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Whole flour and protein hydrolysate from common beans reduce the inflammation in BALB/c mice fed with high fat high cholesterol diet

Sâmara Letícia Silva de Lima[a], Mariana Juste Contin Gomes[a], Bárbara Pereira da Silva[a], Natália Elizabeth Galdino Alves[a], Renata Celi Lopes Toledo[a], Jaqueline Maciel Vieira Theodoro[a], Maria Eliza de Castro Moreira[a], Juliana Aparecida Correio Bento[b], Priscila Zaczuk Bassinello[b], Sérgio Luis Pinto da Matta[c], Elvira Gonzalez De Mejía[d], Hercia Stampini Duarte Martino[a,*]

[a] Department of Nutrition and Health, Federal University of Viçosa, Viçosa, Minas Gerais 36570000, Brazil
[b] Embrapa Rice and Bean, Rodovia GO-462, Km 12. Zona Rural, Santo Antônio de Goiás, Goiás 75375000, Brazil
[c] Department of Biology, Federal University of Viçosa, Viçosa, Minas Gerais 36570000, Brazil
[d] Department of Food Science and Human Nutrition, University of Illinois Urbana-Champaign. 228 ERML, MC-051, 1201 West Gregory Drive, Urbana, IL 61801, USA

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ABSTRACT

Common bean (*Phaseolus vulgaris* L.) is a source of bioactive peptides, but little is known about its effects on hypercholesterolemia, oxidative stress, and the inflammatory process. Therefore, the aim of this study was to evaluate the effect of whole flour and bean protein hydrolysate of common bean variety Carioca on inflammation and oxidative stress in BALB/c mice. Four experimental groups were included in the study: standard diet (SD), high fat high cholesterol diet (HFC), high fat high cholesterol diet and whole bean flour (HFC-F); and high fat high cholesterol diet and bean protein hydrolysate (HFC-PH). Animals fed with bean protein hydrolysate showed lower weight gain and food intake. Animals fed with whole bean flour showed lower alanine aminotransferase and low-density lipoprotein cholesterol levels than animals fed with bean protein hydrolysate. SOD mRNA was lower in HFC, HFC-F and HFC-PH groups whereas SOD concentration was higher in HFC-F and HFC-PH groups. HSP72 mRNA expression was lower in the HFC-F group in relation to HFC-PH. IL-10 and PPARα mRNA expression was lower in HFC-F and HFC-PH groups in comparison with SD. The whole bean flour and bean protein hydrolysate reduced inflammation and the risk factors for cardiovascular diseases in BALB/c mice.

1. Introduction

Cardiovascular diseases (CVD) are the main causes of morbidity and mortality worldwide. It is estimated that 17.9 million people died from CVD in 2016, which represented 31% of deaths worldwide (WHO. World Health Organization, 2017). Hypercholesterolemia is one of the leading behavioural risk factors for CVD (Lammi, Zanoni, & Arnoldi, 2015; Nelson, 2013).

Hypercholesterolemia results in changes in the structure and function of blood vessels (Granger, Rodrigues, Yildirim, & Senchenkova, 2010) and lipid accumulation in liver tissue (Andrade et al., 2013; Nair et al., 2014). In response to these changes, there is an increase in lipid peroxidation and the production of reactive oxygen species (ROS) (Kurtel, Rodrigues, Yilmaz, Yildirim, & Granger, 2013). The oxidative stress induces the production of proinflammatory cytokines such as tumor necrosis factor (TNF-α) and activates the inflammatory pathway of nuclear transcription factor kappa B (NFκB) (Cai et al., 2005; Su et al., 2015). This signaling produces more inflammatory ROS and cytokines, continuously increasing oxidative stress and cell damage (Schett, Elewaut, Mcinnes, Dayer, & Neurath, 2013). In this sense, the search for dietary strategies to prevent and control hypercholesterolemia, oxidative stress, and the inflammatory process is of great interest for CVD prevention (WHO. World Health Organization, 2017).

Common bean (*Phaseolus vulgaris* L.) is one of the most consumed and important legumes in the world (Ramírez-Jiménez, Reynoso-Camacho, Tejero, León-Galván, & Loarca-Piña, 2015). Beans are sources of protein, carbohydrates, dietary fiber, starch, phenolic compounds, vitamins and minerals (Martino et al., 2012; Tharanathan & Mahadevamma, 2003). Protein concentration ranges from 20 to 30% (Hayat, Ahmad, Masud, Ahmed, & Bashir, 2014) and its peptide derivatives are related to negative modulation of inflammatory process (Alves, Mejía, Vasconcelos, Bassinello, & Martino, 2016; Alves,

* Corresponding author.
E-mail addresses: hercia72@gmail.com, hercia@ufv.br (H.S.D. Martino).

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Bean proteins after a process of digestion, fermentation or enzymatic hydrolysis, give rise to biologically active hydrolysates and peptides with important physiological functions, such as antioxidant, anti-inflammatory, antihypertensive and anti-carcinogenic activity (Alves, Mejía, et al., 2016; Luna-Vital, Mojica, Mejía, Mendoza, & Loarca-Piña, 2015; Oseguera-Toledo, Mejia, Dia, & Amaya-Llano, 2011; Hernández-Saavedra et al., 2013; Feregrino-Perez et al., 2014; Pereira & Tavano, 2014; Mojica, Chen, & de Mejía, 2015; Mojica and de Mejía, 2015). However, most of the effects aforementioned are from *in vitro* studies. Thus, the aim of this study was to analyze the chemical composition of the whole bean flour and the bean protein hydrolysate, as well as, for the first time to investigate its effect on oxidative stress and inflammation in BALB/c mice fed with high fat high cholesterol diet.

2. Materials and methods

2.1. Samples

Common bean cultivar BRSMG Madreperola (*Phaseolus vulgaris* L.), were cultivated and harvested by EMBRAPA Rice and Bean (Santo Antônio de Goiás, GO, Brazil). Beans were cooked under pressure (1:2 beans/water) for 50 min at 120 °C, oven-dried for 8 h/60 °C and crushed (600 μm sieve size), Grinder Vertical Rotor MA 090 CFT, Marconi Equipment, Piracicaba, SP, Brazil). Protein hydrolysate was obtained by simulated gastrointestinal digestion process (Alves, Mejía, et al., 2016) using pepsin and pancreatin enzymes (Megías et al., 2004). The flour and bean protein hydrolysate were packed under vacuum and kept at −20 °C until analysis.

2.2. Analysis of macronutrients, total dietary fiber, phytate, tannin and phenolic compounds in whole bean flour and phenolic compounds in bean protein hydrolysates

The chemical composition in whole bean flour was determined in triplicate using the analytical procedures recommended by AOAC (2016) for moisture (AOAC 950.46), mineral residue (ash) (AOAC 920.153), protein (AOAC 945.18) and lipid content (AOAC 923.05). Total dietary fiber, soluble and insoluble fractions were determined through the enzymatic gravimetric method (AOAC 900.02), in duplicate. Carbohydrate content was calculated as the difference, using the equation: [100 – (% moisture + % lipids + % proteins + % total dietary fiber + % ash)]. Phytic acid concentration was determined by ion exchange chromatography and spectrometry (Latta & Eskin, 1980) with modifications (Ellis & Morris, 1986). Total tannins content was evaluated by the vanillin reaction (Burns, 1971) with modifications (Maxson & Rooney, 1972; Price, Van Scoyoc, & Butler, 1978).

Extracts from bean flour and bean protein hydrolysate were obtained according to Bloor (2001). Total phenolic compounds were determined by Folin-Ciocalteau reagent (Singleton, Orthofer, & Lamuela-Raventós, 1999). Results were quantified using a standard curve obtained with concentrations of gallic acid from 0 to 250 ppm and expressed as milligrams of gallic acid equivalents per gram of sample (mg GAE g^{-1}).

In order to evaluate the presence of phenolic compounds of bean flour and protein hydrolysate, it was previously extracted in methanol/water/hydrochloric acid (50:48:2, *v*/v/v) solution. The analyses were performed on UPLC-ESI-QTOF-MS/MS (Acquity UPLC/XEVO, Waters, Milford, USA) equipped with QtoF system (Waters) and Masslynx 4.1 software (Waters), under chromatographic conditions (Huang, Chen, Feng, Guo, & Li, 2013; Iswaldi et al., 2013; Journi, Hammouda, Trabelsi-Ayadi, & Chérif, 2015; Singh et al., 2015). The structures of identified phenolic compounds were obtained from PubChem Compound database, hosted on NCBI (National Center for Biotechnology Information) platform (https://pubchem.ncbi.nlm.nih.gov/).

2.3. Animals and diets

A controlled experimental study was used to assess the oxidative stress and inflammation *in vivo*. Forty-eight adult male BALB/c mice with 60 days of age were distributed into 4 groups with 12 animals each, allocated according to the homogeneity of body weight to make an equal dissemination among groups (da Silva et al., 2019; Dias et al., 2018), so that the difference in weight among groups did not exceed 3 g (mean weight: 32.90 g ± 2.96; no statistical difference between the experimental groups). Animals were distributed in individual stainless-steel cages in temperature-controlled room (22 ± 2 °C) and automatically controlled light-dark cycles of 12 h. Deionized water and respective experimental diets were supplied *ad libitum*. The experimental diets were based on AIN-93 M ($n = 12$) (Reeves, Nielsen, & Fahey, 1993) and high fat high cholesterol diet ($n = 36$) (Anandhi, Thomas, & Geraldine, 2014; Colina-Coca et al., 2014

The experimental groups received the following diets: control diet (CD); high fat high cholesterol diet (HFC); high fat high cholesterol diet and whole bean flour (HFC-F); high fat high cholesterol diet and bean protein hydrolysate (HFC-PH). The amount of whole bean flour offered to HFC-F group (346.6 g/kg of diet) was based on its protein composition in order to provide 50% of dietary protein requirement. The other ingredients were added in sufficient amounts to balance the composition of lipids, proteins, carbohydrates, fiber, and calories (Table 1). The diets were kept under freezing temperature (20 °C) and were placed daily for the animals. During the preparation of the experimental diets, whole bean flour was incorporated to the diet, by mixing with sugar, dextrinized starch, mineral mix, vitamin mix, corn starch, soybean oil, casein and the other ingredients. The bean protein hydrolysate (BPH) was offer in the quantity of 700 mg/kg/day. This amount was based on the difficult to find similar investigations, so, it was used higher dose than bean hydrolyzed protein isolate used in a study of glucose tolerance in rats) (Mojica, de Mejia, Granados-Silvestre, & Menjivar, 2017). The bioactive peptides from BPH was previously characterized and presented peptide sequences (LVTTTVDL, QTSTPLFS, VELVGPK and TRGVLV) with functional proprieties, mainly related to control of glucose, antioxidant and anti-inflammatory activities (Alves, Mejía, et al., 2016). The 6-propil-2-thiouracil (PTU) (10 mg/kg/day) (Panda & Kar,

Table 1
Composition of experimental diets (g/kg of diet).

Ingredients (g/Kg)	SD	HFC	HFC-F	HFC-PH
Casein*	170.73	218.19	124.47	218.19
Cooked whole bean flour	0.00	0.00	346.60	0.00
Dextrinized starch	155.00	105.50	0.00	105.50
Sucrose	100.00	300.00	218.63	300.00
Lard	0.00	200.00	200.00	200.00
Celulose	62.01	62.01	0.00	62.01
Soy oil	40.00	40.00	36.00	40.00
Mineral mix	35.00	35.00	35.00	35.00
Vitamin mix	10.00	10.00	10.00	10.00
Cholesterol	0.00	20.00	20.00	20.00
Choline bitartrate	2.50	2.50	2.50	2.50
L-cystine	1.80	1.80	1.80	1.80
Colic Acid	0.00	5.00	5.00	5.00
Corn starch	422.96	0.00	0.00	0.00
Carboydrate (%)	76.29	45.89	44.35	45.89
Protein (%)	19.21	24.69	24.85	24.69
Lipids (%)	4.50	29.42	30.80	29.42
Phenolic compounds (mg GAE)	Nd	nd	48.52	**
Energy (kcal/kg)	3754.76	4834.76	4677.97	4834.76
CD (kcal/g^{-1})	3.75	4.83	4.67	4.83

* Purity of 82%.
** The concentration of phenolic compounds in the protein hydrolyzate is showed in Table 4.
SD: standard diet; HFC = high fat high cholesterol diet; HFC-F: high fat high cholesterol diet and whole bean flour; HFC-PH: high fat high cholesterol diet and protein hydrolyzate; CD = caloric density.

2005; Panda, Kar, & Patil, 2009) was also administered by gavage to all HFC groups to increase cholesterol total, LDL-c and triglycerides, by suppression of thyroid hormones (Liou, Chang, Liaw, & Jiang, 2005). On the 63rd day, in the morning and after 12 h fasting, animals were anesthetized with isoflurane (Isoforine, Cristália), according to the body weight of the animal. This procedure occurred in Experimental Nutrition Laboratory of the Federal University of Viçosa, Brazil. Blood was collected by cardiac puncture and centrifuged under 4 °C at 800 × *g* for 10 min (Fanem-204, São Paulo, Brazil) and the serum was stored at −80 °C. Liver tissue was collected and stored at −80 °C before analysis.

Body weight gain and feed consumption were monitored weekly during the experiment period to determine the feed efficiency ratio (FER) (da Silva et al., 2016). The hepatosomatic index was calculated by the relationship between liver weight and body weight, multiplied by 100 (Kim, Hong, Jeon, & Kim, 2016). Body mass index (BMI) was calculated by the relationship between weight and naso-anal length (cm) squared (Keys, Fidanza, Karvonen, Kimura, & Taylor, 1972). All experimental procedures with animals were performed in accordance with the ethical principles for animal experimentation and the study was approved by the Ethics Committee of the Federal University of Viçosa (Protocol 97/2015; date of approval: March 04th 2016).

2.4. Biochemical analysis

For the determination of biochemical analysis, 0.5 mL of serum was used. Serum glucose concentrations, total cholesterol, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL) and very-low density lipoprotein (VLDL), triacylglycerides (TGL), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured by colorimetric method using commercially available kits following the manufacturer's instructions (Bioclin, Brazil). Analyses were performed on a BS-200 Chemistry Analyzer (Bioclin).

2.5. Homogenate preparation

To obtain liver homogenate, 100 mg of liver was mixed with 1000 µL of phosphate buffer (50 mM) and 1 mM EDTA (pH 7.4). The sample was macerated and centrifuged at 12,000 × *g* for 10 min at 4 °C, and then the supernatant was removed and stored in an ultra-freezer until analysis.

2.5.1. Superoxide dismutase (SOD)

Quantification of the enzyme superoxide dismutase (SOD) was performed on samples of the homogenate as described above. To the aliquotted samples, were added 249 µL of 50 mM Tris-HCl buffer (pH 8.2), containing 1 mM EDTA; 6 µl MTT (1.25 mM) and 15 µl pyrogallol (10 mM). For the standard, it was added 6 µL of MTT, 15 µl of pyrogallol (10 mM) and 279 µl of buffer. To obtain the blank, 6 µl of MTT and 294 µl of buffer were applied to the wells. The samples, standard and blank were incubated at 37 °C for 5 min and the reading was performed on a Multiskan GO (Thermo Scientific) spectrophotometer at 570 nm. Results were expressed as units of SOD / mg protein (Marklund, 1985).

2.5.2. Catalase

Catalase was analyzed in liver homogenate according to the methodology proposed by Aebi (1984). At 0, 30, and 60 s after the reaction was initiated, the absorbance at 240 nm was determined with a spectrophotometer (T70 + UV/VIS Spectrometer). Enzyme activity was reported as µmol per mL of sample. Catalase activity was calculated according to Lambert Beer's law.

2.6. TNF-α and IL-10 quantification

The TNF-α and IL-10 quantification was performed in liver. To determine the concentration of TNF-α and IL-10 in the liver, hepatic tissue samples were homogenized by means of using PBS (pH 7.4). The TNF-α was assessed by immunoassay using the Rat TNF alpha uncoated Elisa (Ref. 88–7340; Invitrogen, USA), and the IL-10 was assessed by immunoassay using the Rat IL-10 (interleukin 10; *E*-EL-R-0016; Elisa kit, Invitrogen, USA), according to the manufacturer's recommendations. The microplates that were provided in the ELISA kits were respectively precoated with anti-a TNF-α and anti-IL10 antibody. Absorbance at 450 nm was measured spectrophotometrically. The concentration of TNF-α and IL-10 was calculated by comparison to corresponding standard curves.

2.7. Extraction of mRNA in liver tissue and cDNA synthesis

The tissue was macerated in liquid nitrogen under RNase free conditions and the samples were aliquoted for total RNA extraction. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) using the manufacturer's recommendations. A 2 µg of extracted mRNA was used to synthesize cDNA using the M-MLV reverse transcription kit (Invitrogen Corp., Grand Island, NY) according to the manufacturer's protocol (Livak & Schmittgen, 2001).

2.8. Determination of gene expression of proteins involved in inflammation by quantitative reverse transcriptase polymerase chain reaction (RT-qPCR)

mRNA expression levels of proteins from liver involved in inflammation process was analyzed by RT-qPCR. SYBR green PCR master mix from Applied Biosystems (Foster City, CA) was used and analyses were performed on the StepOne™ Real-Time PCR System (Thermo Fisher Scientific, USA) using the measurement system by SYBR-Green Fluorescence and Primer Express software (Applied Biosystems, CA). The RT-qPCR involved an initial denaturation cycle of 95 °C (10 min) and then 40 cycles with 1 min denaturation (95 °C), 1 min annealing (62 °C), followed by a standard dissociation curve. Sense and antisense primer sequences (Choma Biotechnologies, Brazil) were used to amplify heat shock proteins (HSP72), superoxide dismutase (SOD), nuclear transcription factor kappa B (NFκB), tumor necrosis factor α (TNF-α); interleukin 10 (IL-10) and peroxisome proliferator-activated receptor alpha (PPAR-α). The relative expression levels of mRNA were normalized by the endogenous control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Table 2). All steps were performed under open conditions with RNase.

2.9. Histomorphometric analysis of liver

Semi-serialized histological liver fragments of 3 µm thickness were obtained on a semi-automated rotating microtome (Leica, Brazil) and stained by toluidine blue technique. Slides were examined under a Axio SCOPE.A1 photomicroscope (Zeiss, Germany). For visualization and quantification of areas of steatosis and hepatic inflammation, a manual

Table 2
Sequence of primers used in the RT-PCR analysis.

Genes	Oligonucleotide (5′-3′)	
	Forward	Reverse
GAPDH	AGGTTGTCTCCTGTCACTTC	CTGTTGCTGTAGCCATATTC
HSP72	AGGCCAACAAGATCACCATC	TAGGACTCGAGCGCATTCTT
SOD	TGTGTCCATTGAAGATCGTGTG	CTTCCAGCATTTCCAGTCTTTG
NFκB	CTTCTGGGCCATATGTGGAGA	TCGCACTTGTAACGGAAACG
TNF-α	ACGGCATGGATCTCAAAGAC	AGATAGCAAATCGGCTGACG
IL-10	ACTACCATAGCCACAACGCA	TTTCTGTTTCCTACGGCGCT
PPAR-α	CCTGCCTTCCCTGTGAACT	ATCTGCTTCAAGTGGGGAGA

GAPDH: glyceraldehyde 3-phosphate dehydrogenase; HSP72: heat shock proteins; SOD: superoxide dismutase; NFkB: nuclear transcription factor kappa B; TNFα: tumor necrosis factor α; IL-10: interleukin 10; PPARα: peroxisome proliferator-activated receptor alpha.

counting was performed on nucleus, cytoplasm, fat vesicles and macrophages in the tissue, comprising twenty random fields per animal (Cavaliere, 2013) and using ImageJ® software version 1.48 (Wayne Rasband, National Institutes of Health, USA).

2.10. Statistical analysis

The treatments were conducted in a completely randomized design, with twelve replicates. The results were subjected to analysis of variance. To determine "F-value" significance, the *post hoc* Newman-Keuls test were carried out to compare means among the diet groups. The main dispersion was expressed as standard deviation. Statistical analyzes were performed GraphPad Prism software® (GraphPad Software, CA), version 5.0. Data with a *P*-value < 0.05 were considered statistically significant.

3. Results

The whole bean flour showed high concentration of protein (24.63 ± 0.36 g/ 100 g) and dietary fiber (17.89 ± 0.51 g/ 100 g). The content of lipids was 1.17 ± 0.18 g/ 100 g and the concentration of carbohydrate was 44.98 ± 0.60 g/ 100 g. The bean flour presented phytate (122.38 ± 0.45 mg/100 g) and tannin (4.47 ± 0.23 mg EC/ g). Total phenolic compounds concentration in whole bean flour and bean protein hydrolysate were 1.06 ± 0.17 mg GAE/ g and 1.40 ± 0.06 mg GAE/g of sample, respectively. Besides we analyze the content of total phenolic, we performed the chromatographic identification of these compounds. The phenolic compounds identified in the whole bean (catechin and kaempferol) and in the bean protein hydrolyzate (kaempferol) are presented in Fig. 1.

Food intake in a HFC-F group did not differ of standard group ($p > 0.05$). However, food intake and total phenolic consumption were higher ($p < 0.05$) in HFC-F group compared to HFC-PH group. In addition, food efficiency ratio (FER) did not differ ($p > 0.05$) among groups. The body mass index (BMI), liver weight and hepatosomatic index were higher ($p < 0.05$) in HFC-F group after the 63 days of intervention (Table 3). The consumption of whole bean flour did not affect weight gain and food consumption, however the phenolic consumption in this group was higher (18.45 ± 1.42 mg GAE) than the HFC-PH group (0.20 ± 0.01 mg GAE).

The histological analysis of hepatic tissue showed no difference between the percentage of nucleus, cytoplasm and macrophages among the groups that received high fat high cholesterol diets ($p > 0.05$). However, in the group fed standard diet (SD) showed lower fat globules than the other groups (fed high fat high cholesterol diet). Beside this, the treatments with the whole bean flour and bean protein hydrolysate was not able to alter ($p > 0.05$) the fat concentration in hepatic cells'cytoplasm in these groups (Fig. 2). Thus, the difference in liver histological analysis was not observed in normal groups fed with high fat high cholesterol diet associate with whole bean or bean protein hydrolysate.

Liver weight and the hepatosomatic index in HFC-F group were higher than in HFC group (Table 3). However, the activity of ALT enzyme was lower (Table 4). HFC-F and HFC-PH presented lower ($p < 0.05$) glucose levels than SD and HFC groups. Total cholesterol was higher in HFC, while HFC-F and HFC-PH presented the lowest

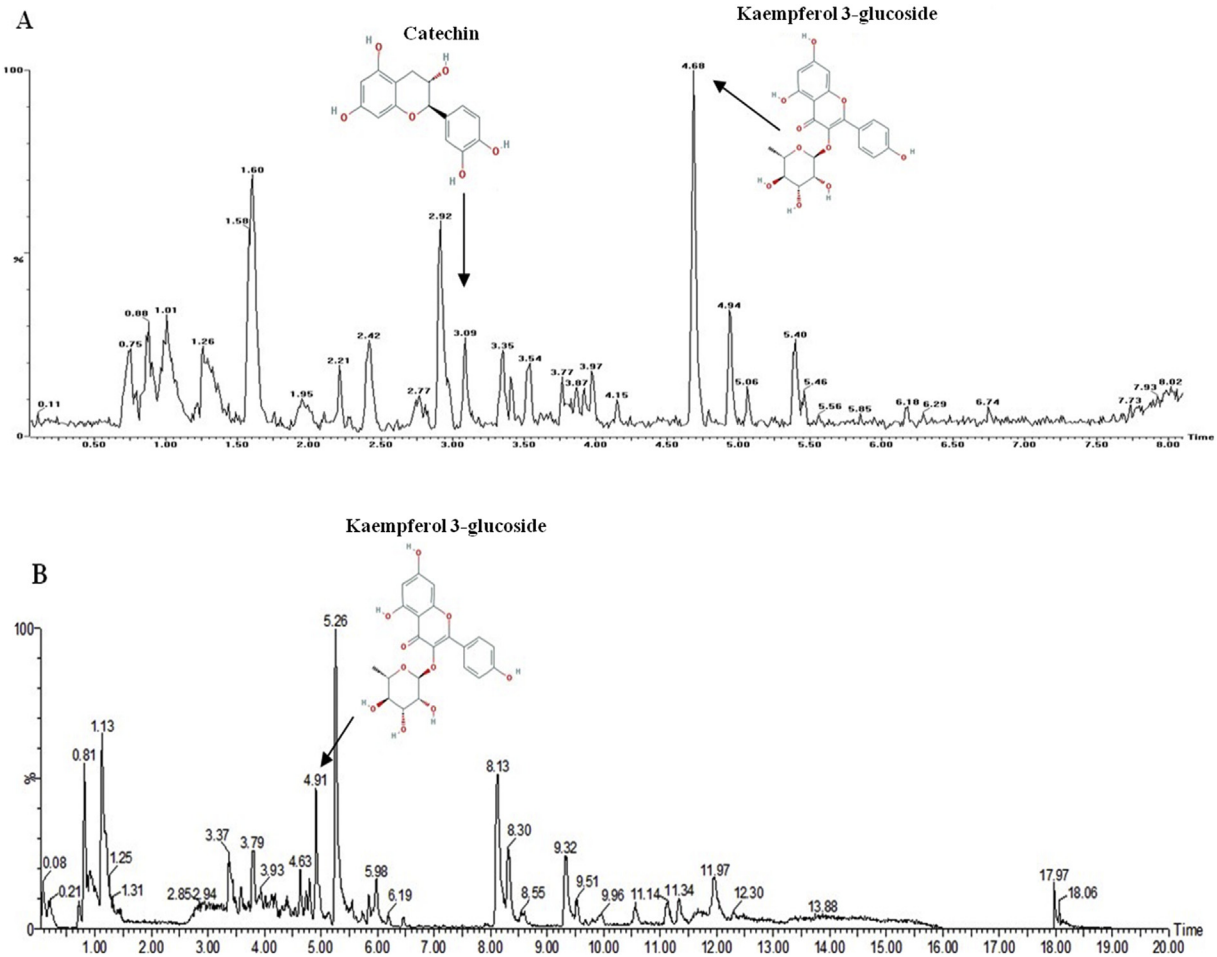

Fig. 1. Chromatograms and chemical structures corresponding to the phytochemicals of the extracts of the whole grain flour (A) and the bean protein hydrolyzate (B) of BRSMG Madrepérola beans. The arrow indicates the retention time of catechin and kaempferol 3-glucoside.

Table 3
Effect of bean flour and bean hydrolysate protein intake on biometric variables and consumption in Balb-c mice (n = 12) for 63 days.

Groups	SD	HFC	HFC-F	HFC-PH
Food consumption (g/week)	37.80 ± 2.47[a]	36.81 ± 3.59[a]	38.19 ± 2.15[a]	32.04 ± 2.38[b]
Phenolic consumption (mg GAE/week)	–	–	18.46 ± 1.42[a]	0.20 ± 0.01[b]
Weight gain (g)	9.80 ± 1.38[a]	10.70 ± 1.99[a]	10.59 ± 2.86[a]	7.72 ± 1.10[b]
FER (g)	0.03 ± 0.00[a]	0.03 ± 0.00[a]	0.03 ± 0.00[a]	0.02 ± 0.00[a]
BMI ($g.cm^{-2}$)	0.37 ± 0.02[b]	0.39 ± 0.02[b]	0.42 ± 0.03[a]	0.38 ± 0.01[b]
Liver weight (g)	1.69 ± 0.11[c]	2.70 ± 0.32[b]	3.35 ± 0.48[a]	2.38 ± 0.28[b]
Hepatossomatic index (%)	4.04 ± 0.36[c]	6.28 ± 0.65[b]	7.49 ± 0.46[a]	6.19 ± 0.89[b]

SD: standard diet; HFC = high fat high cholesterol diet; HFC-F: high fat high cholesterol diet and whole bean flour; HFC-PH: high fat high cholesterol diet and protein hydrolyzate. FER: food efficiency ratio; BMI: body mass index; GAE: gallic acid equivalent. Average scores on the lines followed by different letters differ by Newman-Keuls test ($p < 0.05$).

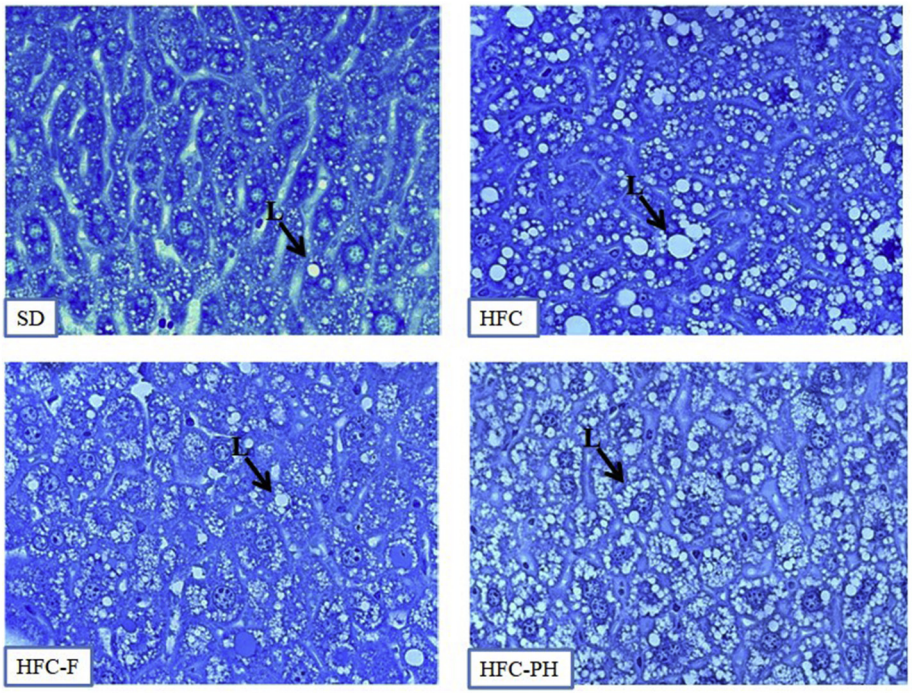

Fig. 2. Liver photo of adult Balb-c mice (n = 12). Effect of bean flour and bean protein hydrolysate intake for 63 days on fat vesicles. SD: standard diet; HFC = high fat high cholesterol diet; HFC-F: high fat high cholesterol diet and whole bean flour; HFC-PH: high fat high cholesterol diet and protein hydrolyzate; L: lipids.

values ($p < 0.05$). All high fat high cholesterol groups presented lower HDL-c and higher ($p < 0.05$) ALT and LDL-c than SD group, but for ALT and LDL-c the values were lower for HFC-F in comparison to HFC and HFC-PH ($p < 0.05$). Triacylglyceride levels were lower in HFC-F and HFC-PH groups in comparison with SD ($p < 0.05$) (Table 4). Glucose, triacylglycerols, total cholesterol and LDL cholesterol levels were lower in HFC-F group compared to HFC group.

mRNA Superoxide dismutase (SOD) expression was lower in high fat high cholesterol groups (HFC, HFC-F and HFC-PH) compared to SD (Fig. 3A). However, the SOD activity was higher ($p < 0.05$) in HFC-F than HFC group (Fig. 3B). mRNA HSP72 expression was higher ($p < 0.05$) in HFC-F and HFC-PH groups compared to SD. Animals fed HFC-F did not differ from SD (Fig. 3C). Catalase concentration did not differ ($p > 0.05$) among HFC groups (HFC, HFC-F and HFC-PH) (Fig. 3D).

The group fed with bean protein hydrolysate (HFC-PH) showed a

Table 4
Effect of bean flour and bean hydrolysate protein intake on the biochemical variables in Balb-c mice (n = 12) for 63 days.

Groups	SD	HFC	HFC-F	HFC-PH
Glucose ($mg\ dL^{-1}$)	233.10 ± 22.82[a]	226.26 ± 26.82[a]	190.27 ± 22.29[b]	187.18 ± 14.18[b]
TC ($mg\ dL^{-1}$)	124.17 ± 11.61[ab]	140.71 ± 21.14[a]	109.06 ± 26.35[b]	115.33 ± 17.15[b]
HDL ($mg\ dL^{-1}$)	68.00 ± 4.00[a]	55.00 ± 7.00[b]	52.00 ± 14.00[b]	47.00 ± 9.00[b]
LDL ($mg\ dL^{-1}$)	12.00 ± 1.00[c]	32.00 ± 9.00[a]	23.00 ± 4.00[b]	34.00 ± 4.00[a]
TGL ($mg\ dL^{-1}$)	45.89 ± 7.85[a]	25.20 ± 4.82[b]	18.16 ± 2.77[c]	18.66 ± 5.66[c]
AST (UL^{-1})	209.17 ± 29.72[a]	263.60 ± 64.90[a]	207.60 ± 41.57[a]	264.25 ± 57.38[a]
ALT (UL^{-1})	34.83 ± 4.20[c]	126.50 ± 18.29[a]	89.40 ± 28.39[b]	137.25 ± 32.10[a]

SD: standard diet; HFC = high fat high cholesterol diet; HFC-F: high fat high cholesterol diet and whole bean flour; HFC-PH: high fat high cholesterol diet and protein hydrolyzate; HDL: high-density lipoprotein; TC: total cholesterol; TGL: triacylglyceride; AST: alanine aminotransferase; ALT: aspartate aminotransferase; LDL: low-density lipoprotein. Average scores on the lines followed by different letters differ by Newman-Keuls test ($p < 0.05$).

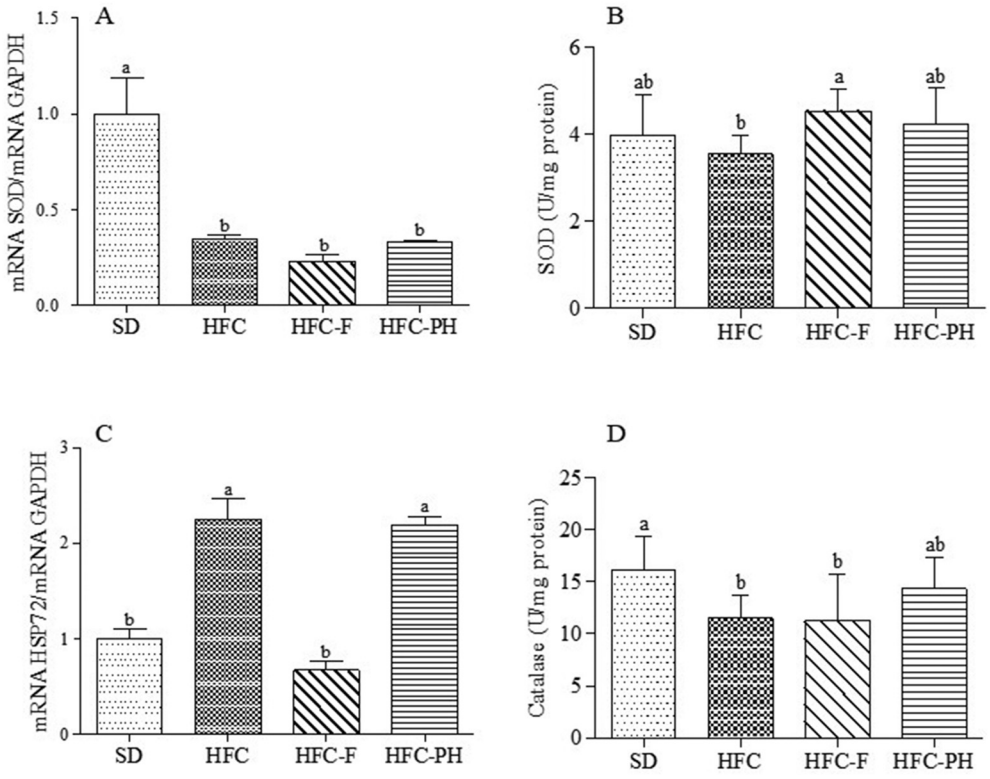

Fig. 3. Effect of bean flour and bean hydrolysate protein intake on oxidative stress in Balb-c mice (n = 12) for 63 days. A: total antioxidant capacity (TAC) in serum; B: malondialdehyde (MDA) in serum; C: superoxide dismutase expression (SOD); D: SOD activity; E: heat shock protein 72 expression (HSP72); F: catalase activity. SD: standard diet; HFC = high fat high cholesterol diet; HFC-F: high fat high cholesterol diet and whole bean flour; HFC-PH: high fat high cholesterol diet and protein hydrolyzate. Mean followed by different letters differed by Newman-Keuls test ($p < 0.05$).

reduction ($p < 0.05$) in inflammation mediating by TNF-α and NFκB expression compared to the group fed only with high fat and high cholesterol diet (Fig. 4A and E). In addition, the consumption of whole bean flour (HFC-F) and bean protein hydrolyzate (HFC-PH) was able to reduce the concentration of TNF-α in relation to the animals fed with high fat high cholesterol diet (HFC) (Fig. 4B). However, the consumption of whole bean flour by the HFC-F group did not affect ($p > 0.05$) TNF-α expression in relation to the HFC group (Fig. 4A), but it reduced ($p < 0.05$) NFκB expression in relation to the other groups (Fig. 4E). mRNA IL-10 expression in the HFC-F and HFC-PH groups did not differ ($p > 0.05$) from the HFC group (Fig. 4C). Nevertheless, the IL-10 concentration did not differ between all the experimental groups ($p > 0.05$) (Fig. 4D). The mRNA PPAR-α expression was lower in HFC-F group than the other groups (Fig. 4F).

Besides this, we observed an antioxidant response in the HFC-F group, indicated by the higher concentration of SOD and the lower expression of HSP72 in relation to the HFC group. TNF-α gene expression in whole bean flour (HFC-F) and bean protein hydrolysate (HFC-PH) groups was similar to standard group (SD) and the treatments reduced NFκB transcription factor expression. In addition, there was no increase in the expression of anti-inflammatory cytokine IL-10 and in the expression of PPAR-α in high fat high cholesterol groups.

4. Discussion

The effect of whole bean flour and protein hydrolysate from common bean variety Carioca on inflammation in adult BALB/c mice fed high fat high cholesterol diet has not been previously reported. The present study focused on the evaluation of the effect on inflammation and oxidative stress in normal adult BALB/c mice fed a high fat high cholesterol diet due to the evidence that the phenolic compounds and bioactive peptides present in beans showed antioxidant and anti-inflammatory effects (Alves, Mejía, et al., 2016; Alves, Vasconcelos, et al., 2016; García-Lafuente et al., 2014; Wang et al., 2016).

We hypothesized that the functional proprieties of bioactive peptides could remain after digestion of whole bean flour in mice and of protein hydrolysates offered by gavage, since proteins have good stability in diet and gavage is a safe process to administered functional products in few amount (Moura et al., 2016). In fact, the antioxidant and anti-inflammatory effect were observed as discussed following, confirming the stability of the bioactive compounds.

The phenolic compounds, namely catechin and kaempferol present in whole bean flour, may act to prevent cardiovascular diseases (Alves, Mejía, et al., 2016) and the weight gain caused by the consumption of high fat high cholesterol diet, since these compounds attenuate DNA damage, prevents the oxidation of LDL cholesterol (de Camargo, Regitano-d'Arce, Biasoto, & Shahidi, 2014) and improves the lipid profile (Kim et al., 2016; Natal et al., 2016). On the other hand, bean protein hydrolysate (HFC-PH) was able to attenuate the negative effects of the high fat high cholesterol diet, reducing weight gain, food intake and FER. These effects can be attributed to the presence of biologically active peptides (LVTTTVDL, QTSTPLFS, VELVGPK, TRGVLV) in the protein hydrolysate of beans cultivar BSRMG Madreperola (Alves, Mejía, et al., 2016).

The increase in glucose concentrations observed in the SD group is related to the high consumption of simple carbohydrates, since approximately 70% of the energy content of the diet comes from cornstarch, dextrinized starch and sucrose. Consumption of simple carbohydrates in excess and in the long term is associated with the development of insulin resistance, the hormone responsible for glycemic control (Gallagher, Leroith, and Karniel, 2010). Biologically active peptides inhibit the enzyme dipeptidyl peptidase IV (DPP-IV) (Alves, Mejía, et al., 2016), which degrades glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1). These compounds are incretins responsible for regulating postprandial glycemia, increasing insulin secretion, reducing glucose production by the liver and delaying gastric emptying (Caron et al., 2016; Engel et al., 2006). In addition, parental proteins such as phytohemagglutinin, alpha and beta phaseolin, alpha-amylase inhibitor 1 and alpha-amylase inhibitor 2 were identified in the protein hydrolysate of BRSMG Madreperola bean (Alves, Mejía, et al., 2016) used in the present study and may exhibit a protective activity on weight gain and food consumption.

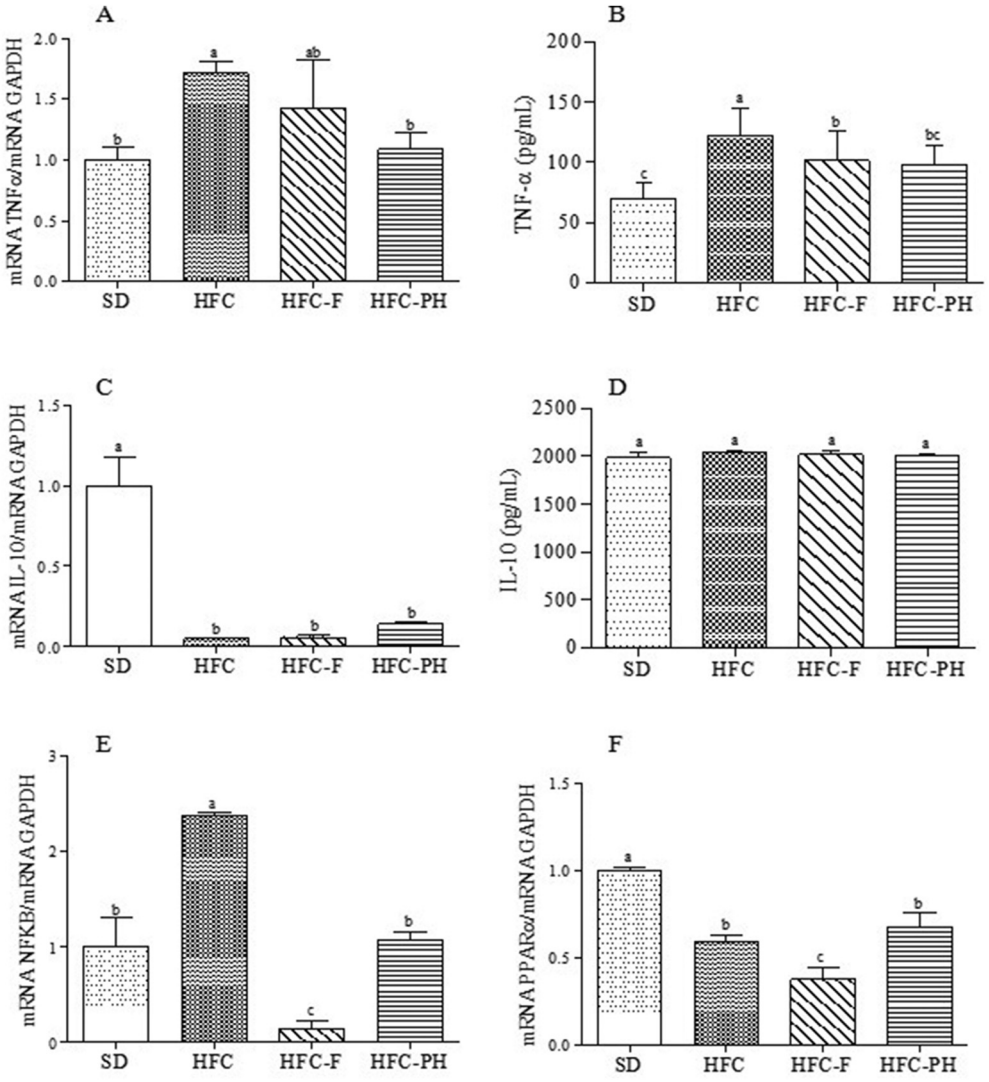

Fig. 4. Effect of bean flour and bean protein hydrolysate intake on inflammation in Balb-c mice (n = 12) for 63 days. A: tumor necrosis factor alpha (TNF-α); B: interleukin 10 (IL 10); C: nuclear transcription factor kappa B (NFκB); D: peroxisomal proliferating receptors alpha (PPAR-α). SD: standard diet; HFC = high fat high cholesterol diet; HFC-F: high fat high cholesterol diet and whole bean flour; HFC-PH: high fat high cholesterol diet and protein hydrolyzate. Mean followed by different letters differed by Newman-Keuls test ($p < 0.05$).

The mechanism involves stimulating the secretion of cholecystokinin (CCK) by enteroendocrine cells, stimulating the secretion of anorexic hormones and reducing the absorption of glucose, which leads to an increased satiety and a reduction of food intake (Baintner, Kiss, Pfüller, Bardocz, & Pusztai, 2003; Fantini et al., 2009; Mccrory, Hamaker, Lovejoy, & Eichelsdoerfer, 2010; Ramírez-Jiménez et al., 2015; Sufian et al., 2011; Sufian, Hira, Asano, & Hara, 2007).

Liver weight and the hepatosomatic index in HFC-F group were higher than in HFC group. However, the activity of ALT enzyme was lower. These results may indicate that there were no possibly hepatic lesions in these animals (Ramaiah, 2007) since the concentrations of liver enzymes are within normal range for rodents (Spinelli, Cruz, Godoy, Motta, & Damy, 2014). These results corroborate with our histological analyses, which showed no difference in fat concentration in hepatic cells'cytoplasm in groups fed high fat high cholesterol diet associate with whole bean or bean protein hydrolysate.

The beneficials effect on lipid profile and glucose concentration may be related to higher content of dietary fiber and other bioactive compounds present in HFC-F, especially the soluble fraction, which decrease glycemia and total cholesterol by the ability to chelate bile acids and reduce the absorption of lipids in the intestinal lumen (Anderson et al., 2009; Jenkins et al., 2006; Sánchez-Muniz, 2012). In the HFC-PH group, the reduction of total cholesterol may be related to the presence of bioactive peptides in bean protein hydrolysate with hypocholesterolemic action. These peptides are found naturally in bean (Alves, Mejía, et al., 2016; Alves, Vasconcelos, et al., 2016; Marques et al., 2015; Marques, Fontanari, Pimenta, Soares-Freitas, & Arêas, 2015) and the mechanism of action involves the reduction of the micellar solubilization of cholesterol, probably by the hydrophobic interaction with the lipids (Hayat et al., 2014; Luna-Vital et al., 2015; Marques, Fontanari, et al., 2015; Marques, Freitas, et al., 2015).

The antioxidant enzymes SOD and catalase are the first mechanisms of antioxidant defense of the organism and have the function of neutralizing the reactive oxygen species (ROS) generated during oxidative stress (Ribeiro et al., 2005). Thus, it can be inferred that the consumption of bean protein hydrolyzate was beneficial in increasing the concentration of these enzymes, and could thus positively influence the inflammatory process, even when ingested with a high fat high cholesterol diet. HSP72 protein belongs to a class of Hsp70 family proteins and it is a molecular chaperone that has broad cytoprotective functions and is upregulated in response to stress (Lin et al., 2018). The antioxidant response in the HFC-F group, indicated that the activity of

antioxidant enzymes was sufficient to reduce the deleterious effects of lipid peroxidation in this group, as well as reduce oxidative damage, indicated by the expression of HSP72 (Mayer & Bukau, 2005; Rodriguez, Alvarado, Vásquez, & Félix, 2006).

The intake of a high fat high cholesterol diet (HFC, HFC-F and HFC-PH) for 63 days causes a reduction in the PPAR-α mRNA expression, indicating that possibly there was no transcriptional regulation of oxidative stress and lipid metabolism through the pathway regulated by PPAR-α (Abdelmegeed, Moon, Hardwick, Gonzalez, & Song, 2010; Kim & Yang, 2013). This result may be related to the increased inflammation present in diet-induced obesity, since a short period of time was enough to affect the PPAR inflammatory marker (Rodríguez, Ribot, Rodríguez, & Palou, 2004). It is known that PPAR-α ligands have anti-inflammatory effects in various cells by means of apoptosis in cytokine activated macrophages, inhibiting NFκB signaling (Monsalve, Pyarasani, Delgado-Lopez, & Moore-Carrasco, 2013). However, the consumption of whole bean flour (HFC-F) and bean protein hydrolysate (HFC-PH) was able to reduce TNF-α mRNA expression and concentration in comparison with high fat high cholesterol group, showing a positive effect on inflammatory process. Besides this, the treatments (whole bean flour and bean protein hydrolysate) attenuated NFκB transcription factor expression.

Interleukin-10 (IL-10) is a key anti-inflammatory cytokine produced by activated immune cells. To know the role of IL-10 in the regulation of metabolic processes is essential for discovery how IL-10 acts to control inflammatory responses such as how this interleukin acts in molecular regulators controlling processes involved in resolution of inflammation (Ip, Hoshi, Shouval, Snapper, & Medzhitov, 2017). It is known that IL-10 is able to reduce the production of pro-inflammatory cytokines in macrophages through the STAT3-dependent pathway (Grant et al., 2014). However, in our research, we observed that, the consumption of whole beans and bean protein hydrolyzate associate with high fat high cholesterol diet was not able to increase IL-10 mRNA expression and IL-10 quantification. Despite this result, we can observe that the antioxidant and anti-inflammatory activities from whole bean flour and its protein hydrolysate were probably enhanced the presence of the phenolic compounds and the bioactive peptides, which have antioxidant action for the body.

The strengths of the work are the presence of controls group (positive and negative) to minimize the impact of the unwanted variables, the appropriate number of animals in each group; the correct use of methods for data analysis and the appropriate use of statistical tests. Also, we can highlight that the dilution of the hydrolyzate was performed at the time of administration to the animal in order to avoid interaction with the PTU. However, our work presented some weaknesses such as the lack of measurement of NFκB and IKB-alpha activation and the lack of analysis of hepatic steatosis, due to the low amount of biological material present in the mice. In addition, it was not possible to synthesize the promising peptides to test which peptide was responsible for the claimed effects in the study.

5. Conclusions

Whole bean flour and bean protein hydrolysate reduced inflammation and may act in a beneficial way to reduce the risk factors for cardiovascular diseases. Both interventions reduced glycemia, high cholesterol levels, lipid peroxidation and increased the antioxidant enzyme activity. In addition, bean protein hydrolysate reduced biometric measurements and food consumption. Therefore, whole bean flour and bean protein hydrolysate may be a promising dietary approach to improve health, which indicates the importance and necessity of studies in humans.

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Author contributions

S.L.S.L and H.S.D.M conceived of the presented idea. S.L.S.L, M.J.C.G, B.P·S and N.*E.G.*A wrote the manuscript with support from H.S.D.M, *E.G.*M and P.Z.B; M.J.C.G, B.P·S, R.C.L.T, J.M.V.T, M.E.C.M., J.A.C·B processed the experimental data and performed the analysis, H.S.D.M and S.L.P.M supervised the experiment. All authors discussed the results and commented on the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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