magalhães et al., 2023), and with the daily requirements established by the Food and Agriculture Organization of the United Nations (FAO, 2013) for children aged 6 months to 3 years.

In Vitro Protein Digestibility and PDCAAS.

The in vitro protein digestibility of CPI was determined in duplicate using the pH-drop method described by Hsu et al. (1977), with modifications by Mendes et al., 2016. CPI was diluted to 6.25 mg protein/mL in 50 mL of distilled water and incubated at 37 °C in a shaking water bath (Marconi, MA093). The pH of the solution was adjusted to 8.0 using NaOH 0.1 N or HCl. Subsequently, 5 mL of an enzymatic solution (2.5 mg/mL trypsin and 1.6 mg/mL pancreatin; 25 BAEE units/mL and 12.8 USP units, respectively) were added. The pH drop was monitored every minute for 10 min using a digital pH meter (Bel Engineering®). Digestibility was estimated using the following equations: $\%D = 93.1359 [1 - e\hat{}(-3.4138 \times (8 - pH))]$ was applied to vegetal proteins (CPI) and the equation $\%D = 97.3704 [1 - e\hat{}(-3.4138 \times (8 - pH))]$ was used for the animal protein reference (casein).

The Protein Digestibility-Corrected Amino Acid Score (PDCAAS) was calculated based on the amino acid composition of CPI and the true in vitro digestibility, using the FAO (2013) reference pattern and the major limiting amino acid.

2.3. Physical properties

Differential Scanning Calorimetry (DSC).

Thermal properties were evaluated in duplicate using a DSC Q200 instrument (TA Instruments, New Castle, USA). Indium (In) standards were used for temperature and energy calibration, and nitrogen was employed as the purge gas. Approximately 2 mg of CPI were mixed with deionized water at a 2:1 (m/m) ratio and placed in hermetically sealed aluminum pans. The pans were sealed and allowed to equilibrate overnight at room temperature. An empty pan was used as the reference. Thermal scanning was performed from 5 to 106 °C at a heating rate of 10 °C/min. The determination of transition temperature (peak) and enthalpy change was conducted using Advantage software, version 5 (TA Instruments, New Castle, USA). The denaturation temperature (Td) was defined as the maximum peak value, while the denaturation enthalpy (ΔHd) was obtained from the integrated area under the peak.

Two concentrations of CPI, 0.01 and 0.03 (*w*/w), were used to evaluate the effect of protein concentration on thermal behavior. These concentrations were selected based on preliminary tests to observe possible shifts in denaturation temperature and enthalpy associated with molecular interactions and protein structural organization at different concentrations.

Scanning Electron Microscopy (SEM).

Morphological analyses of the CPI were performed using a TM-3000 tabletop scanning electron microscope (Hitachi High-Tech, Tokyo, Japan), equipped with a tungsten filament and operating in electron mode with an acceleration voltage of 15 kV. Image acquisition was carried out using the TM-3000 Microscope software, version 02–01 (Hitachi High-Tech, Tokyo, Japan).

Optical Microscopy.

CPI was placed on a glass slide covered with a coverslip and examined using a Leitz Laborlux S optical microscope (Leica, Famalicão, Portugal). A polarizing filter was used to observe the birefringence of starch granules at 100× and 400× magnification. Images were recorded with a digital camera (MShot, Guangzhou, China) attached to the microscope.

Water- and Oil-Holding Capacity.

Water-holding capacity (WHC) and oil-holding capacity (OHC) were determined according to the method described by Stone et al. (2015), with adaptations based on Omura et al. (2021). A 0.05 g of sample was weighed and mixed with 0.5 g of distilled water (for WHC) or soybean oil (for OHC) in microtubes. Samples were vortexed (Phoenix, Recnal, Brazil) for 10 s, with agitation repeated every 5 min for a total of 30 min. The tubes were then centrifuged at 10.000 ×*g* for 15 min. Supernatants were carefully discarded, and the weight of the pellets was recorded to calculate water- and oil-holding capacity.

Color Analysis.

Color variables were evaluated using a colorimeter (Konica Minolta, Tokyo, Japan) based on the CIE Lab scale. The instrument provided values for L* (lightness, ranging from black to white), a* (green to red), and b* (blue to yellow). From the a* and b* values, chroma (C*) – which expresses color saturation, with higher values indicating more vivid colors and lower values indicating duller colors - was calculated. The hue angle (h°), representing the predominant color tone and ranging from 0° to 360° according to its position on the color wheel, was also

recorded.

2.4. Biological assay

Twenty-four male *Wistar* rats (*Rattus norvegicus*, var. *albinus*), 21 days old, were individually housed in stainless-steel cages under controlled temperature (22 °C) and a 12 h light/dark cycle. Throughout the experiment, animals received ad libitum access to deionized water and experimental diets. Animals were randomly assigned to three groups (n = 8 per group) and received the following diets: casein-based control (CC), protein-free diet (aproteic), and cowpea protein isolate diet (CPI) (Table 1). Diets were formulated according to the nutritional recommendations of the American Institute of Nutrition – AIN-93G (Reeves et al., 1993), and containing 9% of protein. Body weight and food intake were recorded weekly for 30 days. The aproteic group was maintained for 17 days to assess protein quality indices, including true digestibility and net protein ratio (NPR), since these variables consider endogenous nitrogen losses. During this period, the feed efficiency ratio (FER)—the ratio between body mass gain and total diet intake—was evaluated (Magalhães et al., 2023). The Lee Index was determined according the criteria proposed by Novelli et al. (2007) and Reynés et al. (2014). At the end of the experimental, after a 6-h fast, animals were anesthetized with isoflurane (Isoforine®, Cristália, Itapira, Brazil) and euthanized by cardiac puncture. Blood was collected and centrifuged at 2865 ×g for 10 min at 4 °C to obtain serum plasma for biochemical analyses. Fragments of the duodenum and colon were collected for histomorphometric evaluation, and cecal feces and a portion of duodenum samples were stored at −80 °C to evaluated intestinal health.

Evaluation of Food Intake, Murinometric Parameters, and Protein Quality.

The evaluation of food consumption was performed using the feed efficiency ratio (FER), which considers the ratio between body mass gain and total diet intake (Magalhães et al., 2023). Murinometric measurements (body weight and naso–anal length) were obtained in the beginning and end of the study for calculating the Lee Index according to the equation proposed by Novelli et al. (2007) and Reynés et al. (2014).

Protein quality was assessed through true digestibility (TD), protein digestibility–corrected amino acid score (PDCAAS), protein efficiency ratio (PER), and net protein ratio (NPR). TD was calculated using the equation proposed by Bender and Doell (1957). PDCAAS was calculated according to methodology proposed by (Schaafsma, 2005); PER was determined according to the equation proposed by Hegsted (1977), and NPR was calculated as described by Bender and Doell (1957). Additionally, the adjusted PER was calculated (Nosworthy et al., 2023). The adjustment was performed using the experimentally obtained PER for each sample and correcting it based on the reference value for casein (2.5), according to Nosworthy et al. (2023).

Table 1
Composition of the experimental diets.

Ingredients (g.100^{-1} g)	CC	CPI
CPI	–	10.41
Casein	10.61	–
Dextrinized Starch	13.20	13.20
Sucrose	10.00	10.00
Soybean Oil	7.00	7.00
Microcrystalline Cellulose	5.14	5.14
Mineral Mix	3.50	3.50
Vitamin Mix	1.00	1.00
L-cystine	0.30	0.30
Choline Bitartrate	0.25	0.25
Corn Starch	49.00	49.20
Diet Composition		
Carbohydrate (%)	84	84
Protein (%)	9	9
Lipid (%)	7	7
Caloric Density (cal. g-1)	4.35	4.35

Biochemical and Metabolic Analyses.

Glucose, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), triglycerides (TGL), uric acid, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein (TP), and albumin concentrations were determined by colorimetric methods using commercial diagnostic kits, following the manufacturer's instructions (Bioclin®, Belo Horizonte, Brazil). Absorbance readings were performed using a Varioskan Lux spectrophotometer (Thermo Scientific, USA).

Atherogenic risk indices were calculated from lipid profile data, as described by Kamoru et al. (2017). The Castelli Risk Index I (CRI—I) was determined as the ratio of total cholesterol to HDL-c, whereas CRI-II corresponded to the ratio of LDL-c to HDL-c. The Atherogenic Index of Plasma (AIP) was calculated as: AIP = $\log_{10}$(TGL/HDLc). Additionally, the Atherogenic Coefficient (AC) was estimated using the formula: AC = (TC - HDLc)/HDLc.

Intestinal Health.

Short-Chain Fatty Acid Concentration.

Approximately 500 mg of feces were homogenized with 1 mL of Milli-Q ultrapure water using a vortex mixer and then centrifuged at 12,000 ×g for 10 min. The supernatant was carefully transferred, and subsequent steps followed the protocol described by Siegfried et al. (1984). Samples were analyzed by high-performance liquid chromatography (HPLC) using a Dionex UltiMate 3000 Dual system (Thermo Scientific) equipped with a refractive index detector (RI-101, Shodex, Germany) maintained at 40 °C. Separation was performed on a Rezex ROA ion-exclusion column (Phenomenex, 300 × 7.8 mm), also maintained at 40 °C, using 5 mM sulfuric acid as the mobile phase at a flow rate of 0.7 mL·min^{-1}. The following fatty acids were used as standards for calibration curve construction: acetic, propionic, and butyric acids.

Bristol Scale and Fecal pH.

Fecal consistency was assessed using the Bristol Stool Scale, a visual and descriptive tool consisting of seven categories, each representing a distinct stool consistency, as follow: type 1: separate hard lumps, resembling nuts; type 2: sausage-shaped but lumpy; type 3: sausage or snake-like with cracks on the surface; type 4: smooth, soft, sausage- or snake-like stools; type 5: soft blobs with clear-cut edges; type 6: mushy stools with ragged edges and type 7: liquid stools with no solid pieces (O'Donnell et al., 1990). Fecal color was also recorded using the following classification: (1) dark brown, (2) light brown, (3) yellowish, (4) discolored, (5) black, (6) red, and (7) green. The validity of the scale has been previously confirmed (Hammer & Phillips, 1993).

Approximately 400 mg of cecal feces were diluted in 4 mL of distilled water and homogenized by vortex agitation for pH determination. The glass electrode of a pH meter (Bel Engineering®) was then inserted directly into the suspension, and readings were taken in duplicate (Grancieri et al., 2017).

Intestinal Morphology.

Semi-serial sections (3 μm thick) of the duodenum and ascending colon were obtained using an automatic microtome (Reichert-Jung®, Germany) and stained with hematoxylin and eosin. Slides were examined under a light microscope (CX31, Olympus®, Tokyo, Japan) using a 10× objective. In the duodenum, the following parameters were evaluated: villus height and crypt depth, thickness of the circular and longitudinal muscle layers, goblet cell count per villus, and individual goblet cell area. In the colon, crypt height and thickness, thickness of the circular and longitudinal muscle layers, goblet cell count per crypt, and goblet cell area were measured. For each animal, 20 random fields were selected, and morphometric analyses were carried out using Image-Pro Plus® software, version 4.5 (Media Cybernetics, Rockville, MD, USA) (da Silva et al., 2019).

Intestinal Oxidative Stress Markers.

Total protein concentrations were quantified in duodenal homogenates using the Bradford method (Bradford, 1976). Superoxide dismutase (SOD) activity was measured in relative units, where one unit was defined as the amount of SOD capable of inhibiting 50% of pyrogallol

auto-oxidation. Absorbance was measured at 570 nm in a spectrophotometer (Varioskan Lux, ThermoFisher Scientific, USA), and results were expressed as SOD units per milligram of protein (Marklund, 1985).

Malondialdehyde (MDA) levels were determined using the TBARS method (Buege & S.D., 1978), with absorbance measured at 535 nm (Varioskan Lux, ThermoFisher Scientific, USA), and results expressed as nmol MDA per milligram of protein. Catalase (CAT) activity was determined by the enzyme's ability to decompose hydrogen peroxide (H_2O_2) into water. Catalase activity was measured at 0, 30, and 60 s after reaction initiation, with absorbance recorded at 240 nm in a spectrophotometer (Varioskan Lux, ThermoFisher Scientific, USA) (Aebi, 1984). Nitric oxide (NO) concentration was quantified using the Griess method (Green et al., 1982). Aliquots of the homogenate were incubated with Griess reagent for 10 min in the dark, and absorbance was measured at 570 nm (Varioskan Lux, ThermoFisher Scientific, USA). Values were expressed in μM based on a standard curve prepared with sodium nitrite.

Determination of Gene Expression of Proteins Associated with Intestinal Health by Real-Time Quantitative PCR with Reverse Transcription (RT-qPCR).

Fragments of the duodenum were ground in liquid nitrogen under RNase-free conditions for total RNA isolation. RNA extraction was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The total RNA obtained was used for complementary DNA (cDNA) synthesis with the M-MLV Reverse Transcriptase kit (Invitrogen, Grand Island, NY, USA) (Livak & Schmittgen, 2001). The duodenal cDNA as used for gene expression analysis, including targets related to intestinal barrier function, such as tight junction proteins.

The reactions were performed with Fast SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on a QuantStudio 1 system (ThermoFisher Scientific, Foster City, CA, USA). Data were analyzed using Primer Express software (Applied Biosystems).

Sense and antisense primers were obtained from Choma Biotechnologies, with the following sequences: Aminopeptidase (AP) forward: TCTCTCCTCAAACCACATGAA and reverse: AGTTCAGGG CCTTCTCATATTC; Sucrase-isomaltase (SI), forward: CCTCCAGAACA-CAATCCCTATAC and reverse: GGAGAGGTGAGATGGGATTAGA; Peptide transporter 1 (PepT1), forward: CCTGGTCGTCTGCATCATATT and reverse: TTCTTCTCATCCTCATCGAACTG; and Sodium–glucose cotransporter 1 (SGLT1), forward: CATCCAGTCCATCACCAGTTAC and reverse: CAATCAGGAAGCCGAGAATCA. Occludin, forward: TTGCTTCATCG CTTCCTTG and reverse: TCCATCTTTCTTCGGGTTTT; Zonulin, forward: GTGGTATCCGATTGTTGT and reverse: TGTCTACTGTCCGTGCTAT; Claudin, forward: GGTTCATCCTGGCTTCG and reverse: ATCCA-CAGTCCCTCGTAG. Relative gene expression was normalized to the reference gene β-actin, using the primers: forward: TTCGTTG CCGGTCCACACCC and reverse: GCTTTGCACATGCCGGAGCC. All procedures were carried out under RNase-free conditions.

2.5. Statistical analysis

The experimental design was randomized, with eight replicates per group. The normality of the data was assessed using the Shapiro-Wilk and Kolmogorov-Smirnov tests. Comparisons between the two experimental groups were performed using the Student's *t*-test for independent samples, adopting a significance level of $p < 0.05$. Statistical analyses were performed using GraphPad Prism software, version 8.0 (Dotmatics, Boston, MA, USA).

3. Results

3.1. Chemical composition

Raw cowpea bean flour (CBF) showed a high carbohydrate content (59.28 ± 0.10 g·100 g^{-1}) and a lower protein content of 23.02 ± 0.10 g·100 g^{-1} compared to cowpea protein isolate (CPI), which exhibited a high protein content (86.46 ± 0.12 g·100 g^{-1}), accompanied by a substantial reduction in carbohydrates (4.05 ± 0.79 g·100 g^{-1}), moisture (5.23 ± 0.40 g·100 g^{-1}), and lipids (1.01 ± 0.01 g·100 g^{-1}). Condensed tannins were not detected in either CBF or CPI. Phytic acid content increased from 0.84 ± 0.04 g·100 g^{-1} in CBF to 3.00 ± 0.10 g·100 g^{-1} in CPI, while oxalic acid increased from 0.60 ± 0.27 g·100 g^{-1} to 1.26 ± 0.51 g·100 g^{-1} following protein isolation. Ash content decreased in CPI relative to CBF. Regarding antioxidant parameters, CBF exhibited higher total phenolic content (4.05 ± 0.16 mg GAE·g^{-1}) and higher antioxidant capacity (10.30 ± 0.40 μmol Trolox·g^{-1}) compared to CPI, which presented values of 1.79 ± 0.04 mg GAE·g^{-1} and 0.73 ± 0.13 μmol Trolox·g^{-1}, respectively. Regarding trypsin inhibition, CBF exhibited higher inhibitory activity (1.57 ± 0.04 UI·mg^{-1}) compared with CPI (0.36 ± 0.01 UI·mg^{-1}) (Table 2).

SDS-PAGE electrophoretic analysis revealed protein profiles with molecular weights between 50 and 75 kDa. The most intense bands indicate major proteins within this molecular weight range. The absence of prominent bands above 75 kDa or below 50 kDa suggests low diversity of high- or low-molecular-weight proteins in CPI (Fig. 1).

3.2. Physical composition

Differential scanning calorimetry revealed two main endothermic events for both CPI concentrations analyzed. For CPI at 0.01 W/g, denaturation peaks occurred with an initial temperature of 83.57 °C and a final temperature of 98.91 °C, with an enthalpy of 9.34 J·g^{-1}, representing the amount of energy required for protein denaturation. In contrast, increasing the CPI concentration to 0.03 W/g resulted in faster denaturation, occurring between 80.97 °C and 99.01 °C, with an enthalpy of 11.45 J·g^{-1} (Fig. 2).

Optical microscopy analysis (Fig. 3) revealed particles with irregular shape and heterogeneous distribution. At 100× magnification (Fig. 3IA), dispersed fragments were observed, some showing colored birefringent reflections under polarized light. At higher magnification (Fig. 3IB), particles of different sizes were visualized, with areas of aggregation and undefined contours.

Scanning electron microscopy of CPI revealed heterogeneous and irregular structures, with particles exhibiting variations in size and shape across magnifications ranging from 50× to 2.000×. In the micrographs obtained at lower magnifications, 50× and 100× (Fig. 3IIA and B), CPI displayed fragmented particles with no defined shape pattern. With higher magnification, 500× to 2.000× (Fig. 3IIC, D, and E), rough and elongated surface features became more evident, along with small points suggestive of residual protein material.

The oil-holding capacity of CPI was higher than its water-holding capacity, with values of approximately 1.99 ± 0.02 g of oil and 1.27 ± 0.16 g of water respectively. In the instrumental color analysis, CPI

Table 2
Chemical composition and bioactive compounds of cowpea flour and cowpea protein isolate.

Compounds (g·100 g^{-1})	CBF	CPI
Total Carbohydrate	59.28 ± 0.10	4.05 ± 0.79*
Protein	23.02 ± 0.10	86.46 ± 0.12*
Lipids	1.58 ± 0.01	1.01 ± 0.01*
Moisture	12.50 ± 0.07	5.23 ± 0.40*
Ash	3.52 ± 0.01	3.25 ± 0.04*
Condensed Tannin	ND	ND
Phytic Acid	0.84 ± 0.04	3.00 ± 0.10*
Oxalic Acid	0.60 ± 0.27	1.26 ± 0.51
TEAC (μmol Trolox·g^{-1})	10.30 ± 0.40	0.73 ± 0.13*
Total phenolic (mg GAE·g^{-1})	4.05 ± 0.16	1.79 ± 0.04*
Trypsin inhibition (IU·mg^{-1})	1.57 ± 0.04	0.36 ± 0.01*

CBF: raw cowpea bean flour. CPI: cowpea protein isolate. ND: not detected. TEAC: antioxidant capacity evaluated by the ABTS method. GAE: Gallic Acid Equivalents. UI: International Unit.

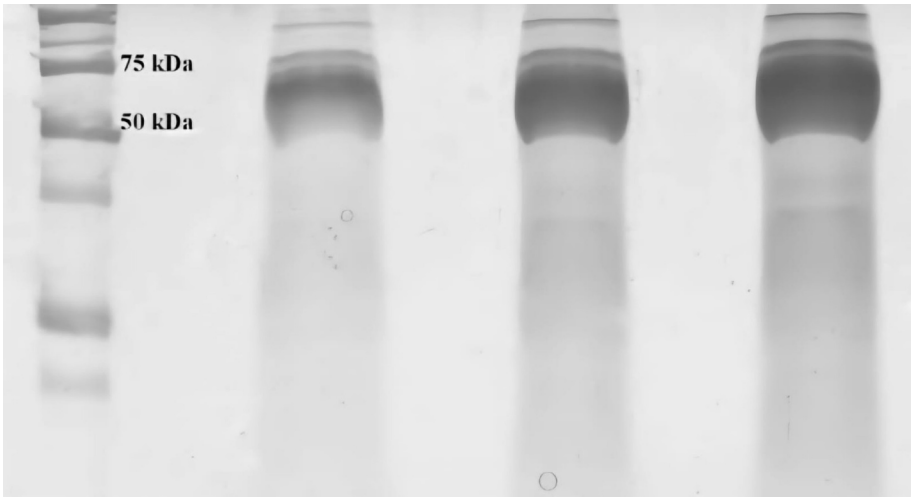

Fig. 1. SDS-PAGE electrophoretic analysis of cowpea protein isolate. Lane M: standard marker proteins; lane 1: CPI cowpea protein isolate; lane 2: CPI cowpea protein isolate; lane 3: CPI: cowpea protein isolate. The bands marked with the arrow in lanes CPI refers protein to 50 KDa e 75 KDa.

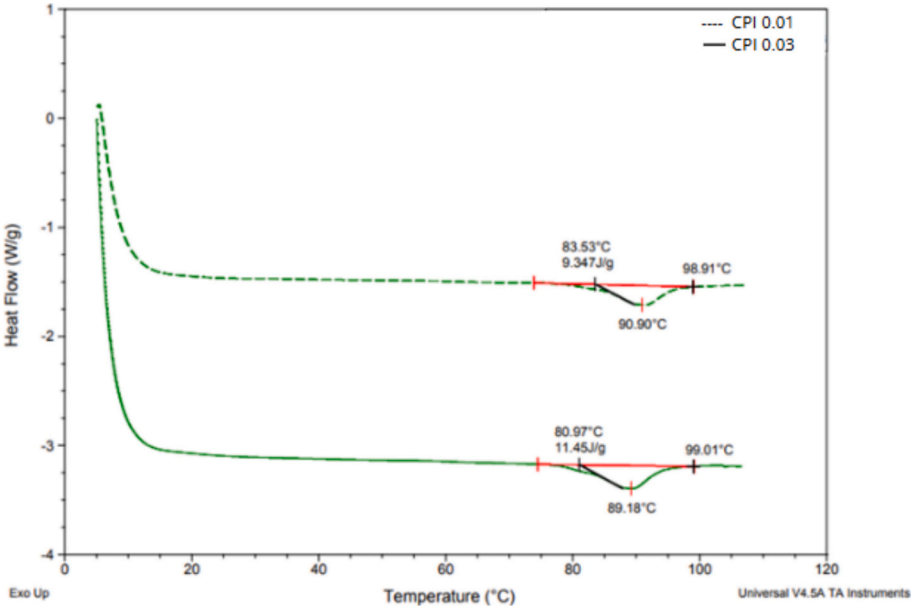

Fig. 2. Thermograms obtained by differential scanning calorimetry of cowpea protein isolate. CPI: cowpea protein isolate.

exhibited high lightness (L* 55.85 ± 0.44), low red intensity (a* 11.65 ± 0.05) and yellow intensity (b* 18.80 ± 0.39), with a chroma (C*) value of 22.20 ± 0.14 indicating low saturation, and a hue angle (h°) of 58.40 ± 0.19, corresponding to a light tonality (Table 3).

3.3. Metabolic effects and in vitro assay

The group fed with CPI showed lower ($p < 0.05$) weight gain, food intake and Lee Index compared with control group. Regarding protein quality indices, protein intake (19.02 ± 0.82 g), PER (2.75 ± 0.23), and NPR (3.55 ± 0.25) were lower ($p < 0.05$) in the CPI-fed group compared with casein control group (CC) (21.61 ± 0.92 g, 3.38 ± 0.50, and 4.09 ± 0.59, respectively) (Table 4).

Fecal weight was similar between groups; however, CPI consumption resulted in greater fecal nitrogen excretion, contributing to lower true digestibility (89.50 ± 2.16%) ($p < 0.05$). The in vitro digestibility of CPI was significantly lower (93.13 ± 0.01%) than that of CC, although the in vivo digestibility values were close to 90% (89.50 ± 2.16) (Table 4).

The composition of indispensable and dispensable amino acids, amino acid chemical score (AA-score), PDCAAS, and IVPDCAAS are presented in Table 5. For CPI, the limiting amino acids were tryptophan and the methionine + cysteine combination, with AA-scores of 0.81 and 0.83, respectively. These values resulted in PDCAAS values of 72.50% and 74.28% and IVPDCAAS values of 75.3% and 77.3%, respectively.

CPI group showed lower ($p < 0.05$) LDL-c (Fig. 4B) and triglyceride levels (Fig. 4D) compared with the CC group, as well as higher ($p < 0.05$) total protein levels (Fig. 4I). No differences ($p > 0.05$) were observed between CPI and CC groups for total cholesterol, HDL, glucose, uric acid, AST, ALT, or albumin.

Regarding Castelli Risk Index II (CRI-II), CPI group showed lower values (0.51 ± 0.18) compared with the CC group (1.41 ± 0.38; $p < 0.05$). No statistical differences were observed for Castelli Risk Index I (CRI—I), Atherogenic Index of Plasma (AIP), or Atherogenic Coefficient (AC).

Fecal moisture was similar between the groups fed with the casein control diet and CPI. Regarding short-chain fatty acids, butyric acid showed a lower concentration in the CPI group (11.46 ± 1.28 mM) ($p < 0.05$), while acetic and propionic acids did not differ between groups.

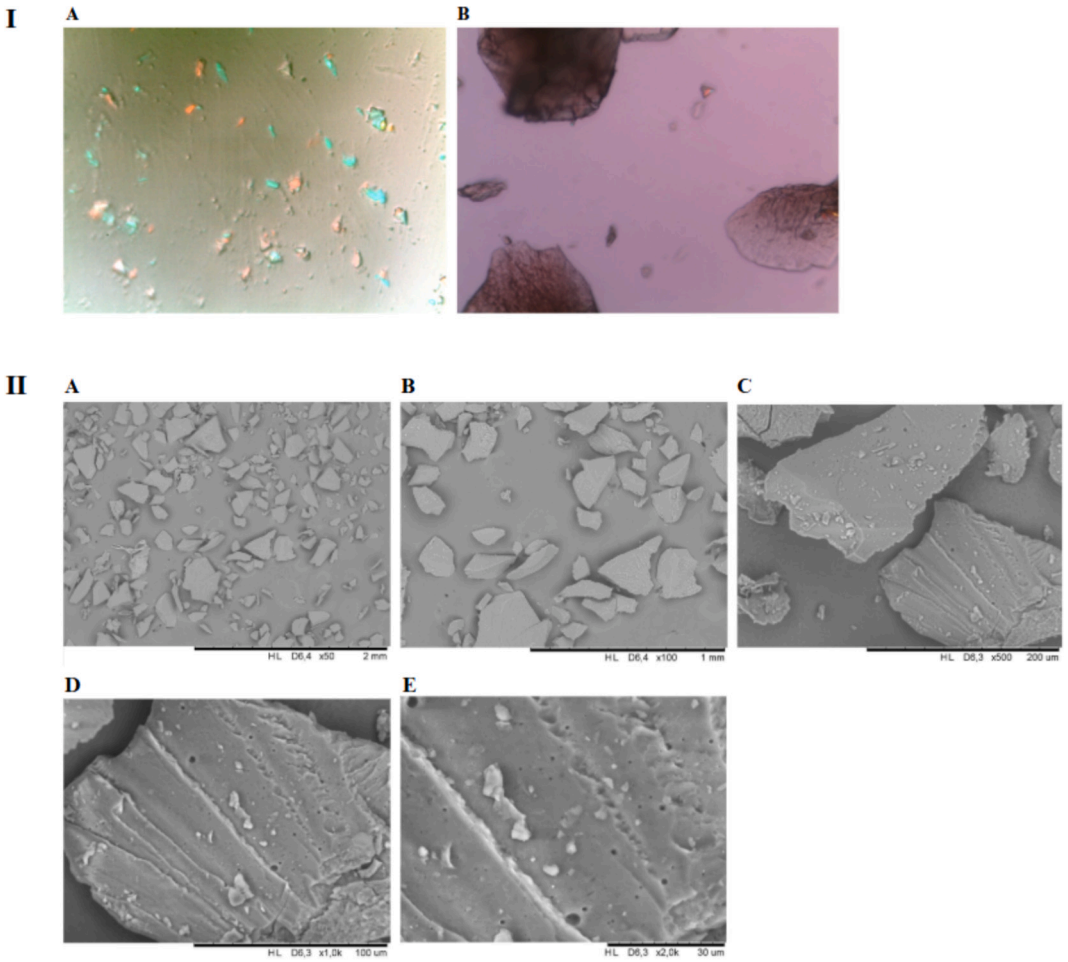

Fig. 3. Images of cowpea protein isolate. I. Optical microscopy. (A) 100× showing increased visualization of particles. (B) 400× showing increased visualization of particles. II. Scanning electron microscopy. A. 50×; B. 100×; C. 500×; D. 1000×; E. 2.000 × .

Table 3
Water- and oil-holding capacity and color parameters of cowpea protein isolate.

Parameters	CPI
WHC	1.27 ± 0.16
OHC	1.99 ± 0.02
Color	
L*	55.85 ± 0.44
a*	11.65 ± 0.05
b*	18.80 ± 0.39
C*	22.20 ± 0.14
h°	58.40 ± 0.19

WHC: Water-holding capacity; OHC: oil-holding capacity; CPI: cowpea protein isolate. L*: lightness; a*: red intensity; b*: yellow intensity; C*: chroma; h°: hue angle. Values expressed as mean ± standard deviation.

Fecal consistency, color, and pH also showed no differences between treatments (Table 6).

The CPI-fed group showed no differences in duodenal villus thickness; however, villus depth (312.01 ± 84.09 μm), crypt thickness and depth (21.75 ± 2.29 μm; 124.81 ± 22.84 μm, respectively), the number of goblet cells per villus (21.43 ± 4.53 μm), and goblet cell area (67.30 ± 5.06 μm^2) were lower compared with the CC group ($p < 0.05$). The thickness of the longitudinal muscle layer and the width of the circular muscle layer did not differ between groups. In colon histology, no differences were observed between groups regarding crypt thickness, crypt

Table 4
Effect of cowpea protein isolate intake on murinometric measurements, dry fecal weight, protein quality indices in Wistar rats ($n = 8$) after 30 days, and in vitro digestibility.

	Group	
Parameter	CC	CPI
Weight gain (g)	146.69 ± 8.53	88.84 ± 5.75*
Food intake (g)	470.12 ± 24.55	404.05 ± 18.78*
Lee index	0.30 ± 0.00	0.28 ± 0.01*
Protein intake (g)	21.61 ± 0.92	19.02 ± 0.82*
FER	31.09 ± 4.86[a]	25.38 ± 2.14*
PER	3.38 ± 0.53	2.75 ± 0.23*
Adj. PER	–	2.03
NPR	4.09 ± 0.59	3.55 ± 0.23*
EER	7.89 ± 1.23	6.44 ± 0.54*
Fecal dry weight	3.19 ± 0.18	2.85 ± 0.42
N total feces (g)	0.04 ± 0.01	0.08 ± 0.01*
TD in vivo (%)	94.84 ± 0.51	89.50 ± 2.16*
TD in vitro (%)	97.33 ± 0.01	93.13 ± 0.01*

CPI: cowpea protein isolate group; CC: casein control group; FER: feed efficiency ratio; PER: protein efficiency ratio; Adj. PER: adjusted protein efficiency ratio; NPR: net protein ratio; EER: energy efficiency.
ratio; gN: fecal nitrogen; TD: true digestibility. Values are expressed as mean ± standard deviation (n = 8). Asterisks (*) indicate statistically significant differences between CC and CPI groups, according to Student's *t*-test ($p < 0.05$).

Table 5
Amino acid composition, amino acid scores, PDCAAS, and IVPDCAAS of casein control and cowpea protein isolate (CPI) samples, based on FAO (2013) recommendations.

	(mg per g protein)			AA – score		PDCAAS		IVPDCAAS	
Amino Acids	**CC**	**CPI**	**Standard** FAO (2013)	**CC**	**CPI**	**CC**	**CPI**	**CC**	**CPI**
Tryptophan	11.54	6.92	8.5	1.36	0.81	–	72.50	–	75.43
Cysteine	–	*	–	–	–	–	–	–	–
Methionine	–	*	–	–	–	–	–	–	–
Methionine + Cysteine	48.60	22.42	27	–	0.83	–	74.28		77.30
Aspartate	–	120.12	–	–	–	–	–	–	–
Serine	–	56.88	–	–	–	–	–	–	–
Glutamate	–	186.14	–	–	–	–	–	–	–
Glycine	–	38.04	–	–	–	–	–	–	–
Histidine	29.96	35.20	20	1.50	1.76	–	–	–	–
Arginine	–	83.37	–	–		–	–	–	–
Threonine	46.38	37.24	31	1.50	1.20	–	–	–	–
Alanine	–	40.39	–	–	–	–	–	–	–
Proline	–	47.37	–	–	–	–	–	–	–
Valine	65.04	52.80	43	1.51	1.23	–	–	–	–
Lysine	68.67	72.44	57	1.20	1.27	–	–	–	–
Isoleucine	51.88	44.84	32	1.62	1.40	–	–	–	–
Leucine	104.72	87.27	66	1.59	1.32	–	–	–	–
Tyrosine	–	36.68	–	–	–	–	–	–	–
Phenylalanine	–	66.45	–	–	–	–	–	–	–
Phenylalanine + Tyrosine	72.90	153.72	52	1.40	2.96	–	–	–	–

CC: casein control; CPI: cowpea protein isolate; AA-score: amino acid score, defined as the ratio between the amount of each indispensable amino acid in the sample (mg/g protein) and the reference value established by the Food and Agriculture Organization (FAO, 2013) for children aged 6 months to 3 years; FAO (2013) pattern: theoretical reference value for essential amino acids (mg/g protein); protein digestibility–corrected amino acid score (PDCAAS): product of the AA-score of the limiting amino acid and the true protein digestibility obtained from rat assays (%); in vitro digestibility–corrected amino acid score (IVPDCAAS): value calculated based on the AA-score of the limiting amino acid and the in vitro protein digestibility. True in vivo digestibility: CC = 93.13%; CPI = 89.50%. True in vitro digestibility: CC = 97.33%; CPI = 93.13%. *Amino acids still under analysis.

depth, goblet cell number, goblet cell area, longitudinal muscle layer width, or circular muscle layer width (Table 6).

Oxidative stress analysis in the duodenum showed that the CPI-fed group showed lower superoxide dismutase (SOD) activity and malondialdehyde (MDA) concentrations (59.47 ± 7.06 U/mg protein and 8.30 ± 1.02 nmol/mg protein, respectively) compared with the CC group (77.02 ± 11.04 U/mg protein and 17.86 ± 3.73 nmol/mg protein, respectively) ($p < 0.05$). Both catalase (CAT) activity and nitric oxide (NO) concentration did not differ between the experimental groups (Table 6).

No difference was observed in *PepT1* gene expression between the CPI and CC groups (approximately 0.90 ± 0.48) (Fig. 5 IA). However, the gene expression of *SI*, *SGLT1*, and *AP* were lower in the CPI group (0.37 ± 0.30, 0.13 ± 0.11, and 0.19 ± 0.25, respectively) compared with the CC group (1.00 ± 0.00) ($p < 0.05$; Figs. 5 IB, IC, and ID, respectively). Besides, claudin-1 (Fig. 5 II-B) and zonulin (Fig. 5 II-C) (3.68 ± 1.03 and 1.85 ± 0.51, respectively) genes expression was higher ($p < 0.05$) in CPI group compared to CC group. In contrast, occludin gene expression reduced (0.63 ± 0.27) in the CPI group (Fig. 5 II-A) relative to CC group (1.00 ± 0.00) ($p < 0.05$).

4. Discussion

The present study evaluated the potential of cowpea protein isolate (CPI) as an alternative source of plant protein, integrating chemical, physical, nutritional, and physiological analyses. CPI exhibited a high protein content and moderate antioxidant capacity, along with low enzymatic inhibitory activity and good thermal stability, characteristics that indicate potential bioactivity. In comparison, raw cowpea bean flour (CBF) showed lower protein content and higher levels of carbohydrates, phenolic compounds, and antioxidant activity, reflecting the composition of the whole grain matrix.

CPI showed a high protein content, surpassing values reported in previous studies using the same legume. Zakki and Aryanti (2025) reported a protein content of 67.6% on a dry basis, while Gómez et al. (2021 observed approximately 81%. According to the Food and Drug Administration (2023), foods that provide 20% or more of the recommended daily value of a given nutrient per serving may be classified as a "high source" of that nutrient. CPI provides approximately 25 g of protein per 30 g serving, qualifying it as a "high source" of protein.

CPI exhibited a protein profile in the 50–75 kDa range, suggesting the presence of storage proteins typical of legumes, such as vicilins (Jayathilake et al., 2018). These findings are consistent with Loushigam and Shanmugam (2023), who identified predominant molecular weight proteins between 55 and 60 kDa in CPI. According to the authors, these bands correspond to the α, β, and γ subunits of vicilins, which account for up to 88% of the total globulins in this legume (Ferreira et al., 2015). In agreement, Teka et al. (2020) observed that globulins and albumins constitute the major protein fractions of cowpea, which globulins with a compact quaternary structure, presenting a negative correlation with in vitro protein digestibility, as well as between in vitro digestibility and phytic acid content. However, this association was not observed in our study, since the CPI demonstrated good protein quality parameters.

The phytic acid content was similar to that reported by Wintersohle et al. (2023) for mung bean protein isolate, indicating that the extraction process of the isolate does not remove this compound, but increased its concentration compared to CBF. Phytic acid, characterized by six phosphate groups and a high negative charge, exhibits high solubility and a strong capacity to chelate proteins and minerals. Under alkaline pH, electrostatic repulsion reduces direct interactions; however, complexes may still form indirectly through multivalent cations. In the isoelectric point, charge neutralization favors protein–phytate complex formation, affecting the protein solubility and digestibility (Wintersohle et al., 2023). Phytate levels considered safe for human consumption range from 250 to 500 mg·100^{-1} g (Kunatsa et al., 2020). Similarly, the oxalic acid content found in the isolate (1.26 g·100^{-1} g) also exceeds levels considered safe for human intake, which are around 250 mg·100^{-1} g (Kunatsa et al., 2020). These higher values observed in CPI reflect a concentration effect inherent to protein isolation process. Therefore, subjected the CPI to heat treatment is recommended to reduce oxalate concentrations in foods to acceptable levels (Anyiam et al., 2025). The estimated daily intake in our study, equivalent to

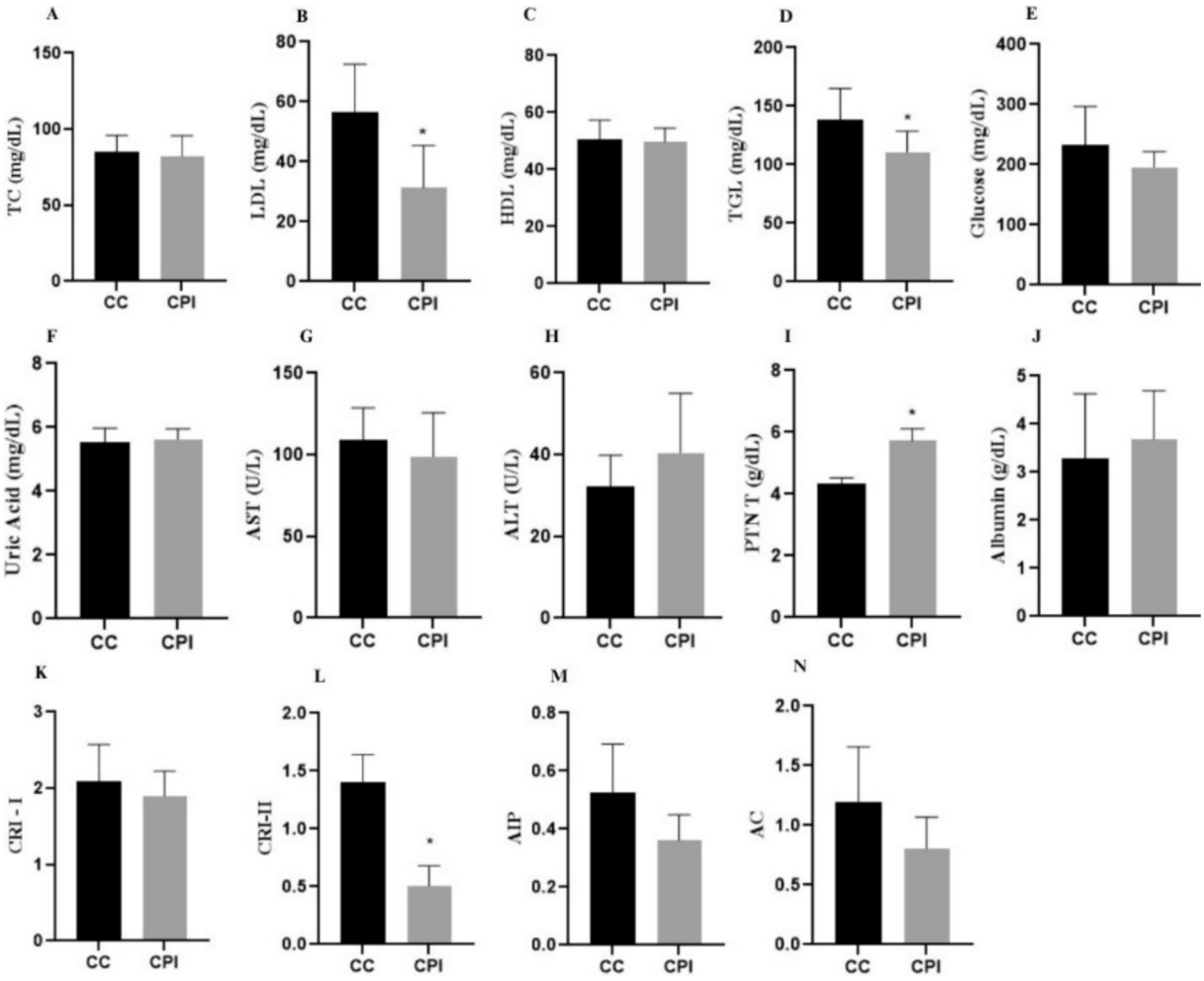

Fig. 4. Biochemical parameters and atherogenic index evaluation in rats fed cowpea protein isolate or casein. Cowpea protein isolate group (CPI); casein control group (CC); Total cholesterol (TC); Low-density lipoprotein (LDL); High-density lipoprotein (HDL); Triglycerides (TG); Aspartate aminotransferase (AST); Alanine aminotransferase (ALT); Total protein (PTN T); Castelli Risk Index (I & II) (CRI); Atherogenic Index of Plasma (AIP); Atherogenic Coefficient (AC). Asterisks (*) indicate statistically significant differences compared with the CC group, according to Student's *t*-test ($p < 0.05$). Values expressed as mean ± standard deviation ($n = 8$).

approximately 16 g of CPI, or about two level tablespoons, falls within the limits considered safe for human consumption, provided that the isolate also undergoes thermal treatment. In addition, condensed tannins were not detected in the CBF and CPI, since cowpea bean seed contain low tannin content, undetectable after protein isolation (Affrifah et al., 2022).

The concentration of total phenolics in CPI (1.8 mg GAE·g^{-1}) was moderate and in CBF was high (4.0 mg GAE·g^{-1}) based on the classification proposed by Andresen et al. (2018), who categorize phenolic levels in legumes as low (<1.0 mg GAE·g^{-1}), moderate (1.0–2.0 mg GAE·g^{-1}), and high (>2.0 mg GAE·g^{-1}). This reduction in CPI is consistent with previous reports indicating that phenolic compounds are partially lost during protein isolation due to their association with non-protein fractions. Nevertheless, the phenolic content observed in CPI remains within the range reported for cowpea-based products (López-Morales et al., 2020).

The trypsin inhibitory activity was lower in CPI compared to CBF, indicating a partial removal or inactivation during protein isolation. The inhibitory activity observed in CPI can be considered low and remains below the range of 6.85–8.25 IU·mg^{-1}, previously reported for cowpea protein isolates (Frota et al., 2018). This decrease is likely associated with the alkaline extraction and isoelectric precipitation, during which some inhibitors remain soluble under acidic conditions (pH 4.0–4.5) and are discarded in the supernatant (Honig et al., 1987; Frota et al., 2018).

The formation of complexes between proteins and these phenolic compounds, common in plant proteins, can create physical barriers that further hinder enzymatic access, reducing digestion efficiency. These structural interactions result in lower in vitro digestibility of plant proteins compared with animal proteins (Günal-Köroğlu et al., 2023). This phenomenon is consistent with our in vivo findings, in which CPI showed reduced true digestibility, which may be associated with the presence of phytic acid and phenolic compounds, the limitation of essential amino acids, as well as the predominant protein profile—globulins—which hampers enzymatic action. In agreement with this, the higher fecal nitrogen excretion observed in the CPI group led to lower true digestibility (TD%) compared with the CC group, corroborating the in vitro digestibility results. Phytate is capable of forming phytate–protein complexes that modify protein structure, potentially leading to inhibition of digestive enzymes such as lipase, amylase, and pepsin, thereby affecting digestion (Kumar et al., 2022; Zayed et al., 2025).

In addition, the lower food intake was observed in the CPI group, which resulted in lower FER and EER, reflected in reduced weight gain compared with the casein group (CC). This reduced food intake may be associated with decreased palatability, as the characteristic “beany” flavor of legumes (Affrifah et al., 2022). Further, vicilins, the major globulins in this legume, may generate peptides that stimulate the secretion of gastrointestinal hormones associated with increased satiety and reduced food intake (Ferreira et al., 2015). Protein quality indices of

Table 6
Evaluation of intestinal health in rats fed cowpea protein isolate and casein.

	Group	
Parameter	**CC**	**CPI**
Fecal moisture content (%)	20.82 ± 5.62	20.63 ± 5.85
Short chain fatty acids (mM)		
Acetic acid	31.23 ± 4.82	29.18 ± 4.91
Propionic acid	15.42 ± 1.38	16.66 ± 1.50
Butyric acid	13.46 ± 1.52	11.46 ± 1.28*
Consistency. Bristol scale	3.88 ± 0.35	4.00 ± 0.00
Color. Bristol scale	1.63 ± 0.52	2.00 ± 1.85
Feces pH	7.03 ± 0.44	6.99 ± 0.37
Duodenum Morphology		
Villus thickness (μm)	79.07 ± 14.93	67.41 ± 17.52
Villus depth (μm)	451.53 ± 70.18	312.01 ± 84.09*
Crypt's thickness (μm)	29.40 ± 2.35	21.75 ± 2.29*
Crypt's depth (μm)	183.08 ± 16.03	124.81 ± 22.84*
Number of caliciform cells (per villus)	26.08 ± 1.97	21.43 ± 4.53*
Goblet cell area (μm^2)	88.57 ± 7.91	67.30 ± 5.06*
Longitudinal mucus layer width (μm)	23.11 ± 6.09	28.34 ± 3.84
Circular mucus layer width (μm)	42.84 ± 6.47	35.25 ± 5.72
Colon Morphology		
Crypt's thickness (μm)	27.76 ± 3.40	25.88 ± 2.33
Crypt's depth (μm)	155.14 ± 54.20	147.77 ± 25.15
Number of caliciform cells (per crypts)	20.06 ± 3.24	17.91 ± 1.76
Goblet cell area (μm^2)	99.47 ± 41.95	80.83 ± 8.58
Longitudinal mucus layer width (μm)	30.22 ± 12.21	34.86 ± 4.10
Circular mucus layer width (μm)	174.51 ± 26.93	155.12 ± 33.38
Oxidative Stress		
SOD (U/mg protein)	77.02 ± 11.04	59.47 ± 7.06*
MDA (nmol/mg protein)	17.86 ± 3.73	8.30 ± 1.02*
CAT (U/mg protein)	30.79 ± 6.19	30.63 ± 6.10
NO (μM)	7.58 ± 2.18	9.63 ± 5.03

Intestinal health parameters of rats fed cowpea protein isolate (CPI) or casein (CC). SOD: superoxide dismutase; MDA: malondialdehyde; CAT: catalase; NO: nitric oxide. Values are expressed as mean ± standard deviation (n = 8). Asterisks (*) indicate statistically significant differences compared with the CC group, according to Student's *t*-test ($p < 0.05$).

CPI, such as PER and NPR were lower compared to CC group. However, the PER value of CPI was higher than 2.0, allowing it to be considered a protein with good quality (FAO/WHO, 1991; Mendel Friedman, 1996). These results indicate that, despite not reaching the levels of the casein control, CPI was effective in supporting growth and body weight maintenance.

In the CPI group, tryptophan and the sulfur-containing amino acids methionine and cysteine were identified as the primary limiting amino acids, as also reported in previous study (Prachi 2010). Even so, the available levels were able to supply 70% of the recommended intake, indicating high digestive efficiency and biological availability for a plant-derived product (FAO, 2013). Combining CPI with foods rich in tryptophan, such as cereals (wheat, rice, barley, rye, and sorghum) (Sudheesh et al., 2022), or dairy products (Nongonierma & FitzGerald, 2012), may enhance protein quality (Shuluwa et al., 2021).

The phytochemical compounds concentration in CPI, exogenous antioxidants, probably improved a redox status, as observed by a decrease of the activity of superoxide dismutase (SOD) and malondialdehyde (MDA) concentration and maintenance of catalase (CAT) activity and nitric oxide (NO) content, indicating a oxidative homeostasis (Anwar et al., 2024; Theodoro et al., 2021).

Changes in the intestinal oxidative environment and epithelial morphology may directly affect the regulation of digestive enzymes and transporters, which together determine nutrient absorption efficiency. Animals fed CPI showed reduced gene expression of the *sucrase–isomaltase* (SI), sodium-dependent glucose transporter (*SGLT1*), and *aminopeptidase* (AP), whereas peptide transporter (*PepT1*) gene expression did not differ from the control group. Maintenance of *PepT1* expression suggests that the generation of absorbable peptides was sufficient to sustain peptide absorption in the small intestine, even with lower levels of free amino acids (Spanier & Rohm, 2018). The reduction in butyrate levels observed in animals that received CPI may have contributed to the lower expression of *SGLT1*, since this short-chain fatty acid is recognized as the main energy source for enterocytes and as a positive modulator of genes involved in nutrient transport. The decrease in butyrate, together with the duodenal morphological alterations (reduced villus depth, reduced crypt length and thickness, and decreased goblet cell number and area), may have compromised the absorptive function of the intestinal epithelium and, the expression of transporters such as *SGLT1*. The lower expression of *SI* observed in

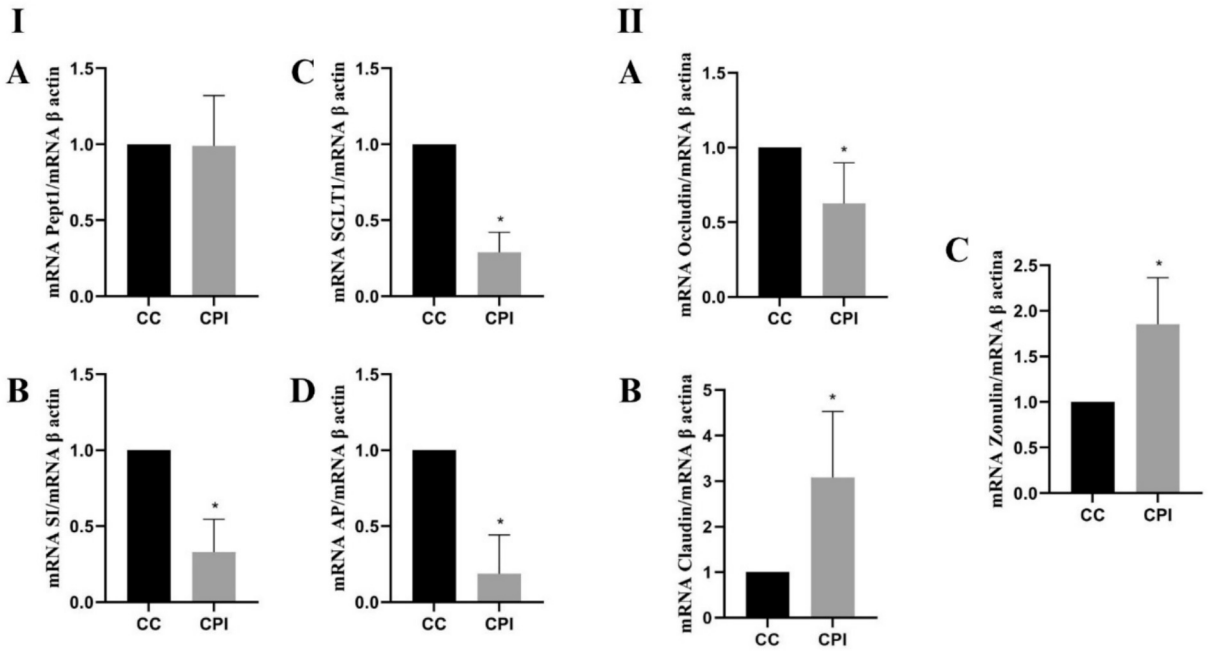

Fig. 5. Effects of cowpea protein isolate on intestinal health in Wistar rats. CPI: Cowpea protein isolate group; CC: casein control group. (A) PepT1: peptide transporter; (B) SI: sucrase–isomaltase; (C) SGLT1: sodium-dependent glucose transporter; (D) AP: aminopeptidase. Data represent the ratio of target gene expression normalized to β-actin. Values are expressed as mean ± standard deviation ($n = 5$). Asterisks (*) indicate statistically significant differences between CC and CPI groups, according to Student's *t*-test ($p < 0.05$).

animals receiving CPI may indicate reduced sucrose digestion capacity in the small intestine, possibly due to modifications in luminal content or regulatory effects of bioactive compounds (Senftleber et al., 2023), such as phytic acid or phenolics. This reduction may alter intestinal modulation, negatively affecting bacterial fermentation and the production of short-chain fatty acids (Senftleber et al., 2023), such as butyrate, which production was decreased.

The lower gene expression of aminopeptidase (AP) may be related to the high phytate:zinc molar ratio in the CPI. Excess phytate can form insoluble complexes with zinc, reducing its bioavailability in the intestinal lumen and consequently limiting the activity of zinc-dependent enzymes such as aminopeptidases. This imbalance may compromise the synthesis, activation, and functional activity of these brush border membrane enzymes. This mechanism is supported by Liu et al. (2009), who demonstrated the chelating effect of phytate on zinc and its negative impact on the activity of mineral-dependent enzymes.

In the context of intestinal integrity, the CPI group showed increased gene expression of claudin and zonulin, which are involved in the regulation and maintenance of epithelial tight junctions. This upregulation may reflect a physiological response to increased epithelial demand or early signs of disruption. The reduced expression of occludin associated with the improve of the oxidative balance supports the hypothesis of a compensatory adaptation in the intestinal epithelium (Kuo et al., 2022). In the colon, no morphological alterations were observed between groups. Additionally, fecal pH, color, and consistency also remained similar to those of the CC group.

Consumption of CPI promoted a reduction in plasma LDL-c and triglyceride levels, without changes in total cholesterol, HDL-c, or glucose concentrations. These results were supported by the Castelli Risk Index II, which corresponds to reduction in LDL-c/HDL-c ratio, reinforcing the potential hypolipidemic effect of CPI. Similar findings were reported by Weththasinghe et al. (2014) e Frota et al. (2015), who described improvements in lipid profiles in response to legume consumption. Although these matrices differ from CPI, such findings suggest that legume products, in general, may exert beneficial effects on lipid metabolism. According to de Lima et al. (2019), this effect may be associated with the presence of bioactive peptides with hypocholesterolemic action, naturally found in beans and released during processing or enzymatic digestion. The suggested mechanism involves the reduced micellar solubilization of cholesterol in the intestinal lumen, possibly due to hydrophobic interactions between peptides and lipids, which may impair intestinal cholesterol absorption and contribute to lower serum LDL-c and triglyceride levels.

CPI exhibited a favorable hepatic and renal safety profile, as evidenced by the absence of changes in plasma ALT and AST and creatinine and urea levels, respectively, indicating preserved liver and kidney integrity. The increase in total plasma protein (PTN T) was not accompanied by alterations in hepatic markers.

Taken together, the physiological responses highlight important considerations for the production and application of CPI as a food ingredient. Physical parameters such as thermal stability, evaluated by differential scanning calorimetry, showed an endothermic peak around 83 °C, even after extraction at pH 9, indicating the presence of protein fractions that resist alkaline denaturation and undergo structural transition only with increasing temperature (de Oliveira et al., 2024). Thus, extraction at pH 9 provided protein purity, as observed in optical and electron microscopy analyses. Regarding water-holding capacity (WHC) and oil-holding capacity (OHC), CPI exhibited higher OHC than WHC, which may be related to the physical entrapment of oil and to non-covalent interactions—including hydrophobic and Van der Waals forces—involved in the formation of protein–lipid complexes (Lima, 2018). The extraction and purification conditions using pH 9 in our study favored the exposure of hydrophobic regions of proteins (Lima, 2018). Therefore, our results indicate that CPI has functional properties relevant for applications in food products that require fat retention, such as emulsions and meat analogues. These interactions are essential for achieving functionalities such as emulsification, hydration, gel formation, viscosity, texturization, and moisture retention—characteristics that are determinant for structure, juiciness, and sensory acceptance of alternative meat products (Bohrer, 2019; Ismail et al., 2020). Regarding color, high lightness values were observed, corresponding to a light coloration of CPI. The low a* and b* values indicate low intensity of reddish and yellowish tones, respectively. The chroma (C*) value further supports the low color saturation, while the hue angle (h°) indicates the predominance of pale-yellow tones. Therefore, CPI exhibited a neutral color profile, a desirable visual characteristic for the production of meat analogues (Ahmad et al., 2022).

In this context, future studies should explore the use of CPI in plant-based meat analog formulations subjected to thermal processing, assessing the stability of its functional properties, nutrient bioavailability, and in vivo physiological responses. This approach will support the validation of CPI as a promising plant protein ingredient for the development of health-promoting products and viable alternatives to animal protein.

5. Conclusion

Cowpea protein isolate (CPI) demonstrated physicochemical characteristics favorable to its application as a food ingredient, including high protein content, thermal stability, and the presence of bioactive compounds. In the in vivo study, CPI showed good performance in biological indices such as PER and PDCAAS. Its intake improved lipid profile and oxidative balance, but decreased butyrate concentration and marks related to intestinal functionality and epithelial integrity, suggesting potential limitations in gastrointestinal functionality. Future studies should investigate the CPI application in thermally processed food products to evaluate the potential beneficial metabolic effects.

Experimental diets formulated with casein (84.79% protein concentration) control (CC) and cowpea protein isolate (CPI) (86.46% protein concentration), expressed in $g \cdot 100^{-1}$ g of diet. Both formulations were normocaloric, containing 9% protein, adapted from Reeves et al. (1993).

CRediT authorship contribution statement

Andressa Alvarenga Silva: Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Valéria Aparecida Vieira Queiroz:** Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Valeria Silva de Lana:** Writing – original draft, Methodology. **Monique Barreto Santos:** Writing – review & editing, Writing – original draft, Methodology. **Renata Celi Lopes Toledo:** Writing – review & editing, Writing – original draft, Methodology. **Edimar Aparecida Filomeno Fontes:** Writing – review & editing, Writing – original draft, Methodology, Data curation. **Carlos Wanderlei Piler de Carvalho:** Writing – review & editing, Writing – original draft, Methodology. **Barbara Pereira da Silva:** Writing – original draft, Methodology, Investigation, Data curation. **Hercia Stampini Duarte Martino:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Compliance with ethical standards

All procedures involving animals performed in this study complied with the ethical standards of the Federal University of Viçosa. The study was approved by the Ethics Committee of Animal Research of the Federal University of Viçosa, Brazil (Protocol 34/2024). Conflicts of interest. The authors declare that they have no conflicts of interest.

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.

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Data availability

The data that has been used is confidential.

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