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Effects Of Oral Supplementation of Black-Eyed-Bean (*Vigna Unguiculata*) In ICR Mice: A Long-Term Study

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ABSTRACT
Grain legumes are a main source of nutrients in some countries. Black-eyed-bean (BEB) (*Vigna unguiculata*), a member of theFabaceae family, is a grain legume consumed worldwide due to its high content of bioactive molecules that promote human health. This study aimed to evaluate the effects of 70% BEB flour (BEBF) (*w/w*) supplementation in ICR healthy mice for 13 weeks. Body and organs weights, as well as food and water intake, were measured. Hematological, biochemical, genotoxicity and stress oxidative analysis were also performed. Oral administration of BEBF revealed increased spleen relative weight and microhematocrit levels. BEBF has been shown to increase superoxide dismutase levels, suggesting an antioxidant effect. Furthermore, it also increased the glutathione-S-transferase levels, indicating that the percentage administered to the mice could be excessive. We observed that the BEBF did not promote physiological changes or signs of toxicity, suggesting that the current dose is not harmful, and, in the future, it can be used as a functional food supplement, and to prevent the development of diseases, namely cancer.

Keywords: Cowpea, flour, functional food, *in vivo*, long-term effects, oral administration

Introduction

Vigna unguiculata (L.) Walp. is a grain legume from the Fabaceae family, commonly known as black-eyed-bean (BEB), cowpea, black-eyed peas, pink-eyes or southern peas (English), “feijão-frade” (Portugal), “carilla” (Spain) and “lobia” (India). It is one of the most ancient food sources used since Neolithic times [1]. Despite being native to Africa, it is traditionally cultivated in the Mediterranean region [2]. Indeed, the BEB has high productivity and tolerance to environmental stress such as drought [3]. According to Food and Agriculture Organization Corporate Statistical Database (FAOSTAT), in 2019, 96.8% of global dry BEB production was in Africa, with Nigeria and Niger being the largest producers with 3.5 and 2.3 million tons produced, respectively [4].

It is widely known that grain legumes are a source of nutrition, diversifying agriculture, and guaranteeing ecological sustainability, apart from a source of proteins, micro- and macronutrients [5, 6]. Like other legumes, although the entire plant of *V. unguiculata* is suitable for the human diet, the most economically profitable part is the dry grain due to its medicinal and nutritional properties [1]. The grain may be consumed crude, boiled, parched, fried, or roasted, and it is a high-quality plant source (203-394 g/kg) in many parts of the world, feeding millions of people in underdeveloped and developing countries [2, 3, 7, 8, 9]. It is also a good source of nutraceuticals compounds as fiber, antioxidants,

polyunsaturated fatty acids, quercetin and kaempferol (flavonols) and p-coumaric, protocatechuic acid and gallic acid (phenolic acids) [3, 10].
High protein and carbohydrate contents with relatively low-fat content and a better amino acid profile, when compared to cereal grains, make BEB an important functional food in the human diet [2]. The agronomic, environmental, and economic BEB advantages, as well as excellent nutritional and nutraceutical properties, make the food industry and producers grow on a large scale [3].
Despite all the benefits, to the best of our knowledge, there are no studies in animal models assessing the effects of prolonged intake of BEB. For this reason, the present study aimed to evaluate the long-term effects of (BEBF) supplementation in healthy mice.

Materials and methods

Ethics statement

This study was performed following the European (Directive 2010/63/EU) and Portuguese (Decree-Law 113/2013) legislation on the protection of animals used for scientific purposes. All experiments procedures were approved by the University of Trás-os-Montes and Alto Douro Ethics Committee (no. 7/2018) and the national competent authority (010535, *Direção Geral de Alimentação e Veterinária*, Lisbon, Portugal). The experiments with live animals were conducted by certified researchers.

BEB characterization

The only Portuguese variety of BEB ('Fradel') was used in this study. This variety is characterized by erect growth habit and grains with cream color and black eye. 'Fradel' dry grains were provided by a local producer in Arrifana (0°13'34"N, 8°16'19"W), Coimbra District, Portugal during spring-summer season of 2017. The protein and phenolics contents were determined in four biological replicates. The protein content was determined using estimated N content, which was obtained by the Kjeldahl method, by the following formula: Protein content (g kg^{-1}) = N content × 6.25 [11].
Following the methodology described by Carvalho *et al.* [12], three hydro-methanolic extracts were prepared of each replicate. Briefly, 40 mg of dry grains lyophilized powder was mixed with 1.5 ml of methanol/distilled water (70:30, *v/v*), vortexed and finally extracted by agitation at room temperature for 30 min. The mixture was centrifuged for at 4ºC for 15 min at 11,200 × *g* to separate the supernatant, repeating this procedure three times and the supernatants stored together in a 5 ml volumetric flask. Total phenolic, flavonoids and ortho-diphenols contents were determined according to spectrophotometric methodology previously described by Carvalho *et al.* [12] for 96-well microplates (Nunc, Roskilde, Denmark) and a Multiscan FC microplate reader (Thermo-Fisher Scientific, Inc., Waltham, MA, USA). The total phenolic content was evaluated by the Folin-Ciocalteu spectrophotometric method and gallic acid was used as standard. A total of 20 µL of sample and 100 µL of Folin-Ciocalteu reagent were mixed and vortexed. After, 80 µL of Na2CO3 (7.5 %) were added. The mixture was vortexed once again and incubated in an oven at 40-45 °C for 30 min, protected from light. Absorbance was read at 750 nm. To determine the ortho-diphenols content, 40 µL of Na2MoO4 (50 g L^{-1}) were added to 160 µL of each sample and gallic acid was used as standard. The mixtures were vortexed and incubated at room temperature for 15 min, protected from light. The absorbance was recorded at 375 nm. For the assessment of flavonoid content, 24 µL of the samples were mixed with 28 µL of NaNO2 (50 g L^{-1}). After 5 min precisely, 28 µL of AlCl3 (100 g L^{-1}) were added and after 6 min, 120 µL of NaOH (1M) were added to the mixture, the absorbance immediately recorded at 510 nm. The flavonoid content was quantified using catechin as standard. A total of three technical replicates were assessed and the results were expressed in mg of gallic acid per mg of dry weight (mg GA g^{-1} dw) for total phenolic and ortho-diphenols content, while for flavonoid content in mg of catechin per mg of dry weight (mg catechin g^{-1} dw).
The minerals (phosphorus, potassium, calcium, magnesium, iron and zinc) contents wet digestion with HNO_3 + H_2O_2 assisted by microwaves [29] and determined by inductively coupled plasma emission spectrometry (ICP-OES).

Animal diet preparation

To preparation of the diet, BEB was powdered in a Vorwerk Thermomix (Wuppertal, Germany), following the same procedure was performed for the rodent standard diet (Mucedola 4RF21 Diet Standard, Settimo MilanesseItaly). BEBF was then mixed with the standard diet at the percentage of 70% (*w/w*) and 5% of water (*v/w*) was added to the mixture. The pellets were prepared using a laboratory pellet press (CPM, C-300 model, Zaandam, The Netherlands) with a 4.8 mm diameter. Control pellets (without BEBF) were prepared using only the standard diet. Both types of pellets were dried at 40ºC for 48h and stored at 4ºC until use..

Animals

Twenty healthy male ICR mice (*Mus musculus*) with 4-weeks-old were obtained from the Charles River Laboratories (France). The animals were housed at the animal facility of UTAD under controlled conditions of temperature (23 ± 2°C), relative humidity (50 ± 10%) and dark/light cycle (12h/12h). All animals were maintained in polycarbonate cages (1284L Eurostandard Type II L cages, Tecniplast, Buguggiate, Italy), with corncob for bedding (Corncob Ultra 12, Ultragene, Santa Comba Dão, Portugal) and, environmental enrichment (cardboard rolls and paper). The cages were

changed weekly, and food and water were provided *ad libitum.* Mice were acclimatized for one week before the start of the experiments.

Then, the animals were randomly allocated into 2 experimental groups (10 animals per group) using a computer-generated randomization and each group was housed in two cages (5 animals/cage). The group I (Control) was fed with standard diet and group II (70% BEBF) was fed with BEBF diet, for 13 weeks. Animals' health status was checked once a week, with the evaluation of the following parameters: body condition, body posture, hair appearance, grooming, mucous color, eyes, ears and whiskers, mental status, response to external stimuli, hydration status, stool appearance and convulsions.

The body weight, food and water consumption were also registered weekly using a top-loading scale (KERN® PLT 6200-2A, Dias de Sousa S.A., Alcochete, Portugal). Moreover, the surface temperature was measured weekly on the back using an infrared thermometer (Uni-T Series UT300B, Uni-Trend Group, China), according to manufacturer instructions.

After 13 weeks of the protocol, all animals were humanely sacrificed through intraperitoneal overdose of xylazine (20 mg/mL) (Rompun® 2% Bayer, Healthcare S.A., Kiel, Germany) and ketamine (100 mg/mL) (Clorketam 1000, Vetoquinol, Barcarena, Portugal) at a ratio of 2:1. Blood samples were collected directly from the heart and were divided into 75-µL sodium-heparin microhematocrit capillary tubes (Hirschmann Laborgeräte GmbH & Co., Eberstadt, Germany), lithium-heparin tubes (FL MEDICAL, Torreglia, Italy) and microtubes for microhematocrit, biochemical and genotoxicity analysis, respectively.

After blood collection, a complete necropsy was performed and internal organs were quickly removed and individually weighed on a precise scale (KERN® PLT 6200-2A, Dias de Sousa S.A., Alcochete, Portugal). The organs were immersed in 10% neutral buffered formalin for histological analysis and part of the liver was stored at -80ºC for oxidative stress study.

Blood analysis

The microhematocrit capillary tubes were centrifuged at 1200 rpm for 5 min, using a centrifuge (PrO-Vet, Centurion Scientific Limited, Chichester, UK) with an adapter this type of tubes. A ruler was used to determine the ratio (%) of the volume packed red blood cells to the volume of whole blood.

The lithium-heparin tubes were centrifuged at 3000 rpm for 15 min (Heraeus Labofuge 400R, Thermo Fischer Scientific, Waltham, MA, USA) to separate plasma. Plasma samples were stored at -80ºC for further biochemical analysis of creatinine, urea, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT) using an autoanalyzer (Prestige 24i, Cornay PZ, Tokyo, Japan).

Alkaline comet assay

The alkaline comet assay was performed following the methods of Collins [13]. One hundred µL of blood was diluted in 200 µL of ice-cold phosphate-buffered saline. Sixty µL of cell suspension was added to 600 µL of 1% low-melting-point agarose, gently mixed with the micropipette, and placed two drops of 70 µL in each slide (precoated with 1% normal melting point agarose and dried). Slides were placed at 4ºC for 5 min to allow the gels to set. Slides were incubated with a lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton X-100, pH 10) at 4°C, for 1h and rinsed three times with the buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL bovine serum albumin, pH 8.0). The slides were then placed in an electrophoresis tank with an alkaline electrophoresis solution (0.3 M NaOH and 1 mM EDTA, pH>13 at 4°C) for 30 min before electrophoresis was carried out (at 25 V, 300 mA for 30 min). The samples were finally neutralized with PBS left to dry at room temperature. Gels were stained with 1 µg/mL of 4,6-diamidino-2-phenylindole solution (Sigma-Aldrich Chemical Company, Spain) and visualized using a fluorescent microscope Olympus BX41 (Olympus America, Inc., Hauppauge, NY, USA) at x400 magnification. Visual scoring was used to categorize the nucleoids into five comet classes based on tail length and intensity, ranging from 0 (no tail) to 4 (almost all DNA in tail) [13]. Fifty nucleoids were classified per gel (100 per case) and Genetic Damage Indicator (GDI) was calculated according to the formula:

$GDI = \sum \%\ \text{nucleoids class}\ i \times i$

Where *i* is the number of each class (ranging from 0-4). GDI was expressed on an arbitrary scale of 0 to 400 (arbitrary units, AU).

Histopathology

After fixation, liver, kidney and colon samples were routinely processed for light microscopy and embedded in paraffin. Two µm thick sections were cut and stained with hematoxylin and eosin (H&E). Slides were examined by a veterinary pathologist using a light microscope with a digital camera.

The degree of inflammation of the animals' colon was evaluated according to Tian *et al.* [14]. Briefly, the severity of inflammation was assessed on a scale of 0 to 3 (0 = no inflammation, 1 = mild, 2 = moderate and 3 = severe) and the thickness of inflammatory involvement scored from 0 to 3 (0 = no inflammation, 1 = mucosa, 2 = mucosa plus submucosa and 3 = transmural). Furthermore, the severity of epithelial damage was evaluated from 0 to 3 (0 = intact epithelium, 1 = disruption of architectural structure, 2 = erosion and 3 = ulceration) and extent of inflammatory lesions was also considered (0 = no lesions, 1 = punctuate, 2 = multifocal and 3 = diffuse). The degree of inflammation of the

liver and kidney was evaluated on a scale from 0 to 2 (0 = no inflammatory infiltrate, 1 = one to four multifocal inflammatory aggregates and 2 = five or more multifocal inflammatory aggregates).

Oxidative stress

Flash frozen portions of the liver were homogeneized in cold buffer (0.32 mM of sucrose, 20 mM of HEPES, 1 mM of MgCl2 and 0.5 mM of phenylmethyl sulfonylfluoride, pH 7.4) on a TissueLyser II (Qiagen, Hilden, Germany). Homogenates were centrifuged at 15000×g at 4ºC for 20 min (Sigma model 3K30, Osterode, Germany) and supernatants were collected for the oxidative stress biomarker assays described below. Determination of total reactive oxygen species (ROS) was performed at 485 nm and 530 nm for excitation and emission wavelengths, respectively, using the fluorescent probe DCFH-DA, as previously described [15] and ROS accumulation was estimated based on a DCF standard curve (0-6.25 nM). The activity of superoxide dismutase (Cu/Zn-SOD) was evaluated based on the nitroblue tetrazolium (NBT) reduction generated by the xanthine/xanthine oxidase system at 560 nm [16]. Superoxide dismutase (SOD) from bovine erythrocytes was used to construct a standard curve (0-3.75 U mL^{-1}). The activity of catalase (CAT) was measured as the decrease of hydrogen peroxide concentration at 240 nm in accordance with the method previously described and was calculated using bovine catalase as a standard (0-5 U mL^{-1}). Glutathione peroxidase (GPx) activity [17] was determined at 340 nm by a coupled assay based on the glutathione reductase catalyzed oxidation of NADPH [18] and using the extinction coefficient of 6.22 mM^{-1} cm^{-1}. Glutathione-S-Transferase (GST) activity was evaluated using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate at 340 nm [19] and using the extinction coefficient of 9.6 mM^{-1} cm^{-1}. The glutathione levels were determined by measuring both the reduced (GSH) and the oxidized states (GSSG) using the fluorochrome ortho-phthalaldehyde (OPA) at 320 nm and 420 nm for excitation and emission wavelengths, respectively [20]. Concentrations were estimated based on GSH and GSSG standard curves (0-10 µM), whereas the ratio between GSH and GSSG was calculated as the oxidative-stress index (OSI). Malondialdehyde (MDA) content, an indicator of lipid peroxidation (LPO), was measured at 535 nm (excitation) and 550 nm (emission) wavelengths, by chromogenic assay via a thiobarbituric (TBA) acid-based method described elsewhere [20]. MDA was estimated based on a standard curve (0-4 nM) of malonaldehyde bis(dimethyl acetal). Data was normalized by the amount of protein in the homogenates performed at 595 nm according to the Bradford method [21] with bovine serum albumin as a standard (0-1.4 mg mL^{-1}). All samples were performed in duplicate using 50 µg of each sample and measured against a reagent blank using a PowerWave XS2 microplate scanning spectrophotometer (Bio-Tek Instruments, USA) or a Varian Cary Eclipse (Varian, USA) spectrofluorometer, equipped with a microplate reader at 30°C.

Statistical analysis

The homogeneity index was calculated according to the following formula HI = 2Wi/(Wi + Wh), with Wi being the lowest animal weight and Wh the highest animal weight [22]. The ponderal gain was calculated according to the following formula PG = Wf – Wi/Wf x 100 with Wi being initial body weight and Wf final body weight [23]. Relative organ weight was determined by applying the following formula: organ weight/body weight x 100.

The statistical analysis was performed using Statistical Package for Social Sciences (SPSS) version 25 (Chicago, IL, USA). The data were analysed for normality using the Shapiro-Wilk normality test. The differences between groups were determined using an unpaired Student's *t*-test. Data are expressed in mean and standard error (SE). *n*-denote the number of individual animals. Data was considered statistically significant for p values lower than 0.05 and show as significant $p \leq 0.05$ (*), $p \leq 0.01$ (**), or $p \leq 0.001$ (***).

Results

BEB grains characterization

The mean values of protein, total phenolic, flavonoids and ortho-diphenols contents obtained for four biological replicates of BEB dry grain used in this study collected are shown in the Table 1.

Table 2 presents the mean values obtained for six mineral contents (P, K, Ca, Mg, Fe and Zn) of BEB dry grains.

Table 1. Means and standard deviation of protein, total phenolic, flavonoids and ortho-diphenols contents of 'Fradel' variety.

Contents	'Fradel' BEB variety
Protein (g kg^{-1})	277.2 ± 3.36
Total phenolic (mg GA g^{-1} dw)	1.31 ± 0.18
Flavonoids (mg catechin g^{-1} dw)	1.19 ± 0.17
***Ortho*-diphenols (mg GA g^{-1} dw)**	2.99 ± 0.92

mg of gallic acid *per* mg of dry weight (mg GA g^{-1} dw)

Table 2. Means and standard deviation of six mineral contents (P, K, Ca, Mg, Fe, Zn) of 'Fradel' variety.

Contents	'Fradel' BEB variety
P (g kg^{-1})	3.27 ± 0.40
K (g kg^{-1})	9.51 ±0.28
Ca (g kg^{-1})	0.57 ± 0.14
Mg (g kg^{-1})	1.41 ± 0.06
Fe (mg kg^{-1})	40.91 ± 6.55
Zn (mg kg^{-1})	32.61 ± 2.24

Physiological parameters

No mortality or signs of toxicity were observed during the experimental period. The mean body weight was not statistically different between groups throughout the study ($p > 0.05$) (Figure 1). The mean body weight of animals from both groups increased from the initial to the final of the experiment, with mice from group II presenting a significantly higher ponderal gain (PG) when compared with mice from group I ($p = 0.036$) (Table 3).

Food and water consumption at the beginning and at the end of the experimental protocol can be observed in Table 4. Although food and water consumption were slightly higher in group II when compared with group I, these differences did not reach the level of statistical significance ($p > 0.05$). Body temperature was similar between groups throughout the experiment ($p > 0.05$) (data not shown).

In Table 5, the relative weights of different organs in both experimental groups are summarized. In a general way, the relative organs' weight was similar between groups, except for the spleen that was higher in group II when compared with group I ($p = 0.017$).

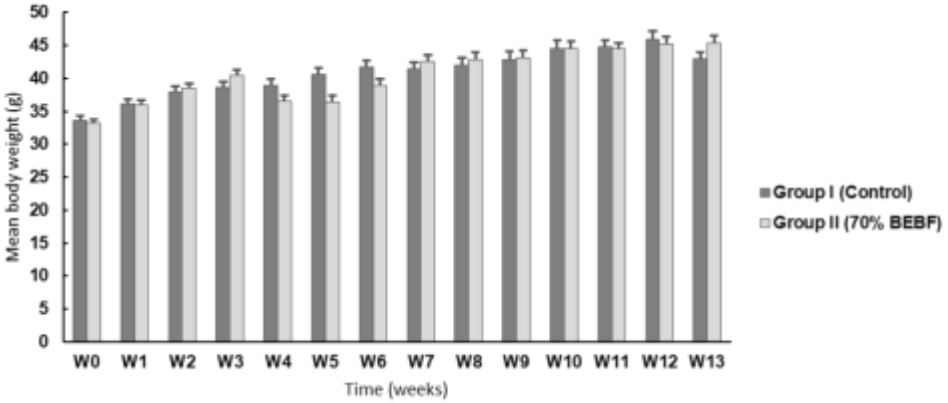

Figure 1. Body weight of both groups over the 13 weeks of the experimental protocol. Data are presented as mean ± SE. No differences were found between groups ($p > 0.05$). W- week.

Table 3. Homogeneity index (HI), initial and final mean body weights (g) and ponderal gain (PG) in both groups.

Group (*n*=10)	HI	Initial body weight	Final body weight	PG
Group I (Control)	0.920	33.63 ± 0.65	42.98 ± 1.00	0.21 ± 0.02
Group II (70% BEBF)	0.916	33.23 ± 0.52	45.37 ± 1.09	0.26 ± 0.01*

Initial and final mean body weight and ponderal gain are presented as mean ± standard error (SE).* Statistically different from group I: significant at $p \leq 0.05$.

Table 4. Initial and final food and water consumption (g).

Group (*n*=10)	Food consumption		Water consumption	
	Initial	Final	Initial	Final
Group I (Control group)	5.37 ± 0.20	6.36 ± 0.19	7.05 ± 0.04	5.13 ± 0.02
Group II (70% BEBF)	5.86 ± 0.02	6.40 ± 0.14	7.42 ± 0.01	6.26 ± 1.11

All data are presented as mean ± SE. Statistically significant differences were not observed ($p > 0.05$).

Table 5. Relative weights (%) of different organs in both groups.

Organs											
Groups (*n*=10)	**Heart**	**Lungs**	**Spleen**	**Liver**	**Thymus**	**Right kidney**	**Left kidney**	**Right gastrocnemius muscle**	**Left gastrocnemius muscle**	**Right adrenal**	**Left adrenal**
Group I (Control)	0.45 ±0.02	0.64 ±0.03	0.26 ±0.09	5.10 ±0.09	0.12 ±0.02	0.85 ±0.02	0.83 ±0.03	0.48 ±0.01	0.48 ±0.01	0.02 ±0.003	0.02 ±0.001
Group II (70% FBEB)	0.52 ±0.20	0.56 ±0.02	0.32 ±0.02*	5.13 ±0.17	0.11 ±0.08	0.88 ±0.03	0.85 ±0.03	0.46 ±0.01	0.45 ±0.02	0.064 ±0.045	0.07 ±0.039

All data are presented as mean ± SE. * Statistically different from group I: significant at $p \leq 0.05$.

Hematocrit and biochemical parameters

The group BEBF supplemented (group II) presented a higher hematocrit when compared to group control (group I) ($p = 0.049$). Although the serum levels of urea, ALT, AST and GGT were higher in group II, the differences did not reach the level of statistical significance ($p > 0.05$) (Table 6).

Table 6. Hematocrit and biochemical markers in both groups.

Groups (*n*=10)	Ht (%)	Creatinine (mg/dL)	Urea (mg/dL)	ALT (U/L)	AST (U/L)	GGT (U/L)
Group I (Control)	45.41±0.76	0.25±0.07	55.69±8.25	34.92±5.72	98.26±12.33	16.38±2.85
Group II (70% BEBF)	47.54±0.66*	0.25±0.08	83.76±15.68	41.78±4.43	131.73±18.29	24.05±2.65

All data are presented as mean ± SE. * Statistically different from group I: significant at $p \leq 0.05$.

DNA damage in mononuclear peripheral blood cells

The alkaline comet assay was performed to determine DNA damage in mononuclear peripheral blood cells. As depicted in Figure 2, GDI was similar between groups [group I: 127.26 ± 6.82 AU, group II: 125.05 ± 8.66 AU; $p = 0.057$].

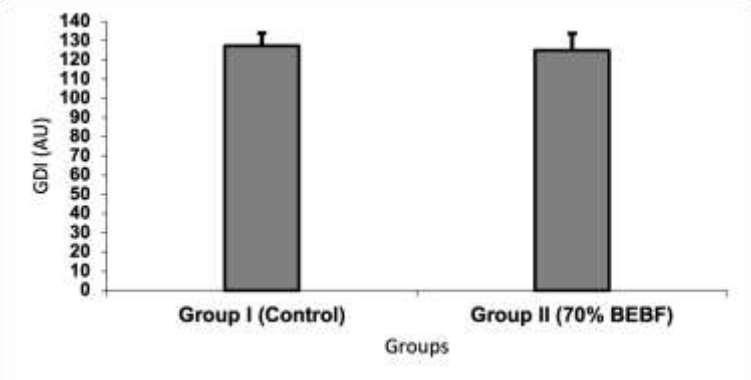

Figure 2. Genetic damage indicator (GDI) measured by comet assay in peripheral mononuclear blood cells. Data are presented as mean ± SE and expressed as arbitrary units (AU). No differences were found between groups ($p > 0.05$).

Histopathological analysis

The histopathological analysis of the colon, liver and kidney was performed for both groups. Most of the mice from both groups (7 animals from group I and 8 animals from group II) presented some degree of inflammation in the colon. Epithelial damage was not observed in any group. Although the differences were not statistically significant, the thickness of inflammatory involvement and extent of inflammatory lesions were slightly lower in animals BEBF supplemented ($p > 0.05$) (Table 7).

No significant differences were observed regarding the inflammation score attributed to the animals' kidneys and liver ($p > 0.05$) (Table 8). Histopathological images are observed in Figure 3.

Table 7. Histopathological analysis of the colon in both groups.

Group (*n*=10)	Animal	Severity of inflammation	Thickness of inflammatory involvement	Severity of epithelial damage	Extent of inflammatory lesions
Group I (Control)	1	0	0	0	0
	2	0	0	0	0
	3	2	1	0	2
	4	1	1	0	1
	5	2	2	0	1
	6	0	0	0	0
	7	1	1	0	2
	8	1	1	0	2
	9	2	1	0	2
	10	2	2	0	2
	Mean ± SEM	1.10 ± 0.28	0.90 ± 0.23	0.00 ± 0.00	1.20 ± 0.29
Group II (70% BEBF)	1	1	1	0	1
	2	2	1	0	2
	3	1	1	0	1
	4	1	1	0	1
	5	1	1	0	1
	6	2	1	0	2
	7	0	0	0	0
	8	1	1	0	1
	9	2	1	0	2
	10	0	0	0	0
	Mean ± SEM	1.10 ± 0.23	0.80 ± 0.13	0.00 ± 0.00	1.10 ± 0.23

Statistically significant differences were not observed ($p > 0.05$). SEM- standard error of the mean

Table 8. Degree of inflammation of liver and kidneys in both groups.

Group (*n*=10)	Animal	Kidney inflammation	Liver inflammation
Group I (Control)	1	0	0
	2	0	0
	3	1	0
	4	0	0
	5	0	0
	6	0	0
	7	0	0
	8	0	0
	9	0	0
	10	0	0
	Mean ± SEM	0.10 ± 0.10	0.00 ± 0.00
Group II (70% BEBF)	1	1	2
	2	0	1
	3	1	0
	4	0	0
	5	1	0
	6	0	0
	7	0	0
	8	0	0
	9	0	1
	10	0	0
	Mean ± SEM	0.30 ± 0.15	0.40 ± 0.22

Statistically significant differences were not observed ($p > 0.05$). SEM- standard error of the mean

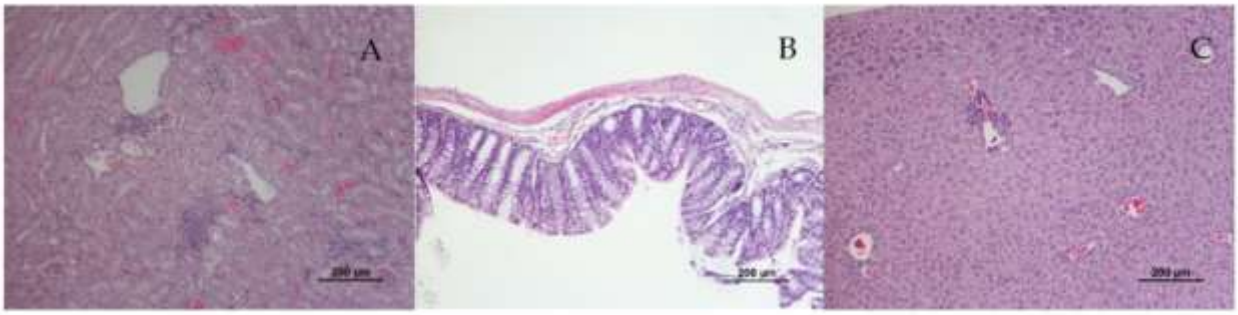

Figure 3. Histopathological analysis of the BEBF supplemented group. (**A**) Colon showing slight inflammatory infiltrate; (**B**) Liver showing a focal inflammatory infiltrate of mononuclear inflammatory cells; (**C**) Kidney with the presence of multiple foci of mononuclear inflammatory cells (H&E staining).

Hepatic oxidative stress

In Figure 4 are summarized the results of hepatic oxidative stress analysis. The group BEBF supplemented (group II) presented a higher activity of SOD (p = 0.0064) and activity of GST (p = 0.0003) when compared to group control (group I). The activity of CAT, GPx and GST enzymes, the levels of ROS, GSH and GSSG, as well as OSI and LPO were similar between the two groups ($p > 0.05$).

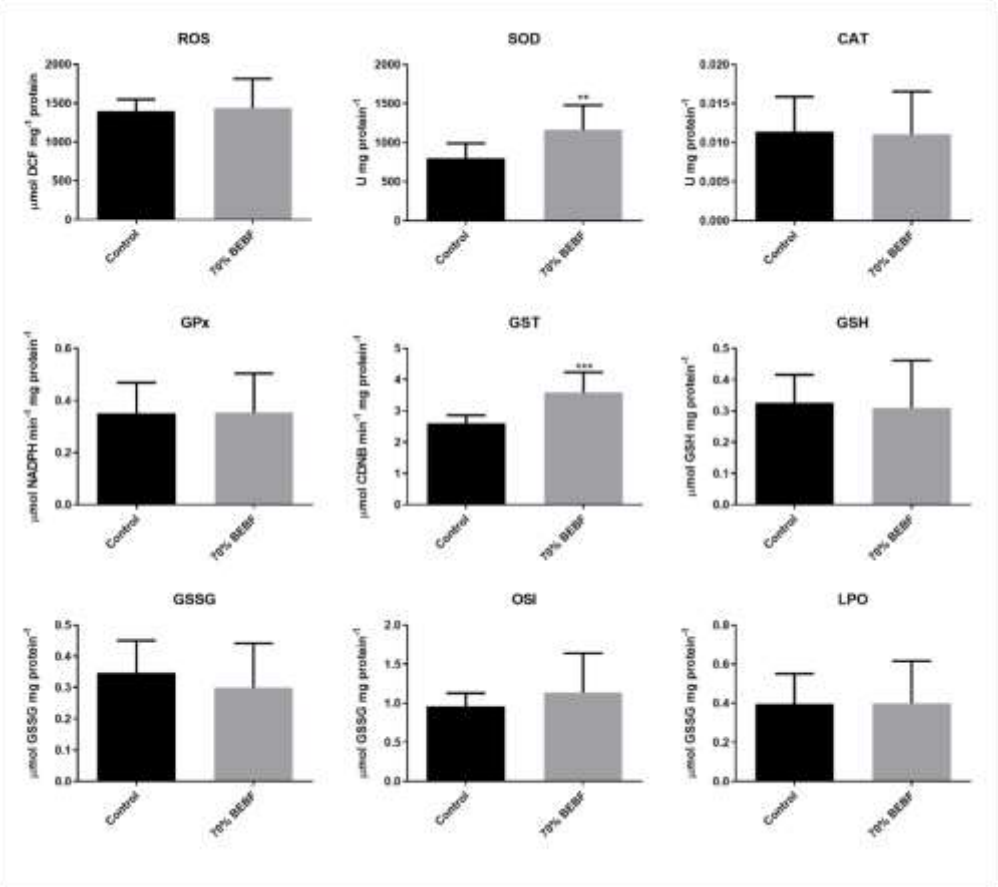

Figure 4. Oxidative stress analysis in the liver. Data are presented as mean ± SE. **Statistically different from group I: very significant ($p \leq 0.01$). *** Statistically different from group I: highly significant ($p \leq 0.001$).

Discussion

The BEB is an excellent source of protein, vitamins, minerals, soluble and insoluble fiber [1]. Protein is one of the major nutrients in BEB; our results showed the high protein content of the variety 'Fradel' (~280 g kg^{-1}) being this value in agreement with other studies [2, 7, 24]. A previous study also revealed that 'Fradel' BEB variety has good levels of free essential and non-essential amino acids including glutamine, asparagine, tyrosine and phenylalanine suggesting that meals with BEB have the potential to cover human nutritional requirements [25]. Also, the total phenolic, flavonoids and ortho-diphenols contents obtained (1.31 mg GA g^{-1} dw, 1.19 mg catechin g^{-1} dw and 2.99 mg GA g^{-1} dw, respectively) were relatively high comparatively with other BEB varieties [26]. Some reports refer that BEB

dry grains are a good source of minerals essential for human health and nutrition, especially phosphorus, potassium, calcium, magnesium, iron and zinc [7]. Our results have shown BEB's high content in these minerals (P = 3.27 g kg^{-1}; K = 9.51 g kg^{-1}; Ca = 0.57 g kg^{-1}; Mg = 1.41 g kg^{-1}; Fe = 40.91 mg kg^{-1}; Zn = 32.61 mg kg^{-1}). In this way, it can be used as a functional food in the human diet due to its antidiabetic, anti-inflammatory, anticancer and antihypertensive properties [2]. To explore the possible influence of BEB on mice, they were fed with a diet containing 70% of BEBF for 13 weeks. As far as we know, this is the first experimental study to evaluate the effects of long-term supplementation of BEBF in mouse physiological parameters.

No physiological or behaviour changes, as well as mortality were recorded in the present study. The food consumption was similar between groups, suggesting that the BEBF-diet was accepted and well-tolerated by the animals and that palatability was not affected. Mean body weight was similar between the groups throughout the experimental protocol. As the animals were young at the beginning of the study and were growing, the final body weight was greater than the initial body weight. The weight gain of the BEBF-supplemented group was significantly greater when compared to the control group, suggesting a positive influence of the BEBF diet on body weight. As food consumption was similar between groups, the BEBF diet can be more caloric than the control diet. Further investigation using animals of different ages and food analysis is required to clarify this point.

The spleen was the only organ with a significant increase of relative weight in BEBF group when compared to the control group, suggesting splenomegaly development in animals BEBF-supplemented. Previous studies associated splenomegaly with leucocytosis, extramedullary hematopoiesis, erythrophagocytosis or anemia [27, 28]. Also, a significant increase in microhematocrit was observed in animals fed with BEBF. In a previous study with mice, the authors found that splenomegaly and increased microhematocrit levels may be associated with extramedullary hematopoiesis in the spleen [30]. Thus, in the future histological examination of the spleen was desirable to confirm the presence of extramedullary hematopoiesis, justifying the enlarged spleen, as well as the increased values of the erythrocyte volume in BEBF-supplemented group.

Panzeri *et al.* [31] tested *V. unguiculata* seeds extract in four human colorectal cancer cell lines (E705, DiFi, SW480 and Caco-2) and observed a reduction in cell viability and proliferation. Moreover, they observed that the extract did not affect normal cells (using CCD841 healthy cell line), suggesting that BEB can prevent colorectal cancer without affecting health. Although not statistically significant, a slight reduction of inflammation was observed in the colon of group BEBF-supplemented. Additional studies using an animal model of colon cancer may help to assess the preventive effect of BEB.

In terms of the comet assay, BEBF intake for 13 weeks had no effect on the animals. The histological analysis of the liver and kidney revealed a slight increase in inflammation in animals fed with BEBF ($p > 0.05$). Furthermore, the biochemical analysis also demonstrated a slight increase of hepatic biomarkers (AST, ALT, GGT) and renal damage (increased urea levels) in the same group ($p > 0.05$). However, both histological and biochemical analyses did not reveal any significance to affirm that BEBF induces hepatic and renal lesions. In fact, Iyanda showed that BEB did not cause hepatic or renal damage in Wistar rats [32].

Exposure to a variety of chemicals causes oxidative stress in various species, increasing ROS and, as a result, activation of the antioxidant system to protect organisms through the induction of antioxidant enzymes such as CAT, SOD, and others [33]. Thus, these enzymes protect the body against oxidative damage [34]. The SOD increased levels could result from the stimulation of antioxidant activity by BEBF. GSTs scavenge free radicals by catalyzing reduced GSH, combining it with other side products of lipid peroxidation for detox purposes [35]. Thus, its increase could mean that BEBF incorporation at 70% may be excessive, however, the increase of GST may be due to stimulation of the detoxification capacity and not hepatic damage. New studies with lower percentages should be carried out to clarify this point. Ibrahim Sayeed and colleagues observed that SOD, GSH, CAT and LPO levels were increased in group supplemented with an aqueous extract of *V. unguiculata* and demonstrated that the extract has hepatoprotective activity in Wistar albino rats with ethanol-induced acute liver damage [36]. In addition, flavonoids exhibited antioxidant effects and their ability to increase SOD in normal mice has already been demonstrated [37]. Also, flavonoids can be influencing the increase in GST in the liver [38].

We are fully aware that our study presents some limitations. First, other concentrations of BEBF and other times of exposure would be desirable. Moreover, BEB could be prepared in another way: 1) cooked before incorporation into the diet or 2) not crushed. Indeed, the cooking process creates chemical and physical changes in the food, which can degrade certain compounds or otherwise increase their bioavailability [39]. Furthermore, we are aware that the pelleting process could degrade some compounds due to the humidity and heat it requires [40]. In the future it would also be interesting to analyse the photochemical profile of the pellets to find out whether the chemical composition is maintained after the pelleting processes. Finally, a more detailed phytochemical analysis of BEB could be relevant but is not crucial. The aim of this study was the evaluation of BEBF as a functional food supplement, and made sense to be ingested as a whole, and not as an extract with pharmacological properties. Thus, it is worth to note that the observed effects were probably due to synergistic actions between several *V. unguiculata* compounds.

Conclusions

The BEB is a source of macronutrients, micronutrients and bioactive molecules that benefit human health. BEB is important to feed the population of developing countries, especially African countries, not only for its economic

importance but also for its adaptability and easy cultivation. This study in healthy mice showed that dietary with BEBF does not have any adverse long-term effects on health, an important finding. In terms of translational medicine, the results suggested that it can be used as a functional food with health benefits (such as colon lesions prevention) and has no harmful effects in long-term use.

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Conflict of interest statement
None to declare.

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