



Extruded sorghum (*Sorghum bicolor* L.) improves gut microbiota, reduces inflammation, and oxidative stress in obese rats fed a high-fat diet



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ABSTRACT

The aim of this study was to investigate the effects of extruded sorghum flour (ESF) on rats fed with a high-fat diet (HFD) on gut microbiota modulation, inflammation, and oxidative stress. Male *Wistar* rats were fed a normal diet (AIN-93 M, n = 8), HFD (n = 8), or HFD plus ESF replacing 50% cellulose and 100% corn starch (HFDS50, n = 8) or HFD plus ESF replacing 100% cellulose and 100% corn starch (HFDS100, n = 8) for eight weeks. ESF improved the composition of intestinal microbiota by increasing the proportion of the *Bacteroidetes* phylum instead of the *Firmicutes* phylum. Furthermore, ESF reduced the concentrations of p65 NF-κB in liver, seric resistin, lipids peroxidation and increase the total antioxidant capacity of plasma, the expression of superoxide dismutase, and the heat shock protein 72. In conclusion, ESF improves the intestinal dysbiosis, inflammation and oxidative stress in obese rats fed a hyperlipid diet.

1. Introduction

Obesity reaches epidemic proportions worldwide and is considered a marker for several chronic noncommunicable diseases (NCDs) (Kaur, 2014). There are many factors involved in the genesis of obesity, however, the main factor is the sedentary lifestyle associated with a high-calorie diet with high amounts of saturated fats and low in dietary fiber (Calder et al., 2011). In addition, there are evidence demonstrating the role of diets, especially those rich in saturated fats, affecting the composition of the gut microbiota, and this in turn, in development of metabolic disorders (Lam et al., 2015; Zhou et al., 2017).

The consumption of high-fat diet, a rich source of saturated fats, can increase not only the body fat mass, but also affect the inflammatory and metabolic properties of the intestinal microbiota and, therefore, alter host physiology (Caesar, Tremaroli, Kovatcheva-Datchary, Cani, & Bäckhed, 2015; Requena, Martínez-Cuesta, & Peláez, 2018). HFD induces gut dysbiosis by an increase of *Firmicutes* and reduction of *Bacteroidetes* phylum, which is a partner associated with obesity condition (Clarke et al., 2012). In addition, HFD may increase energy storage, damage gut barrier function, drive to low-grade chronic inflammation,

and consequently to insulin resistance and obesity (Xu et al., 2019).

Considering the high risk for NCDs, especially obesity, nutritional strategies need to be investigated to prevent/treatment of these disorders. Foods rich in bioactive compounds are widely studied once these compounds are beneficial to health and its biological activity has been proven (Han et al., 2015; Kim, Kim, & Park, 2015). The beneficial effects of bioactive compounds are direct by absorption of their metabolites and indirectly by non-absorbed compounds that modify the gut microbiota and, consequently, all the metabolism (Fernandes, Faria, Calhau, de Freitas, & Mateus, 2014; Floegel et al., 2010; Sharma, Meeran, & Katiyar, 2007).

In this context, sorghum (*Sorghum bicolor* L.) is a rich source of bioactive compounds, especially 3-deoxyanthocyanines (3-DXAs), tannins and phenolic acids, as well as dietary fibers, minerals, such as calcium, phosphorous, iron, zinc, and magnesium, unsaturated lipids, and complex B vitamins. These compounds guarantee nutritional quality and a powerful functional action (Cardoso, Pinheiro, Martino, & Pinheiro-Sant'Ana, 2015; Prasad, Benhur, Kommi, Madhari, & Patil, 2016). There are many genotypes of sorghum, the SC 319 genotype with brown pericarp has a high content of dietary fiber and bioactive

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compounds, including proanthocyanins (tannins) and 3-DXA (Cardoso, Pinheiro, de Carvalho, et al., 2015). Some *in vitro* and *in vivo* studies have demonstrated the benefits of non-extruded sorghum and its compounds in gut microbiota as well as in variables associated with NCDs development (Burdette et al., 2010; Moraes et al., 2012; Ritchie et al., 2015).

In order to facilitate the consumption of sorghum by humans, some processes, such as cereal extrusion, are necessary. It was demonstrated that the extrusion process increases total phenol content in sorghum bran (Lopez et al., 2018; Salazar Lopez et al., 2016), also the antioxidant capacity was enhanced which improve the capacity of RAW cells inhibit the nitric oxide (NO) production (Salazar Lopez et al., 2016). In addition, the extrusion process may bring down the level of anti-nutritional factors in cereals and improves their digestibility (Patel, Patel, & Singh, 2016). A study with *Wistar* rats fed with HFD showed that ESF reduced the body mass index, liver weight, hepatic steatosis, and lipogenesis by increasing adiponectin 2 receptor and peroxisome proliferator-activated receptor α , and sterol regulatory element-binding transcription factor 1 gene expression (de Sousa et al., 2018). In addition, it has been shown that the sorghum-extrusion process increased the iron bioavailability in rats (Gomes et al., 2017) and the content of proanthocyanidin monomers and dimers-3 fold, which improve the bioavailability and effectiveness against oxidative stress (Morais Cardoso et al., 2015). Furthermore, the consumption of ESF associated with unfermented probiotic milk alleviates the inflammation and oxidative stress in individuals with chronic kidney disease (Lopes et al., 2018) and reduced body fat percentage and increased daily carbohydrate and dietary fiber intake on overweight men (Anuniação et al., 2019).

Despite other studies analyzed sorghum effects, it is still needed to determine the preventive and therapeutic effects of sorghum grain in the extruded form against the HFD. Furthermore, since the sorghum consumption by humans is not very common, the extrusion process can improve the sensory characteristics of sorghum (Jafari, Koocheki, & Milani, 2018), which may stimulate the human consumption, leading health benefits. Then, the objective of the study was to evaluate the effects of extruded brown pericarp sorghum flour (ESF) on the modulation of intestinal microbiota, inflammatory profile and oxidative stress of obese animals fed a high-fat diet.

2. Material and methods

2.1. Extruded sorghum flour

A brown pericarp sorghum SC 319 genotype from “Embrapa Milho e Sorgo” research station, located at Nova Porteirinha, Minas Gerais, Brazil (15°47'S 43°18'W and 516 m above sea level) were extruded by Vargas-Solórzano, Carvalho, Takeiti, Ascheri, and Queiroz (2014) on Cereal Laboratory (Embrapa Agroindústria de Alimentos, Rio de Janeiro, Brazil). The extruded grains were ground until flour, then packed in polyethylene bags, and stored at -20°C until use.

ESF chemical composition data, including carbohydrates, proteins, lipids, dietary fiber, luteolinidin, apigeninidin, 5-Metoxi-luteolinidin, 7-Metoxi-apigeninidin, total proanthocyanins, and total phenolic compounds were previously determined by our research group (Arbex et al., 2018) and the data was used to inform the design of experimental diets in this study (Table 1).

2.2. Animals and diets

Thirty-two adult males *Wistar* rats (*Rattus norvegicus*) (60 days old) from Universidade Federal de Viçosa, Brazil, were housed in individual cages and kept in a room at $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$, under a light-dark cycle of 12 h and water *ad libitum*. The experiment was approved by the Ethics Committee of Animals Use of Universidade Federal de Viçosa, protocol No. 06/2014.

Table 1

Composition of the experimental diets (g·100 g⁻¹).

Ingredients	AIN-93M ^A	HFD ^B	HFDS50 ^C	HFDS100 ^D
Casein	14	19,50	17,67	15,84
Maltodextrin	15,50	10	3,10	0
Corn starch	46,57	5,32	0	0
Sucrose	10	34,10	34,10	25,04
Soybean oil	4	1	0,66	0,32
Lard	0	20	20	20
Cellulose	5	5	2,5	0
Vitamin Mix	1	1	1	1
Mineral Mix	3,5	3,5	3,5	3,5
Bitartrate choline	0,25	0,25	0,25	0,25
L-cystine	0,18	0,18	0,18	0,18
Cholesterol	0	0,15	0,15	0,15
BHT	0,0008	0,0004 48	0,0004	0,0004
Extruded sorghum flour	–	–	16,86	33,72
Total 3- DXAS (μg)	Nd	nd	16,92	33,85
Luteolinidin (μg)	Nd	nd	4,45	8,90
Apigeninidin (μg)	Nd	nd	2,39	4,78
5-Metoxi-luteolinidin (μg)	Nd	nd	6,82	13,65
7-Metoxi-apigeninidin (μg)	Nd	nd	3,27	6,54
Total proanthocyanins (mg EC)*	Nd	nd	8,20	16,41
Total phenolic compounds (mg AG)**	Nd	nd	0,08	0,16
Calories (kcal):				
Carbohydrates (%)	72,58	42,42	41,09	39,82
Protein (%)	17,96	16,74	17,12	17,58
Lipids (%)	9,47	40,85	41,79	42,61
Caloric density (kcal/g)	3,80	4,66	4,56	4,44

nd: not determined; BHT: Butylated hydroxytoluene.

^A Standard diet for rodents.

^B High fat diet.

^C High fat diet + 50% substitution of cellulose and 100% of corn starch by extruded sorghum flour.

^D High fat diet + 100% substitution of cellulose and corn starch for extruded sorghum flour.

* Equivalent of catechin.

** Gallic acid equivalent.

The study was divided into two phases. In phase I, during 49 days, the rats were kept into two groups: The HFD group (n = 24) received high-fat diet which composition was based on commercial diet D12079B (Research Diets®) for obese induction. The AIN-93M group (n = 8) received a standard diet for adults animals (Reeves, Nielsen, & Fahey, 1993) as a lean control. At the end of this phase, to confirm the obesity in Group 1, the rats have fasted for 12 h and blood samples were collected by caudal puncture for biochemical analyzes and biometric measurements were also performed (data not shown).

In phase II, animals from HFD group were redistributed into three groups (n = 8) according to body weight average (303.8 g \pm 1.4):

- HFD group: high-fat diet (D12079B, Research Diets®)
- HFDS 50 group: high-fat diet + 50% of the amount of cellulose and 100% of corn starch were replaced by extruded sorghum flour
- HFDS 100 group: high-fat diet + 100% of the amount of cellulose and corn starch were replaced by extruded sorghum flour

The AIN-93M group diet was maintained. The other ingredients of the diet (casein, maltodextrin, sucrose and soybean oil) were adjusted to provide isocaloric hyperlipidic diets with or without ESF addition between the obese groups (Table 1). The animals received them respectively treatments during 8 weeks and weight and food intake of each animal were monitored weekly.

At the end of phase II, the feces were collected and stored at -80°C and after a 12-h fast, the animals were anesthetized with 100% isoflurane (Isoforine, Cristália®) and the blood was collected by cardiac into appropriate tubes, 16 \times 100 mm (BD Vacutainer®) and centrifuged

Table 2
Primers sequence for qPCR.

Group	Primers (F and R)	Standard Genomic DNA	References
Total Bacterias	F-GCAGGCCTAACACATGCAAGTC R-CTGCTGCCTCCCGTAGGAGT	<i>Escherichia coli.</i>	Castillo et al. (2006)
Bacteroidetes	F-CATGTGGTTTAATTTCGATGAT R-AGCTGACGACAACCATGACG	<i>Bacteroides vulgatus</i>	Guo et al. (2008)
Firmicutes	F-ATGTGGTTTAATTCGAAGCA R-AGCTGACGACAACCATGACG	<i>Lactobacillus delbrueckii</i>	Guo et al. (2008)
Proteobacteria	F-CATGACGTTACCCGAGAAAG R-CTCTACGAGACTCAAGCTTGC	<i>Escherichia coli.</i>	Friswell et al. (2010)

F: Foward; R: Reverse. All oligonucleotides were obtained from Alpha DNA and Molecular Diagnostics LTDA.

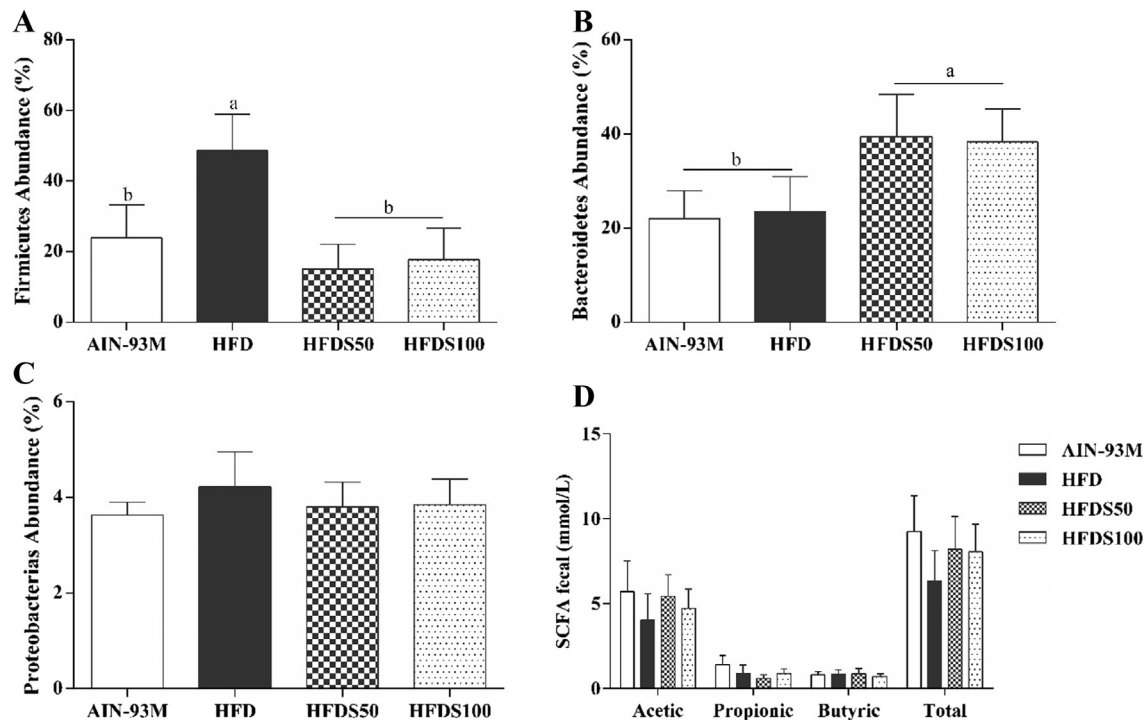


Fig. 1. Intestinal composition of groups treated or not with extruded sorghum flour and or not HFD. *Firmicutes* percentage (A), *Bacteroidetes* percentage (B), *Proteobacterias* percentage (C) and concentration of short chain fatty acids in feces (D) of rats treated with different diets for the 8 weeks. Each value is expressed as mean \pm standard deviation. ^{a,b}Different letters indicate statistically significant difference ($p < 0.05$) and the absence of letters means similarity between groups ($p > 0.05$) analyzed by ANOVA *post-hoc* Tukey's test. (A), (B) and (C) were analyzed by Quantitative Real-Time Polymerase Chain Reaction (qPCR), and (D) was analyzed by High Performance Liquid Chromatography (HPLC). AIN-93M: lean control group; HFD: obese control group; HFDS50: test group with extruded sorghum flour replacing 50% cellulose and 100% corn starch in a high-fat diet; HFDS100: test group with extruded sorghum flour replacing 100% cellulose and 100% corn starch in a high-fat diet.

(10 min, 1006g, 4 °C) to obtain serum. The liver was removed, weighted, and immediately frozen in liquid nitrogen and stored at -80 °C.

2.3. DNA preparation and gut microbiota analysis

Microbial DNA was extracted from fecal samples using the QIAamp DNA stool mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The gut microbiota composition of total bacterias, *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* was evaluated by quantitative real-time polymerase chain reaction (qPCR; CFX96 Touch™ Real-Time PCR Detection System; Bio-Rad®, Berkeley, California). Every analysis were performed in duplicate using SYBR® Green PCR Master Mix (Promega®) containing in each well 2 μ L sample or standard, sense, and antisense oligonucleotides (Table 2) at concentrations of 300 nM and nuclease-free water for a total of 25 μ L. The initial DNA denaturation was at 95 °C (10 min), followed by 40 cycles of denaturation at 95 °C (10 s), annealing of the oligonucleotide was at 60 °C

(20 s) and extension at 72 °C (15 s) (Castillo et al., 2006).

Standard curves were constructed for each experiment using five sequential dilutions of bacterial genomic DNA from pure cultures (Table 2) ranging from 20 ng to 0.032 ng. The abundance of bacteria phyla from each fecal sample was calculated according to the relative quantification method described by Stevenson and Weimer (2007). The different strains used were obtained from the American Type Culture Collection (ATCC) (*Bacteroides ovatus* ATCC 8483; *Escherichia coli* ATCC 11775) and from the Tropical Cultures Collection (*Lactobacillus delbrueckii* UFV H2b20 CCT 3744).

2.4. Determination of short chain fatty acids

Rats feces samples (500 mg) were homogenized with ultrapure water and centrifuged (12,000g, 10 min, 4 °C) and the cell-free supernatants were treated as described by Siegfried, Ruckemann, and Stumpf (1984). Organic acids (acetic, propionic and n-butyric) were determined by HPLC in a Dionex Ultimate 3000 Dual detector HPLC

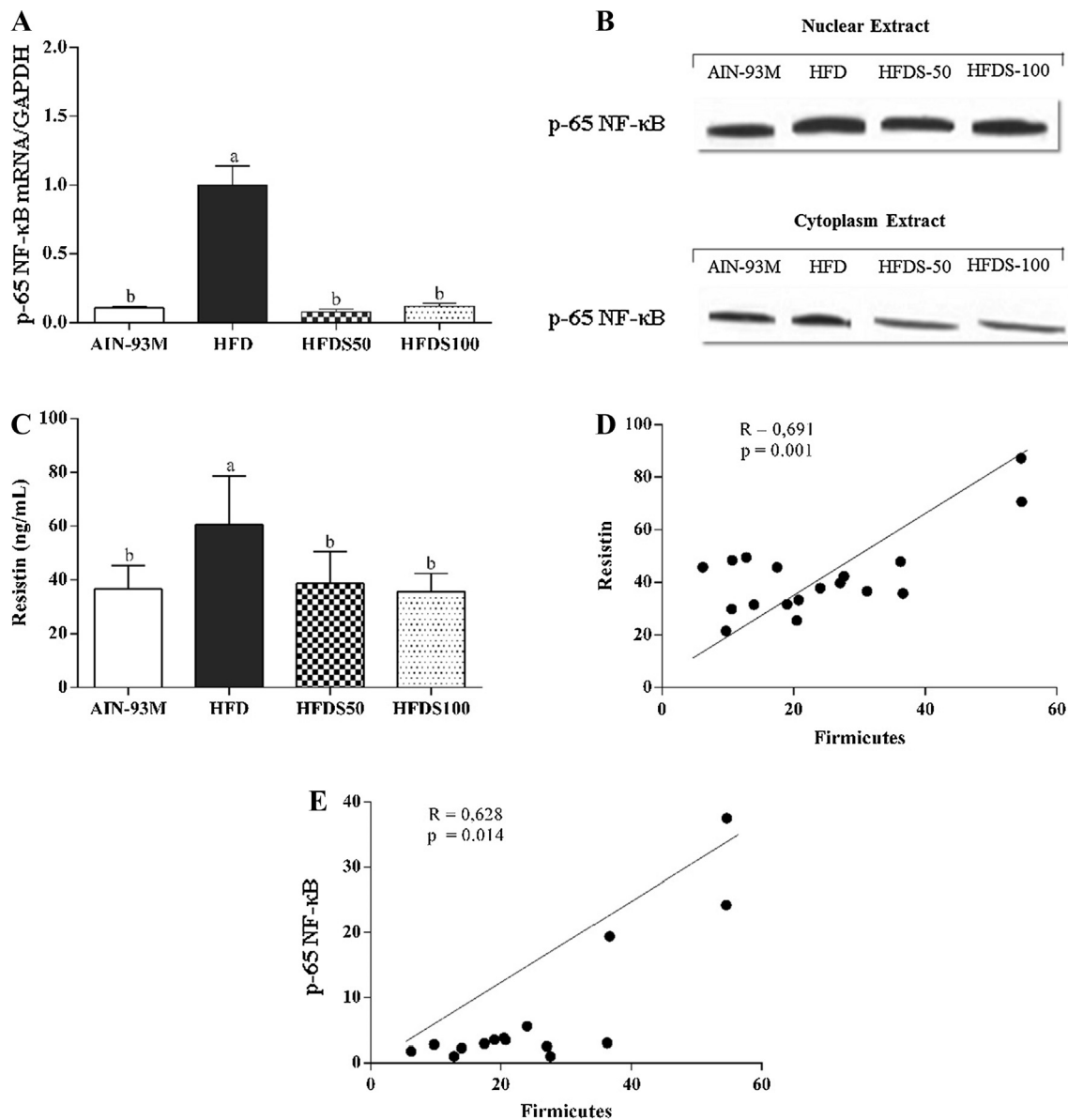


Fig. 2. The inflammatory markers produced by groups treated or not with extruded sorghum flour and or not HFD. Expression (A) and protein quantification (B) of NF-κB on liver tissue, resistin seric levels (C), correlation of *Firmicutes* phylum with resistin seric (D) and NF-κB (E) of rats treated with different diets for the 8 weeks. Each value is expressed as mean \pm standard deviation. ^{a,b} Different letters indicate statistically significant difference ($p < 0.05$) analyzed by ANOVA *post-hoc* Tukey's test. Correlation between variables was measured by Pearson's regression analysis, with a 95% confidence interval. (A) was analyzed by RT-PCR, (B) was analyzed by western-blot, (C) was analyzed by ELISA, and (D) and (E) by Pearson's regression. AIN-93M: lean control group; HFD: obese control group; HFDS50: test group with extruded sorghum flour replacing 50% cellulose and 100% corn starch in a high fat diet; HFDS100: test group with extruded sorghum flour replacing 100% cellulose and 100% corn starch in a high fat diet.

(Dionex Corporation, Sunnyvale, CA, USA) coupled to a refractive index (RI) Shodex RI-101 maintained at 40 °C using a Phenomenex Rezex ROA ion exchange column (300 \times 7.8 mm) maintained at 45 °C.

Analyses were performed isocratically in duplicate samples using a mobile phase was prepared with sulfuric acid 5 mmol/l, the flow rate of 0.7 ml/min, and injection volume 20 μ L. Five calibration standards were prepared by diluting the stock solutions 2, 4, 8 and 16 times in the mobile phase. Acetic, propionic, and butyric organic acids were used for the calibration of the standard curve. All stock solution of acids were prepared with a final concentration of 10 mmol/l, except acetic acid (20 mmol/l). The correlation coefficients of the external standards were 0.999, 0.999 and 0.998 for acetic, propionic and butyric acids, respectively. Quantitation limits were calculated based on the standard deviation of the response and the slope of the calibration curves. The LOD and LOQ values were obtained (mmol/l): $LOD_{Acetic} = 0.023$ and

$LOQ_{Acetic} = 0.069$; $LOD_{Propionic} = 0.232$ and $LOQ_{Propionic} = 0.077$; $LOD_{Butyric} = 0.013$ and $LOQ_{Butyric} = 0.038$.

2.5. Cytokine

Serum resistin concentrations were analyzed by immunoassay using a commercial ELISA specific kit (Resistin Cat. # SEA847 RA, USCN, USA) followed the manufacturer's instructions. The absorbance was read at 450 nm wavelength in reader (Awareness®, Stat Fax 2100) and the amount of resistin was determined by standard curves that were run at the same time as the samples. Results were expressed in ng/ml.

2.6. Analysis of genomic expression in liver by RT-qPCR

RNA total extraction was performed using Trizol Reagent

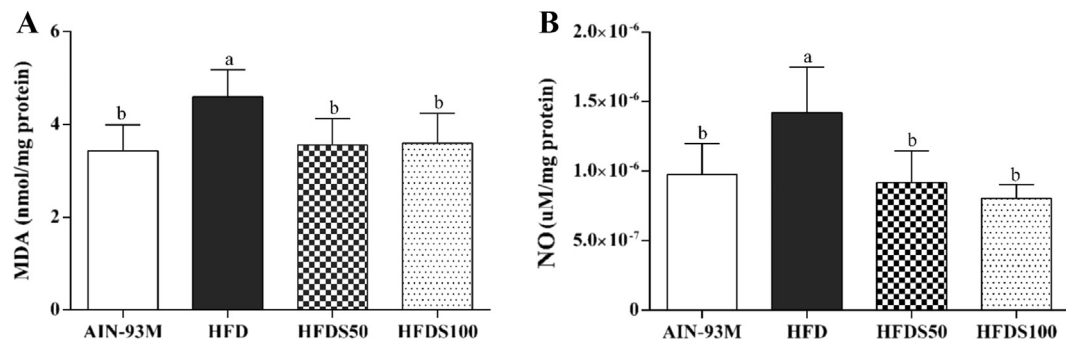


Fig. 3. The inflammatory and oxidative markers produced by groups treated or not with extruded sorghum flour and or not HFD. Oxidative stress markers by Malondialdehyde (A) and nitric oxide (B) of rats treated with different diets for the 8 weeks. Each value is expressed as mean \pm standard deviation. ^{a,b}Different letters indicate statistically significant difference ($p < 0.05$) and the absence of letters means similarity between groups ($p > 0.05$) analyzed by ANOVA *post-hoc* Tukey's test. (A) was analyzed by TBARS test and (B) by absorbance. AIN-93M: lean control group; HFD: obese control group; HFDS50: test group with extruded sorghum flour replacing 50% cellulose and 100% corn starch in a high fat diet; HFDS100: test group with extruded sorghum flour replacing 100% cellulose and 100% corn starch in a high fat diet; MDA: Malondialdehyde; NO: Nitric Oxide.

(Invitrogen®, USA) and a Mirvana™ mRNA Isolation Kit (Ambion® by Life Technologies™) according to the manufacturer's protocols. The RNA concentration and purity were evaluated by IDrop plate spectrophotometer Multiskan™ GO (Thermo Scientific®, USA), and its integrity was confirmed by electrophoresis agarose gel. A M-MLV Reverse Transcriptase Kit (Invitrogen®) was used to cDNA synthesis. The gene expression relative quantification was performed by RT-qPCR using AB StepOne Real Time PCR System equipment and Fast SYBR Green Master Mix (Applied Biosystems®, USA) reagent. The initial conditions to run were 95 °C (20 s) and then 40 cycles at 95 °C (3 s), 60 °C (30 s) followed by melting curve analysis.

The primers used on amplification were: p65 NF- κ B (Nuclear Transcription Factor Kappa B): CTG CGC GCT GAC GGC (Forward); TCG TCG TCT GCC ATG TTG AA (Reverse); HSP72 (Heat Shock Protein 72): AGG CCA ACA AGA TCA CCA TC (Forward); TAG GAC TCG AGC GCA TTC TT (Reverse); Zn-SOD (Superoxide dismutase coupled to zinc): GAG CAG AAG GCA AGC GGT GAA (Forward); CCA CAT TGC CCA GGT CTC (Reverse); and the housekeeping gene GAPDH (Glyceraldehyde-3-phosphate dehydrogenase): AGG TTG TCT CCT GTC ACT TC (Forward); CTG TTG CTG TAG CCA TAT TC (Reverse). All primers were designed using Primer 3 Plus program and obtained from Sigma-Aldrich®, Brazil Ltda. Gene expression was calculated using 2-Delta-Delta C (T) (2- $\Delta\Delta$ Ct) method (Livak & Schmittgen, 2001).

2.7. Western blotting

Hepatic tissue was homogenized using NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Thermo Scientific®) for separation of nuclear and cytoplasmic protein fractions, according to manufacturer's instructions. Then, 15 μ g of protein from nuclear and cytoplasmic fraction and 5 μ L of the reference standard (Broad Range Markers – Santa Cruz Biotechnology®) were separated by SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and transferred to PVDF (Polyvinylidene Fluoride) membrane (BioRad®). The membranes were blocked with a 5% blocking solution (Blotting-grade blocker, BioRad®) and incubated overnight with the anti-p65-NF- κ B monoclonal primary antibody (ab13594; Abcam®, Cambridge, UK). The membranes, after successive washes, were incubated with horseradish peroxidase conjugated secondary antibody (A-9044, Sigma-Aldrich Brazil Ltda.) for 2 h at 4 °C. Bands were detected with 3,3'-diaminobenzidine tetrachloride.

2.8. Oxidative stress, activity of antioxidant enzymes and total antioxidant capacity

Oxidative stress was determined by malondialdehyde (MDA) in liver

homogenates by thiobarbituric acid reactive substances (TBARS) (Buege & Aust, 1978) and nitric oxide concentrations (NO) (Green et al., 1982). The activity of antioxidant enzymes Superoxide Dismutase (SOD), Catalase, and Glutathione-S Transferase (GST) was determined in hepatic tissue according to Marklund (1985), Aebi (1984) and Habig, Pabst, and Jakoby (1974), respectively. Protein concentrations in liver homogenates were quantified according to the Bradford (1976) method. The Total Antioxidant Capacity of Plasma (TAC) was assessed by enzyme immunoassay using Sigma Aldrich® specific kit (St. Louis, MO, USA) according to the manufacturer's instructions.

2.9. Statistical analysis

The normality of the data was evaluated by Shapiro-Wilk test. The non-normal distribution data were transformed into Log₁₀. Data were analyzed by variance analysis (ANOVA) followed by Tukey test using the SPSS (Statistical Package for Social Sciences) software, version 20.0. Differences were considered significant at $p < 0.05$. Results were expressed as the mean \pm standard deviation.

Correlation between variables was measured by Pearson's regression analysis, with a 95% confidence interval. The graphs were elaborated in Graphpad Prism software, version 6.0.

3. Results

3.1. Extruded sorghum flour effects on gut microbiota and short chain fatty acids.

The *Firmicutes* phylum was more abundant in the HFD group (49% \pm 10) when compared to the AIN-93M group (24% \pm 9). ESF consumption in both groups, HFDS50 and HFDS100, reduced the percentage of *Firmicutes* (20% to HFDS50 and 18% to HFDS100) (Fig. 1A).

The proportion of *Bacteroidetes* phylum was similar between HFD group (24% \pm 7) and AIN-93M group (22% \pm 6). In contrast, the occurrence of the *Bacteroidetes* phylum in both ESF groups was superior to AIN-93M and HFD groups (Fig. 1B). Every group showed similar occurrence of *Proteobacteria* (Fig. 1C) and to short chain fatty acids: acetate, butyrate, and propionate (Fig. 1D).

3.2. Effects of extruded sorghum flour on NF- κ B signaling and resistin levels

The HFD group had the highest expression of p65 NF- κ B compared to other groups. AIN-93M and ESF groups had similar values. The consumption of ESF by HFDS50 and HFDS100 groups decrease p65 NF- κ B expression to 0.08 and 0.12 times, respectively (Fig. 2A). These results are confirmed by more p65 NF- κ B protein detection on nucleus of

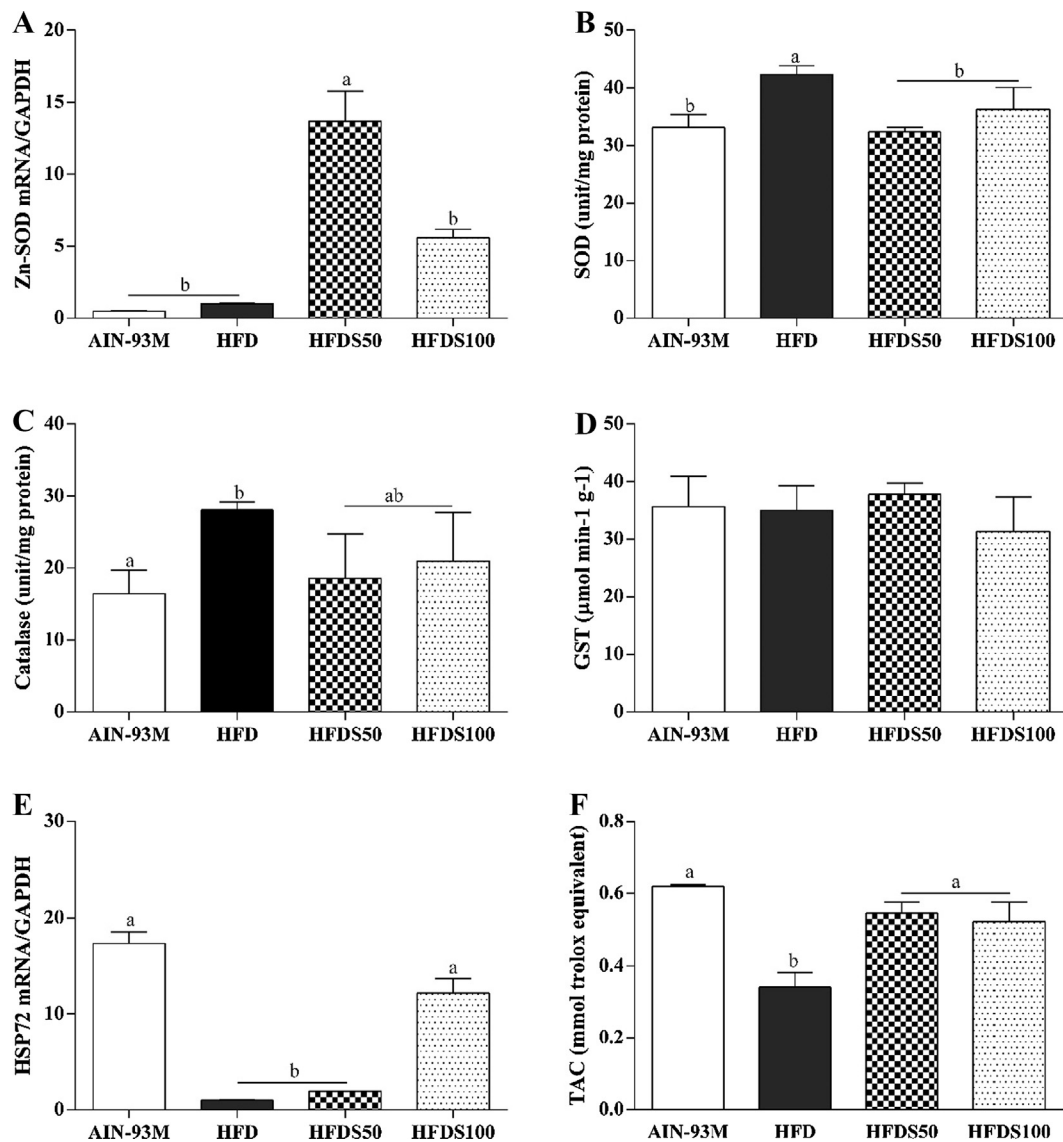


Fig. 4. The oxidative markers produced by groups treated or not with extruded sorghum flour and or not HFD. Gene expression of endogenous antioxidant Zn-SOD (A), activity of antioxidant enzymes SOD (B), Catalase (C), Glutathione S-Transferase (D), and gene expression of heat shock protein 72 (E), and total antioxidant capacity (F) of rats treated with different diets for the 8 weeks. Each value is expressed as mean \pm standard deviation. ^{a,b}Different letters indicate statistically significant difference ($p < 0.05$) analyzed by ANOVA *post-hoc* Tukey's test. (A) and (E) were analyzed by RT-PCR, (B), (C), (D) were analyzed by colorimetric test, (F) was analyzed by ELISA test. AIN-93M: lean control group; HFD: obese control group; HFDS50: test group with extruded sorghum flour replacing 50% cellulose and 100% corn starch in a high fat diet; HFDS100: test group with extruded sorghum flour replacing 100% cellulose and 100% corn starch in a high fat diet; SOD: Superoxide Dismutase; GST: Glutathione S-Transferase; HSP72: heat shock protein 72; TAC: Total antioxidant capacity.

HFD group that AIN-93M and ESF groups (Fig. 2B).

Resistin serum levels were higher in HFD group ($56.5 \text{ ng/mL} \pm 18.08$) than in the AIN-93M group ($36.68 \text{ ng/mL} \pm 8.6$). HFDS50 and HFDS100 groups reduced resistin levels (38.7 ± 11.9 and 35.7 ± 6.6 , respectively) and the values were similar to AIN-93M group (Fig. 2C).

A significant correlation was observed between the percentage of *Firmicutes* and the levels of resistin ($r = 0.69$) (Fig. 2D) and p65 NF- κ B expression ($r = 0.62$) (Fig. 2E), considering the results by every group.

3.3. Effects of extruded sorghum flour on the markers of oxidative stress and antioxidant activity

The HFD group had the highest values, compared to AIN93-M, HFDS50, and HFDS100, to MDA (Fig. 3A) and NO (Fig. 3B). Then, ESF was able to reverse the lipid peroxidation associated with oxidative stress caused by hyperlipid diet.

The expression of the Zn-SOD antioxidant enzyme in the HFD group was similar to AIN-93-M group. However, the consumption of ESF increased Zn-SOD expression by 13.6 and 5.6 times in the HFDS50 and HFDS100 groups, respectively, in relation to HFD group (Fig. 4A). However, SOD showed higher hepatic activity in the HFD group, whereas ESF consumption promoted a reduction in the activity of these enzymes in both the HFDS50 and HFDS100 groups, similar to AIN93-M group (Fig. 4B).

The groups treated with ESF, HFDS50, and HFDS100, had the Catalase enzyme activity similar to HFD and AIN93-M groups (Fig. 4C). GST had activity similar between every group (Fig. 4D).

The expression of HSP72 in the liver was reduced in HFD group and HFDS50. However, HFDS100 group increase in 12.2 times the expression of this protein, compared to HFD group, and its value was similar to AIN93-M group (Fig. 4E).

The total antioxidant capacity (TAC) in the plasma of HFD group was lowest compared to the animals of AIN-93M group. However, the

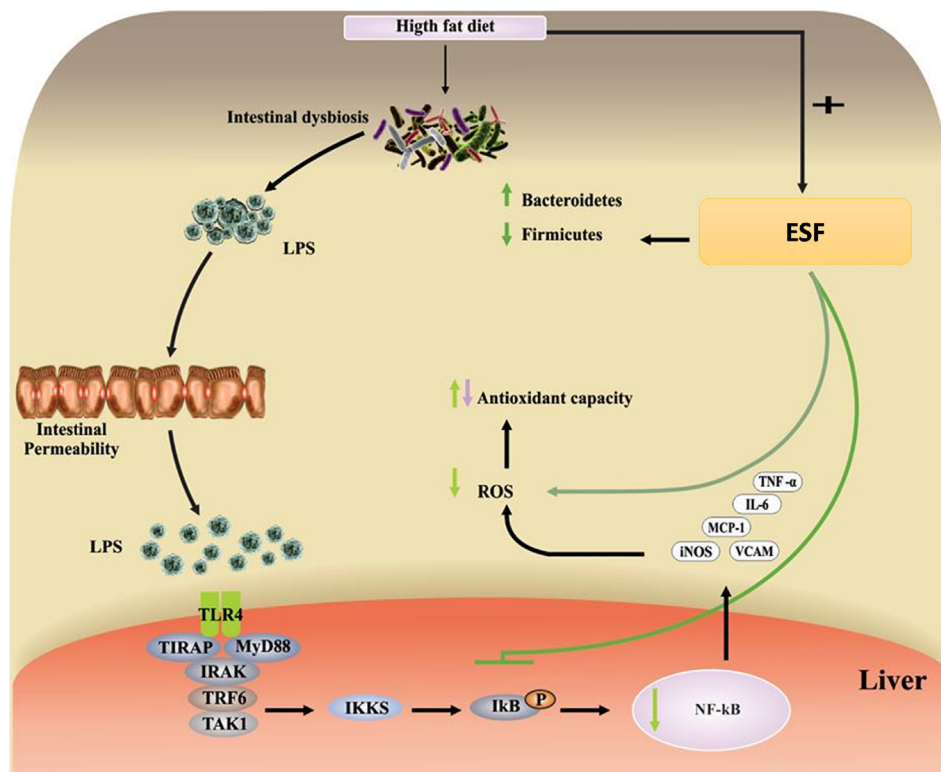


Fig. 5. Performance of high-fat diet and bioactive compounds of extruded sorghum flour in the modulation of intestinal microbiota, inflammation and oxidative stress. The HFD increase the intestinal dysbiosis, which is high LPS levels are released, also its damage the intestinal permeability. This scenario favors the LPS translocation to blood and tissue, as liver and its binding on TLR4 receptor, acting the downstream NF- κ B pathway and further the secretion of pro-inflammatory cytokines, chemokines, and enzymes. In addition the HFD increase the oxidative state by high ROS production. ESF was effective to increase the *Bacteroidetes* and reduced *Firmicutes*, which generate less LPS resulting in less activation of the inflammatory pathways and consequent lower systemic inflammation. Finally, ESF had antioxidant effect and improved the oxidative state. ESF: extruded sorghum flour; IKK: I κ B kinase; IL-6: Interleukin 6; iNOS: inducible nitric oxide synthase; IRAK: IL-1R-associated kinase; LPS: lipopolysaccharides; MCP-1: monocyte-attractive protein; MyD88: Myeloid differentiation factor 88; NF- κ B: Nuclear factor kappa B; ROS: reactive oxygen species; TAK1: Transforming growth factor β -activated kinase 1; TIRAP: TIR domain containing adaptor protein; TLR4: toll-like receptor 4; TNF- α : tumor necrosis factor α ; TRF6: tumor necrosis factor receptor-associated factor 6; VCAM: vascular adhesion molecule.

consumption of ESF by both groups, HFDS50 and HFDS100, increased CAT and the values were similar to AIN-93M group (Fig. 4F).

4. Discussion

The brown pericarp extruded sorghum is a cereal rich in dietary fiber and an excellent source of bioactive compounds such as 3-deoxyanthocyanines, tannins and phenolic acids (Cardoso, Pinheiro, Martino, et al., 2015). Phenolic compounds may reach the large intestine and to be metabolized by gut microbiota influencing its composition. The consumption of foods rich in phenolic compounds has been shown anti-obesity effects via modulating gut microbiota community structure and diversity (Xu et al., 2019). On another hand, dietary fiber is related with benefits against obesity, such as increase satiety and intestinal transit, lower lipids and glucose absorption, and it is the main determinant of gut microbiota composition and function (Benítez-Páez et al., 2016). Therefore, dietary fiber may regulate the abundance, complexity, and stoichiometry of certain strains on gastro-intestinal tract, keeping the gastrointestinal harmony (Heinritz et al., 2016).

Extruded sorghum flour changed the gut microbial community structure, especially by reducing the abundances of *Firmicutes*. Intestinal dysbiosis in obesity has been broadly correlated with the increase of phylum *Firmicutes* due to *Bacteroidetes* and in addition, this ratio is associated with considerable metabolic disturbances (Hildebrandt et al., 2009; Ismail et al., 2011; Ley, Peterson, & Gordon, 2006). On the other hand, the higher ratio of *Bacteroidetes* to *Firmicutes* is indicated by regulating energy balance and reducing adiposity (Bervoets et al., 2013; Woting, Pfeiffer, Loh, Klaus, & Blaut, 2014). However, the benefits effects occur when *Bacteroidetes* are a highly compartmentalized microbe-dense environment of the GI tract and are not stressed which drive to produce endotoxins, exotoxins, lipopolysaccharides, between others (Zhao, Jaber, & Lukiw, 2017). These findings, therefore, suggest the role of ESF, as a fiber and phenolic source, in modulating of composition and metabolic activity of the intestinal microbiota, which in turn can positively affect health.

Besides that, in this study, animals from the HFD group showed

increased of inflammatory markers p65 NF- κ B and resistin, and both were positively associated with the *Firmicutes* phylum. These results were expected since the increase of *Firmicutes* is associated with intestinal dysbiosis in the obese and this, in turn, with increased intestinal permeability (Kim, Gu, Lee, Joh, & Kim, 2012). This condition allows the influx of endotoxins (lipopolysaccharides, flagellins, peptidoglycans) which favors the activation of the signaling pathways, mainly NF- κ B. Then, the expression and secretion of inflammatory markers are stimulated, such as TNF- α , resistin, interleukins (IL-1 β , IL-6, IL-8), and inducible nitric oxide synthase (iNOS) (Caesar et al., 2015; Saad, Santos, & Prada, 2016).

On another hand, animals that consumed HFD plus ESF had reduced expression and protein levels of NF- κ B, as well as lower levels of serum resistin. The phenolic compounds found in ESF, especially anthocyanins, are able to inhibit NF- κ B activation and consequently the production and release of inflammatory cytokines and oxidant molecules (Burdette et al., 2010; Domitrovic, 2011; Rahman, Biswas, & Kirkham, 2006; Zhang et al., 2010). Besides that, the results of this research may be also attributed to the fiber fermentation, which promote the hypertrophy of intestinal mucosal cells, leading to an increase of surface area in the intestine (Weaver, Martin, Story, Hutchinson, & Sanders, 2010), reducing the intestinal permeability (Grancieri et al., 2017), and increase the production of mucus and antimicrobial peptides, such as defensins and cathelicidins (Barton & Kagan, 2009). All these factors avoid the translocation of endotoxins and consequent inflammation (Klingbeil & de La Serre, 2018).

Although not observed in this study differences in the concentrations of SCFA in feces, the consumption of dietary fiber is associated with increasing levels of short-chain fatty acids (SCFA), mainly acetate, propionate, and butyrate by microbiota fermentation, especially *Bacteroidetes* (De Vadder et al., 2014; Grancieri et al., 2017). These fatty acids exercise beneficial effects against high-fat diet and obesity complications (Carvalho et al., 2019). Acetate regulates the energy and metabolism and reduces the appetite through the central homeostatic mechanism. Butyrate and propionate regulate the release of intestinal hormones, reduce food intake, and are the energy source of

colonocytes, mainly butyrate, which also exhibits anti-inflammatory properties (Koh, De Vadder, Kovatcheva-Datchary, & Bäckhed, 2016).

However, the absorption of SCFA in the cecum and colon is a very efficient process, and only 5 to 10% (19–50 mmol) are excreted in the feces and the remainder used by colonocytes, hepatic and muscular tissue (Roediger & Moore, 1981), this fact may explain the lack of results in this research. Other studies that evaluated the production of SCFA in cecal material found higher production of these metabolites after consumption of dietary fiber, tannins and other phenolic compounds (Fotschki, Juśkiewicz, Sójka, Jurgoński, & Zduńczyk, 2015; Frejnagel & Juskiwicz, 2011; Goverse et al., 2017). However, overweight and obese adults that consumed sorghum bran extracts alone had no difference on SCFA levels to normal and overweight/obese controls (Ashley et al., 2019). In addition, the consumption of sorghum and unfermented probiotic milk by hemodialysis individuals increase the SCFA after 7 weeks of treatment (Lopes et al., 2019).

Another complication associated with obesity is the oxidation molecules or reactive oxygen species (ROS) development (Włodarczyk & Nowicka, 2019). ROS is associated with DNA damage, protein oxidation, lipid peroxidation, necrosis, and cell apoptosis (Stamler, Toone, Lipton, & Sucher, 1997). Obese animals that consumed ESF had lower hepatic concentrations of ROS such as nitric oxide (NO) and lipid peroxidation products, such as malondialdehyde when compared to animals in the HFD control group. Previous studies have demonstrated the association between sorghum and antioxidant capacity, suggesting that this effect is due to the presence of phenolic acids capable of inhibiting the NF- κ B activation pathway and consequently the production of iNOS and NO, as previously mentioned (Kim, Min, et al., 2012; Salazar Lopez et al., 2016). Furthermore, it has been showed that extrusion processing seems to increase the retention of low molecular weight proanthocyanidins (monomers and dimers), favoring the higher bioavailability of these compounds and, therefore, greater efficacy against oxidative stress (Cardoso, Pinheiro, de Carvalho, et al., 2015).

In addition, the consumption of ESF by animals reflected in higher total antioxidant capacity (TAC) in the plasma, as well as, they presented higher gene expression of the enzyme SOD (endogenous antioxidant) and heat shock protein HSP72 (in the HFDS100 group) cell chaperone that acts on the repair or degradation of impaired proteins (Kiang & Tsokos, 1998). However, the activities of hepatic antioxidant enzymes were reduced, such as SOD, or were not different, as Catalase and GS-T, in the animals that consumed ESF compared to the animals of the HFD-control group. These results may be associated to polyphenols content in ESF, which can eliminate ROS by direct acting on detoxification of oxidant radicals, through chelate redox metals (Khan, Yousif, Johnson, & Gamlath, 2015; Khosravi, Poursaleh, Ghasempour, Farhad, & Najafi, 2018). At this condition, the necessity of endogenous antioxidant enzymes, such as SOD, catalase, glutathione, between others, is less, which driving to reduced production of these enzymes in order to equilibrate the redox state of the organism (Khosravi et al., 2018).

Then, this research showed the contribution of the non-enzymatic antioxidants present in ESF, which together with dietary fiber functioned directly as radical scavengers, promoting redox cellular rebalancing in obese animals. Another experiment with mice observed an increase of antioxidant activity of a procyanidin-rich extract from sorghum (Wu et al., 2011), however, an investigation with *Wistar* rats fed with extruded whole-grain sorghum had any antioxidant systemic effect (Llopert et al., 2017).

Fig. 5 synthesised the effects of hyperlipidic diet on an organism and as ESF can act. The HFD increase the intestinal dysbiosis and consequently, the LPS from pathogenic bacteria. In addition, the intestinal permeability is also increased, allowing the LPS translocation to blood and tissue, such as liver. The LPS can bind on TLR4, acting the NF- κ B pathway and further the secretion of pro-inflammatory markers, like iNOS, cytokines, and MCP-1. Moreover, the HFD increase the oxidative state. The consumption of ESF by rats reduced the dysbiosis and

consequently, less LPS was available to stimulate the inflammatory process, which was proved by reduced nuclear p65- NF- κ B expression and seric resistin. Besides, the ESF had an antioxidant effect and improved the oxidative state in animals.

5. Conclusion

Extruded brown pericarp sorghum flour is a rich source of dietary fiber and phenolic compounds. Its consumption by rats fed a hyperlipidic diet improved intestinal dysbiosis by the increase of *Bacteroidetes* and the decrease of *Firmicutes*. In addition, inflammatory markers such as the NF- κ B transcription factor and the resistin protein had the expression reduced. Moreover, ESF reduced oxidative stress by inhibiting the production of reactive species and contributing to a higher total antioxidant capacity. These effects were observed when sorghum flour replace 50 and 100% of dietary fiber, which highlight the promising contribution of sorghum against obesity and its associated dysbiosis, inflammation, and oxidative stress, demonstrating that extruded sorghum consumption by humans should be stimulated.

6. Ethics statements file

The experiment was approved by the Ethics Committee of Animals Use of Universidade Federal de Viçosa, protocol No. 06/2014, following all the ethical requirements required for animal experimentation.

Declaration of Competing Interest

The authors declare no conflict of interest.

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