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Germinated millet flour (*Pennisetum glaucum* (L.) R. Br.) reduces inflammation, oxidative stress, and liver steatosis in rats fed with high-fat high-fructose diet

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ABSTRACT

This study investigated the effects of germinated millet flour on inflammation, oxidative stress, adiposity, and liver steatosis in Wistar rats fed with a high-fat high-fructose diet. In phase 1 of the experiment (8 weeks) the animals were separated into a control group with an AIN-93M diet (n = 10) and a high-fat high-fructose group (HFHF) (n = 20) with a diet rich in saturated fat (31%) and fructose (20%). In phase 2 (10 weeks), the control group was maintained on the AIN-93M diet (n = 10) and the HFHF group was divided into HFHF group (HFHF diet, n = 10) and the Millet group (HFHF with germinated millet flour replacing 43.6% dietary fiber, 100% starch, 36% protein and 39% oil in the experimental diet, n = 10). The germinated millet flour reduced ($p < 0.05$), in comparison with HFHF group, the adiposity (24.18 ± 4.45g to millet and 32.89 ± 8.46g to HFHF), triglycerides (100.00 ± 17.93g to millet and 147.8 ± 21.57g to HFHF), uric acid, ALT, NF-κB (131.9 ± 97.14 pg/ml to millet and 346.3 ± 58.88 pg/ml to HFHF), TNF-α (98.80 ± 19.90 pg/ml to millet and 141.4 ± 25.12 pg/ml to HFHF), platelet/lymphocyte and neutrophil/lymphocyte ratio, liver steatosis, inflammatory infiltrate (0.1880 ± 0.07 pg/ml to millet and 1.269 ± 0.17 pg/ml to HFHF), MDA levels, and liver collagen deposition. Further, germinated millet increased ($p < 0.05$) IL-10 (1511 ± 528.50 pg/ml to millet and 483.8 ± 91.59 pg/ml to HFHF), PPARα protein, total antioxidant capacity of the liver (0.2825 ± 0.04 pg/ml to millet and 0.2075 ± 0.05 pg/ml to HFHF) and the activity of SOD and catalase, compared to HFHF group. Thus, germinated millet flour was able to reduce adiposity and liver steatosis and presented anti-inflammatory and antioxidant effects, which highlight its functional biological effects.

1. Introduction

The consumption of simple carbohydrates, especially fructose, and saturated fat have increased since the past century, because of its use in processed foods, such as jellies, fast-foods, and soft drinks. The consumption of foods rich in fructose and saturated fat can lead to inflammation and oxidative stress, owing its pro-inflammatory and oxidative characteristics (Ter Horst and Serlie, 2017). Inflammation contributes to the overproduction of liver triglyceride (TG) that increase fat deposition in tissues and inflammatory response through the activation of pathways, such as tumor necrosis factor alpha (TNF-α) and nuclear factor kappa B (NF-κB) (Giriş et al., 2018). The inflammation can be related to platelet/lymphocyte ratio (RPL) and the neutrophil/lymphocyte ratio (RNL), since inflammation increases platelet activation, adhesion and aggregation, increase the neutrophil count, and reduce lymphocytes because of apoptosis and redistribution to lymphatic organs (Kim et al.,

Abbreviations: PPARα, Peroxisome Proliferator Activated Receptor Alpha; SOD, Superoxide Dismutase; CAT, catalase; IL-10, Interleukin-10; NF-κB, Nuclear factor kappa B; TNF-α, Tumor Necrosis Factor Alpha; RNL, Neutrophil/lymphocyte ratio; RPL, Platelet/lymphocyte ratio.

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2016).

The accumulation of liver lipid and inflammation contribute to the synthesis of reactive oxygen species (ROS), culminating in oxidative stress and less antioxidant capacity (Giriş et al., 2018). These changes induce hyperuricemia, due to a compensatory mechanism to neutralize ROS, and can lead to greater production of superoxide anion through the activation of enzymes xanthine oxidase and nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), causing the increase in oxidative stress. Oxidative stress is controlled by the endogenous antioxidant defense system, which includes antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT), and exogenous vitamins (C and E), flavonoids, and carotenoids (Francisqueti et al., 2017). This explains the search for natural foods, rich in bioactive compounds. In this context, millet can be a choice, since it has dietary fibers and bioactive phytochemicals with antioxidant capacity (Sharma and Gujral, 2019).

Millet has shown antioxidant and anti-inflammatory effects as non-germinated grain, once is a source of proteins, lipids, vitamins, minerals, and bioactive compounds, like phenolic acids, flavonoids, gallic acid, syringic acid, p-coumaric and ferulic acid (Chandrasekara and Shahidi, 2012). However, millet contains the compound glycosyl flavone that can inhibit the thyroid enzyme peroxidase (TPO), responsible for the production of thyroid hormones, and also phytates that decrease the bioavailability of nutrients. In view of this, it is suggested that these grains be subjected to processing, such as germination, to reduce these compounds and enable safe consumption. Germination is a simple and inexpensive process in which hydrolytic enzymes promote biochemical changes, structural modification and synthesis of new compounds that can increase the nutritional value and stability of the grains. This type of processing reduces antinutritional factors, such as phytic acid, increasing the bioavailability of vitamins, proteins, minerals, and fibers, showing to be as effective as other methods, such as extrusion and heat (Dias-Martins et al., 2018; Hithamani, Srinivasan, 2014).

Owheruo et al. (2019) demonstrated that germinated millet improves the phytochemical composition of the grains and, consequently, their antioxidant properties. However, no studies have evaluated the effects of germinated millet on metabolic changes *in vivo*. Our hypothesis was that germinated millet flour, as a source of fiber and phenolic compounds, can reduce the inflammation, oxidative stress, adiposity and liver fat in adult rats fed with high-fat high-fructose diet. Thus, the objective of this study was to investigate the effects of germinated millet flour on inflammation, oxidative stress, adiposity, and liver fat in rats, fed with high-fat high-fructose diet.

2. Material and methods

2.1. Germinated millet flour

Millet grains (*Pennisetum glaucum* LR Br.), cultivar BRS1502, with 99% germination rate, were soaked in water (1:3 grain/water ratio) for 3 h for germination. The grains were germinated at 30° ± 2 °C and 90% of relative humidity during 24 h, and dried at 50 °C, for 4 h. The germination rate was calculated as (total numbers of seed germinated/total number of seeds) x100 (Li et al., 2020). Then, the grains were grinding in a hammer mill LM3100 (Perten Instruments AB, Huddinge, Suécia) to pass through 1.0 mm screens.

This flour consists of 64.5% carbohydrate, 15.1% protein, 7.6% dietary fiber, 5.4% lipids, and 3.5 g/100 g of resistant starch. These data were used to calculate the experimental diet and discuss the results.

2.2. Chemical analysis

The analysis of fatty acid methyl esters was carried out in a gas chromatograph, model GC 2010 Plus (Shimadzu, Japan), equipped with a SPLIT injector, capillary column SP-2560 (75 m long × 18 mm, and diameter 14 μm) and flame ionization detector (FID). The working temperature of the detector and injector was 240 °C and the split ratio was 1:50. The initial temperature of the column was 150 °C, maintained for 5 min, then raised to 180 °C at a rate of 2 °C per minute and, maintained in isotherm for 12 min. Finally, the temperature was raised to 220 °C at a rate of 4 °C per minute and then kept in isotherm for 9 min.

A mixture of 37 fatty acid methyl esters (Supelco, USA), from C4:0 to C24:1, was used as standard. The fatty acids were identified according to retention times and quantification was done by normalizing the area (%) (Adapted of Silva et al., 2020).

The resistant starch content (RS) was determined through simulated digestion with pancreatic α-amylase and amyloglucosidase, using a commercial kit (Resistant star assay kit AACC 32–40, Megazyme, Sydney, Australia), as instructions of manufacturer. The content of total phenolic compounds in the millet was determined using the Folin-Ciocalteu method (Singleton et al., 1999). The determination of the total tannin content was performed by the vanillin/HCl reaction method, according to Price et al. (1978).

2.3. Animal study

Thirty male Wistar rats (*Rattus norvegicus*), 21 days old, received a commercial diet for 19 days. Then, the experiment was divided into two phases. In the first phase, to induce metabolic changes, the animals were randomly divided into two groups, according to body weight (wt): control group (wt 155.98 ± 17.05g, n = 10), which received AIN93-M diet and HFHF group (wt 158.03 ± 17.81g, n = 20), which received a diet rich in saturated fat (31%) and fructose (20%) (high-fat high-fructose diet), during 8 weeks (adapted from Marineli et al., 2015). After this period, the animals were fasted for 12 h to collect blood by capillarity for the analysis of the triglycerides concentration, using the commercial kit Accutrend GCT (Roche, Santo Andre, Brazil) device according the manufacturer's instructions. Triglycerides levels were used together with morphometric measurements to confirm the effects of the HFHF diet.

The second phase of the experiment consisted of intervention. The control group (AIN-93M; wt 349.94 ± 30.71g, n = 10) kept receiving normal diet AIN-93M, and the animals from the previous HFHF group were redistributed into two groups: HFHF control (wt 366.89 ± 36.90g, n = 10), which maintained a high-fat high-fructose diet, and millet group (wt 370.12 ± 36.49g, n = 10) with HFHF diet added with germinated millet flour, for more 10 weeks (adapted from Marineli et al., 2015). In all stages, the animals received water and diet *ad libitum*.

The millet diet had replacement of 43.6% of dietary fiber (21.76g), according to recommendation for rodents (Reeves et al., 1993), from germinated millet flour. In this way, 286.3g of flour/kg diet was used, which provided the replacement of 100% starch, 36% protein, and 39% oil. The HFHF group received a diet with high lipid content (4% soy oil plus 31% lard), in addition to 20% fructose (Marineli et al., 2015) (Table 1).

After the 10th week, the animals were anesthetized with isoflurane and euthanized by cardiac puncture. Blood was collected in a heparin tube and centrifuged at 1008 g for 15 min at 4 °C to collect plasma. The adipose tissues (mesenteric, retroperitoneal, and epididymal) were washed with saline solution (PBS), weighed, immersed in liquid nitrogen, and later stored at −80 °C. The liver was also removed, washed in phosphate-buffered saline solution (PBS), part of which was fixed in 10% formaldehyde and kept at room temperature for later histological analysis, and the remaining was stored at −80 °C until the time of analysis.

The study was approved by the Ethics Committee on the Use of Animals of the Federal University of Viçosa (CEUA/UFV), process n ° 39/2019. All the experimental procedures with animals were performed in accordance with Directive 86/609/EEC of November 24, 1986, in compliance with the ethical principles for animal experimentation.

Table 1
Composition of the experimental diets.

Ingredients (g/kg-1)	AIN93-M	HFHF	Millet
Albumin*	136.6	136.6	87.3
Corn starch	463.5	135.0	-
Dextrinized starch	155.0	45.0	-
Sucrose	100.0	28.6	8.6
Lard	-	310.0	310.0
Soy oil	40.0	40.0	24.5
Cellulose	55.8	55.8	34.0
Mineral mix	35.0	35.0	35.0
Vitamin mix	10.0	10.0	10.0
L-cystine	1.8	1.8	1.8
Choline Bitartrate	2.5	2.5	2.5
Resistant starch	-	-	10.1
Germinated millet flour	-	-	286.3
Fructose	-	200.0	200.0
Total phenolics	-	-	0.6
Condensed tannins	-	-	0.1
Composition of diets (%)			
Carbohydrate	73.2	42.5	40.3
Protein	12.0	12.0	12.0
Lipid	4.0	35.0	35.0
Caloric density (kJ)	158.99	221.75	217.57

Composition of the experimental diets based on standard rodent diet (AIN-93M) (Reeves et al., 1993). HFHF: high-fat high-fructose diet; millet: test diet containing HFHF added with germinated millet flour, replacing 43.6% dietary fiber, 100% starch, 36% protein and 39% oil. * Albumin based on 88% protein content.

2.4. Feed efficiency and energy efficiency ratio, and biometric measures

Food consumption and animal weight were obtained weekly. The feed efficiency ratio (FER) was obtained through the relationship between body weight gain (g) and the amount of diet (g) consumed by the animals. The energy efficiency ratio (EER) was obtained by the final body weight gain (g) divided by the total energy consumption (kcal) multiplied by 1000.

An inelastic measuring tape was used to measure waist circumference and naso-anal length, which were used to calculation of Lee index. The total body fat was obtained by the sum of mesenteric, retroperitoneal, and epididymal adipose tissue fat.

2.5. Biochemical analysis

Plasma concentrations of aspartate aminotransferase (AST; code k048-6, Bioclin, Belo Horizonte, Brazil), alanine aminotransferase (ALT; code k049-6, Bioclin, Belo Horizonte, Brazil), and uric acid (code k139-2, Bioclin, Belo Horizonte, Brazil) were evaluated by colorimetric methods. In addition, the platelet/lymphocyte ratio (RPL) and neutrophil/lymphocyte ratio (RNL) were calculated as the ratio between the number of platelets and neutrophils with lymphocytes, respectively.

The concentration of triglycerides was measured at the end of the period of induction of metabolic changes (phase 1) and after 10 weeks of intervention, before euthanasia (phase 2). The blood collection was done by capillarity, using the ACCU-CHEK Active and Accutrend GCT (Roche, Santo Andre, Brazil) device according to the manufacturer's instructions.

2.6. Quantification of p65-Nuclear factor kappa B and peroxisome proliferator-activated receptors alpha and cytokines in liver tissue

The liver homogenate was prepared using a 50 mM phosphate buffer with 1 mM EDTA (pH 7.4), containing protease inhibitor (Protease Inhibitor Cocktail powder, code P2714; Sigma Aldrich, St. Louis, USA), in a 1:10 (wt:v) proportion of tissue and buffer. The concentrations of IL-10 and TNF-α were quantified in liver homogenates using Rat IL-10 kit ELISA (code E-EL-R0016, Elabscience, Houston, USA) and Rat TNF alpha Uncoated ELISA kit (code #88-7340-88, Invitrogen, Vienna, Austria), respectively, as recommended by manufacturers.

The p65-NF-κB and PPAR-α were quantified in the nuclear fraction of liver tissue, which were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (code 78835; Thermo Scientific, Vienna, Austria). To quantify p65-NF-kB was used the commercial kit Rat NF-kB-p65 ELISA (code E-EL-R0674; Elabscience, Houston, USA) and for PPAR-α, the kit Rat PPAR-α ELISA (code E-EL-R0725; Elabscience, Houston, USA), following manufacturers' recommendations. The concentrations of IL-10, TNF-α, p65-NF-κB, and PPAR-α were calculated by comparison to the corresponding standard curves.

2.7. Antioxidant enzymes and total antioxidant capacity

The total protein concentrations were quantified in liver homogenates by Bradford (1976). The quantification of Superoxide Dismutase (SOD) was performed in relative units, and one unit was defined as the amount of SOD enzyme that inhibits the auto-oxidation of pyrogallol. The analysis was carried out on a spectrophotometer (Multiskan GO, Thermo Scientific; Vienna, Austria) at 570 nm, and the results were expressed as units of SOD per milligram of protein (Marklund, 1985).

Catalase (CAT) was determined in the liver by the ability to cleave hydrogen peroxide (H_2O_2) in water. Catalase activity was analyzed at 0, 30, and 60 s after the reaction was initiated and the absorbance was read at 240 nm on a spectrophotometer (T70+UV/VIS Spectrometer) (Aebi, 1984).

The Total Antioxidant Capacity (TAC) was measured in the liver homogenate and plasma using the commercial antioxidant assay kit (code MAK187; Sigma-Aldrich, St. Louis, USA), according to the manufacturer's specifications. Malondialdehyde (MDA) was measured by the method of thiobarbituric acid reactive substances (TBARS), according to the methodology described by Buege e Aust (1978).

2.8. Histological analysis in liver tissue

The liver tissue was fixed in 10% formaldehyde and later embedded in resin. The gomori trichrome was used for counting inflammatory infiltrate and lipid vesicles and the slides were photographed under a bright field microscope (Olympus AX 70 TRF, Tokyo, Japan). Picrosirius staining was used to quantify type I, III, and total collagen, and the slides were photographed under a light microscope (Olympus BX53 monochrome photomicroscope) with a polarizing filter coupled to an image analyzer. The analyses were performed using the Image J® 1.48v software system (National Institute of Health, USA) in a histological field with a reticule of 266 points on the images until reaching the sum of 1064 points per animal, using a 20x objective. The degree of steatosis was assessed semi-quantitatively, according to a scale of 5°: grade 0, if the percentage of fat was absent or less than 5%; Grade 1, if $\geq$ 5% and <25%; Grade 2, if $\geq$ 25% and <50%; Grade 3, if $\geq$ 50% and <75%; and grade 4, if $\geq$ 75% (Turlin et al., 2001).

2.9. Statistical analysis

The results were expressed as means ± standard deviation. The data were submitted to normality tests of Shapiro-Wilk and Kolmogorov-Smirnov. The one-way analysis of variance (ANOVA) was applied, followed by the Newman-Keuls test, at 5% of significance. The statistical analysis and graphic were performed using the GraphPad Prism software system, version 6.0.

3. Results

3.1. Germinated millet flour reduced adiposity and biochemical variables, increase the phenolics and fatty acids consumption, and had no effects on biometric measurements

It was observed that the intake of the HFHF diet, in phase I of the

experiment, caused an increase of 58.92% of the animals' weight. The animals in the control group (standard diet) had a 55.42% increase in body weight in phase 1. After 18 weeks of the experiment (the end of phase 2), there was no difference in body weight (Supplementary Fig. 1 and Table 2), waist circumference, and Lee index among the experimental groups. However, the total body fat was higher ($p < 0.05$) in animals from the HFHF group. Germinated millet flour reduced ($p < 0.05$) adiposity in comparison to the HFHF group, which was similar to the AIN-93M control group (Table 2). Besides, the animals fed with the germinated millet flour decreased ($p < 0.05$) triglycerides, uric acid, and ALT relative to group HFHF, however, AST levels did not differ between the experimental groups (Table 2).

The control group AIN-93M had higher ($p < 0.05$) food consumption and lower ($p < 0.05$) food efficiency ratio (FER), compared to the HFHF and millet groups. However, the energy efficiency ratio (EER) did not differ between the experimental groups.

The germinated millet flour consists of 2.17 mg GAE/g of total phenolics, 0.42 mg CE/g of condensed tannins, and 3.5 g/100 g of resistant starch. In the total diet consumed, throughout the intervention, the animals of the millet group consumed 10.39g of resistant starch, 0.64g of total phenolics, 0.12g of condensed tannins, 132.06 mg of linoleic acid, 63.87 mg of oleic acid, 52.42 mg of palmitic acid, 17.80 mg of stearic acid and 12.68 mg of lauric acid from the flour (Table 2).

Table 2
Effect of germinated millet flour on adiposity, biochemical variables, and profile of food consumption in the animals induced to metabolic changes with the HFHF diet, after 10 weeks of intervention.

Variables	AIN-93M	HFHF	Millet
Adiposity			
Total body fat (g)	20.08 ± 2.67[b]	32.89 ± 8.46[a]	24.18 ± 4.45[b]
Biochemical variables			
Triglycerides (mg/dL)	117.8 ± 20.37[b]	147.8 ± 21.57[a]	100.0 ± 17.93[b]
Uric acid (mg/dL)	1.17 ± 0.51[b]	2.13 ± 0.56[a]	1.17 ± 0.47[b]
ALT (U/L)	31.13 ± 9.67[b]	52.14 ± 11.26[a]	39.29 ± 4.82[b]
AST (U/L)	120.9 ± 17.19[a]	130.3 ± 11.81[a]	120 ± 25.35[a]
Biometric Measurements			
Initial weight (g) *	349.90 ± 30.71[a]	366.90 ± 36.90[a]	370.10 ± 36.49[a]
Final weight (g)	415.00 ± 34.52[a]	438.10 ± 66.65[a]	443.50 ± 28.21[a]
Weight gain (g)	68.00 ± 10.71[a]	64.96 ± 18.07[a]	75.79 ± 21.46[a]
Waist circumference (cm)	18.85 ± 0.78[a]	19.02 ± 0.64[a]	19.41 ± 0.51[a]
Lee index (g/cm^3)	292.80 ± 14.55[a]	297.90 ± 13.79[a]	303.20 ± 8.87[a]
Food Consumption **			
Total food consumption (g)	1367.00 ± 69.07[a]	990.10 ± 59.44[b]	1028.00 ± 23.78[b]
FER (%)	4.73 ± 0.96[b]	7.23 ± 2.04[a]	8.13 ± 1.03[a]
EER (%)	1.40 ± 0.30[a]	1.41 ± 0.50[a]	1.59 ± 0.20[a]
Resistant starch (g)	-	-	10.39
Total phenolics (g)	-	-	0.64
Condensed tannins (g)	-	-	0.12
Linoleic acid (mg)	-	-	132.06
Oleic acid (mg)	-	-	63.87
Palmitic acid (mg)	-	-	52.42
Stearic acid (mg)	-	-	17.80
Lauric acid (mg)	-	-	12.68

* Initial weight: measured at the beginning of the Phase II (intervention). ** Food consumption: Sum of all consumption during the intervention. Values expressed as means and standard deviation (n = 10/group). Different letters in line indicate significant differences by the Newman-Keuls test, at 5% probability. AIN-93M group: Animals fed with standard diet; HFHF group: high-fat high-fructose diet; millet: HFHF diet added with germinated millet flour. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; FER: Food efficiency ratio; EER: Energy efficiency ratio.

3.2. Germinated millet flour increases anti-inflammatory cytokine and reduces the inflammatory markers, collagen deposition, and liver steatosis

The millet group presented higher ($p < 0.05$) concentrations of anti-inflammatory cytokine IL-10 compared to the HFHF group and AIN-93M control (Fig. 1A). Also, germinated millet flour reduced ($p < 0.05$) TNF-α concentrations compared to the HFHF group but remained higher than AIN-93M control (Fig. 1B). Furthermore, the millet group had the lowest values of p65-NF-κB (Fig. 1C), RPL (Fig. 1D), and RNL (Fig. 1E) ($p < 0.05$) than the HFHF group, which was similar ($p > 0.05$) to the AIN-93M group. The germinated millet flour increased ($p < 0.05$) the concentration of PPAR-α protein, which was similar to the AIN-93M control group (Fig. 1F).

As for liver histomorphometry, the animals in the AIN-93M group presented 14.1% lipids into the liver, which was classified as grade 1 steatosis. The HFHF diet increased ($p < 0.05$) this percentage to 30.2% and was classified as grade 2 steatosis. The germinated millet flour reduced this percentage ($p < 0.05$) to 23.7%, thus reverting steatosis to grade 1 (Fig. 2). The millet group presented a lower ($p < 0.05$) amount of inflammatory infiltrates and collagen deposition (type I, type III, and total collagen), compared to the AIN-93M and HFHF groups (Fig. 2).

3.3. Germinated millet flour increases SOD, CAT, and total liver antioxidant capacity

The consumption of germinated millet flour increased ($p < 0.05$) the antioxidant enzymes CAT (Fig. 3A) and SOD (Fig. 3B) compared to the AIN-93M and HFHF groups. In addition, TAC of the liver was higher in the millet group, compared with the HFHF group, and similar to the control group AIN-93M (Fig. 3C). The plasmatic TAC did not differ between the experimental groups (Fig. 3D). However, the MDA levels reduced in comparison with the HFHF group ($p < 0.05$) and became similar to the AIN-93 group (Fig. 3E).

4. Discussion

The present study demonstrated that germinated millet flour increased anti-inflammatory cytokine and reduced inflammatory markers, in addition to presenting antioxidant activity, and reduced adiposity and liver steatosis, thus minimizing the metabolic changes induced by the HFHF diet in adults Wistar rats.

The germinated millet flour reduced the concentration of ALT, triglycerides, and adiposity and increased PPAR-α concentration, compared to the animals fed with HFHF diet. The presence of fatty acids, dietary fiber (7.6%) and resistant starch (3.5g/100g) in the millet flour may have provided these effects, increasing lipid oxidation and reducing body fat and triglycerides. Fatty acids, such as palmitic acid, oleic acid, and linoleic acid, can bind directly to PPAR-α and act as agonists, which increases the expression and activity of this receptor (Han et al., 2017), as observed in our study. Thus, increasing the concentration of PPAR-α may have increased the expression of the genes involved in the peroxisomal and mitochondrial β-oxidation pathways, thus playing an important role in the oxidation of fatty acids. Therefore, the germinated millet flour was able to recover the concentration of PPAR-α and increase the oxidation of fatty acids, which reduced the deposition of fat in tissues and liver and adiposity, besides reverting steatosis to grade 1.

The HFHF diet-induced inflammation and oxidative stress without affecting the weight, waist circumference, Lee index, and EER of the animals. The lower food consumption in groups fed a high-fat high fructose diet (HFHF and Millet) probably was owing to the higher caloric density, which increases the satiety and control food intake. These results corroborate with the study of Stanhope (2016), which also does not observe any difference either in weight gain or total energy intake in animals fed with the HFHF diet. The non-change in AST concentration between the experimental groups can be explained by its short half-life (12h) and rapid normalization in blood.

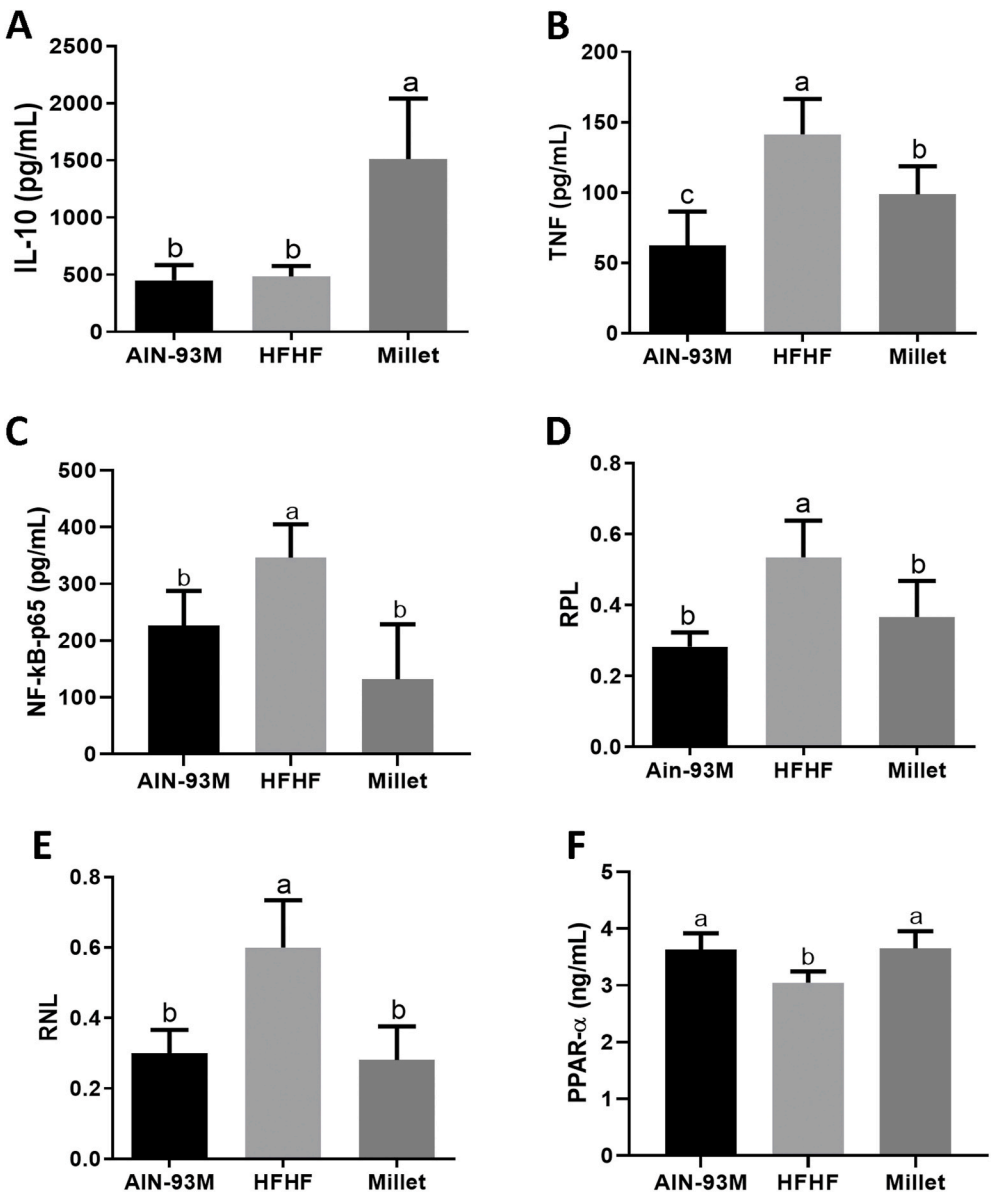

Fig. 1. Effect of germinated millet flour in inflammatory markers in animals induced to metabolic changes with the HFHF diet, after 10 weeks of intervention. Quantification of interleukin 10 (IL-10) (A); tumor necrosis factor (TNF-α) (B); nuclear factor kappa B (p65-NF-κB) (C); platelet/lymphocyte ratio (RPL) (D); neutrophil/lymphocyte ratio (RNL) (E) and peroxisome proliferator activated receptor alpha (PPARα) (F). The concentration of IL-10, TNF-α, p65-NF-κB and PPAR-α were measure in liver tissue by ELISA test. The RPL and RNL were analyzed using a leukogram-calorimetric tests. Values expressed as means and standard deviation (n = 10/group). Different letters indicate significant differences by Newman-Keuls test at 5% probability. AIN-93M group: Animals fed with standard diet; HFHF group: high-fat high-fructose diet; millet: HFHF diet added with germinated millet flour.

The germinated millet flour was able to reduce inflammation by activating the anti-inflammatory cytokine IL-10 and reducing TNF-α, NF-κB, RPL, and RNL, which consequently reduced the inflammatory infiltrate into liver tissue. These effects can be related to the polyphenols and prebiotics (dietary fiber and resistant starch) present in the millet, which gave it an anti-inflammatory and antioxidant potential. In our study, germinated millet flour provided a consumption of 0.72 g/kg/day of dietary fiber and 0.33 g/kg/day of resistant starch. These effects may have been improved with the consumption of 20 mg/kg/day of total phenolics and 4 mg/kg/day of condensed tannins, which are effective in reducing inflammation (Yahfoufi et al., 2018).

Besides, germinated millet flour increased the activity of SOD and CAT, recovered the total antioxidant capacity of the liver, and reduced uric acid concentration. The bioactive compounds and prebiotics present in the germinated millet flour may also have reduced oxidative stress, by modulating inflammation, mainly phenolic compounds. These compounds can inhibit enzymes responsible for ROS generation, such as NADPH oxidase (NOX) and xanthine oxidase, and increase endogenous antioxidant enzymes like SOD, CAT, and glutathione (Yahfoufi et al., 2018), contributing to improving the antioxidant activity in cells, reducing the lipid peroxidation since MDA levels were reduced by germinated millet, as we observed in this study. Plasmatic TAC did not increase in the millet group, probably due to the low half-life of phenolic compounds in plasma, preventing them from being detected in the analyzes (Grgić et al., 2020).

The oxidative stress, in addition to being related to inflammation, can also be caused by liver damage, which in turn produces more ROS, generating a vicious circle. The imbalance of oxidative stress affects the transcription of numerous biochemical mediators, such as cytokines, mainly TNF-α and Transforming Growth Factor (TGF-β), which can modulate the tissue and cellular events that characterize different types of chronic liver damage, such as apoptosis, necrosis, fibrosis, cholestasis, and regeneration (Vitaglione et al., 2005). This evidence wes observed in our study, in which the HFHF induced high inflammation with high levels of TNF-α, decreasing antioxidant defenses and causing liver damage.

The HFHF diet promoted grade II steatosis, which led to a redox imbalance, with local inflammation and collagen deposition. The same was observed by Feillet-Coudray et al. (2019), who demonstrated the presence of steatosis, while Balakumar et al. (2016) observed an increased collagen deposition around the portal triad in the animals fed with HFHF diet. Although liver damage markers AST and ALT did not

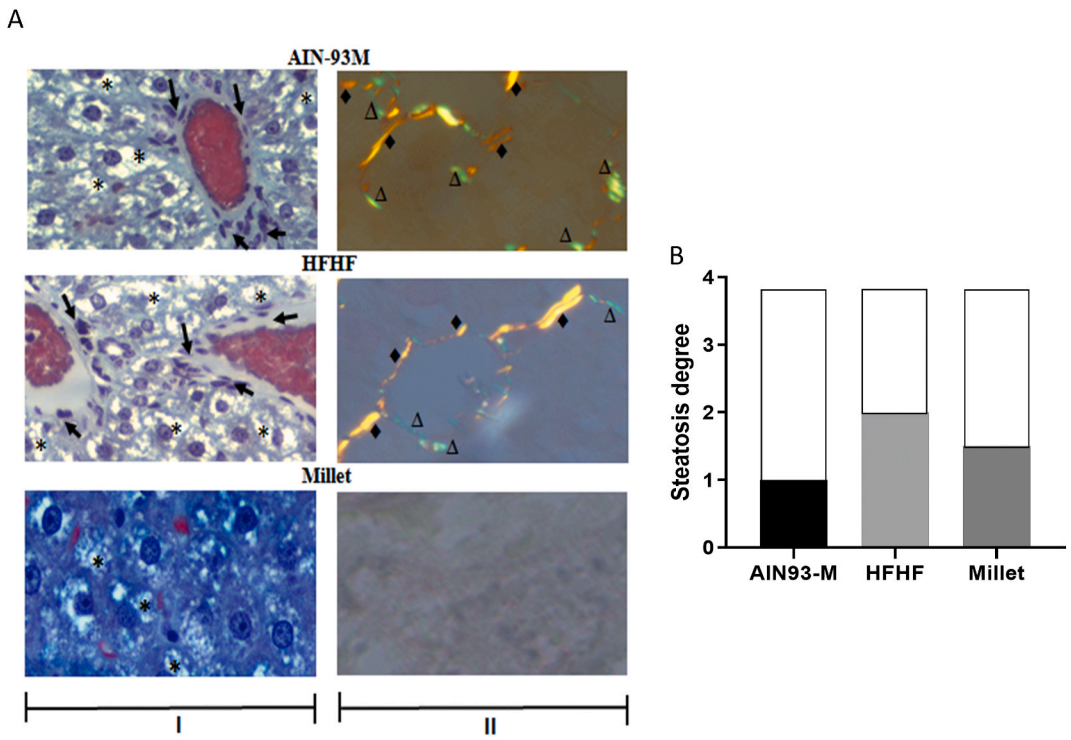

Fig. 2. Effect of germinated millet flour in liver steatosis, inflammatory infiltrate and collagen deposition in the liver of animals induced to metabolic changes with the HFHF diet, after 10 weeks of intervention. Representative photomicrographs of liver cuts. AIN-93M group: Animals fed with standard diet; HFHF group: high-fat high-fructose diet; millet: HFHF diet added with germinated millet flour (A). The percentage of fat was classified in degrees of liver steatosis, according to the classification proposed by Turlin et al. (2001) (B). (*): Lipid vesicles; (→): Inflammatory infiltrate; (◆): Type I collagen; (Δ): Type III collagen. Staining was carried out with gomori (I- steatosis) and picrosirius trichrome (II-collagen). Bar: 20 μm. Objective: 20×.

increase, the control group AIN-93M presented fat and collagen deposition in the liver, in addition to inflammatory infiltrate. These results may have occurred from the increased synthesis of liver fatty acids, through the activation of the *de novo* lipogenesis pathway, because of the large amount of carbohydrate (73%) present in the AIN-93M diet (Ferramosca et al., 2013). Furthermore, the reduction of steatosis and inflammation by germinated millet flour probably prevented the formation of collagen into liver tissue and reduced its damage in animals fed with this germinated grain.

Millet is considered a subsistence crop, which is consumed in human food only in developing countries (Dias-Martins et al., 2018). In the present study, an amount equivalent to a cup of tea of germinated millet flour was used, which corresponds to human consumption of 142.8g per day. This amount can be achieved through the use of this flour in the preparation of cakes, cookies, and pasta, increasing the supply of nutrients and bioactive compounds with beneficial effects on the body. The use of millet in culinary preparations may involve the use of dry heat (roasting), pressure cooking, use of microwaves, among other methods. These processes can be related to changes in the concentration and even in the bioaccessibility of nutritional compounds. It was demonstrated that roasting millet (150 °C, 5min) there was an increase in the total number of polyphenols and flavonoids in the cereal, but the use of microwave (300, 450, and 600 W for 3 min) and open-pan boiling (excess of water in an open-pan at 5, 10 and 15 min) reduced the total of these compounds compared to the raw grain. However, all the processes used in the study, as roasting, germinating (48 h to germination), open-pan boiling, or pressure-cooking (triple distilled water for 15–20 min), led to an increased bioaccessibility of polyphenols and flavonoids. Therefore, although some culinary processes can reduce the content of bioactive compounds, it also makes them more accessible for use by intestinal bacteria and intestinal absorption (Hithamani, Srinivasan, 2014). Thus, the use of thermal processing, after germination, can further favor the bioavailability of bioactive compounds present in millet.

Therefore, in view of our results, we propose that the consumption of germinated millet can increasing the expression of PPARα, which increased the β-oxidation of fatty acids, thus reducing the levels of triacylglycerol and accumulation of lipids into adipocytes, reducing fat boy and consequently hepatic steatosis. With less adipose tissue, the migration of macrophages into it is reduced, leading to less activation of NF-κB and consequently less pro-inflammatory factors were formed, such as TNF-α, in addition to better functionality of the blood immune cells, improving the values of RPL and RNL. On the other hand, anti-inflammatory factors were stimulated, such as IL-10. Furthermore, the phenolic compounds present in millet stimulated the expression of antioxidant enzymes, such as CAT and SOD in the liver, reducing oxidative stress with consequent less inflammatory stimulation in the liver tissue, reducing inflammatory infiltrates and collagen deposition. All of these changes helped to reverse the metabolic damage caused by a diet rich in saturated fat and fructose (Fig. 4).

5. Conclusion

The germinated millet flour showed anti-inflammatory, antioxidant action in high-fat and high-fructose fed rats, besides reducing adiposity and liver steatosis, which open a new field of investigation against the chronic diseases. Given the results, we must promote the insertion of millet in human food, since it worked as a functional food in the present study and can be easily inserted into suitable culinary preparations. However, technological properties of this flour and the impact of food processing must be further investigated to guarantee the preservation of the functional outcomes observed in this study.

CRediT authorship contribution statement

Jaqueline Maciel Vieira Theodoro: Conceptualization, Data

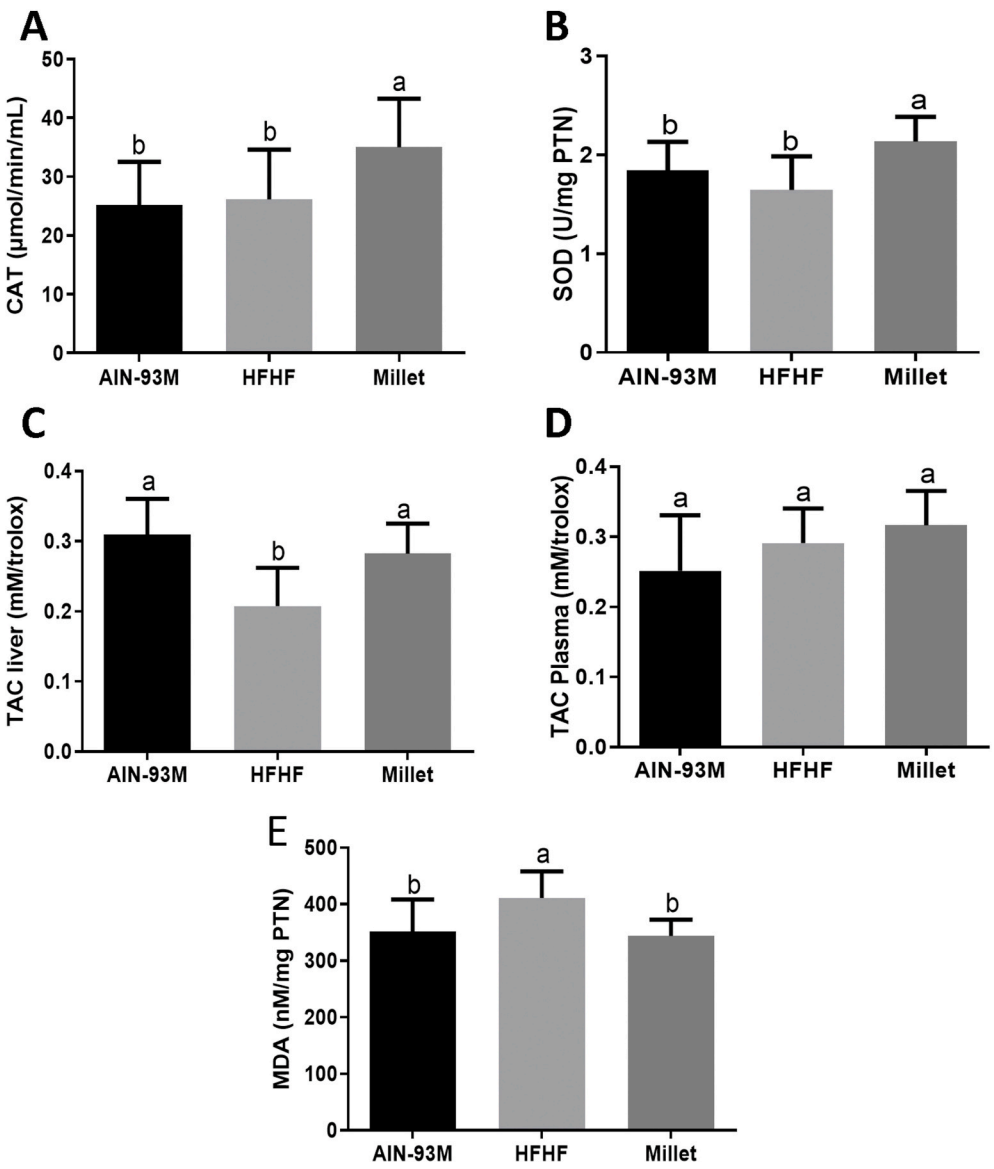

Fig. 3. Effects of germinated millet flour in antioxidant capacity of the liver in animals induced to metabolic changes with the HFHF diet, after 10 weeks of intervention. Catalase (CAT) (A); superoxide dismutase (SOD) (B) Total antioxidant capacity (TAC) in liver (C); TAC in plasma (D); Malondialdehyde (MDA) (E). Values expressed as means and standard deviation (n = 10/group). The CAT, SOD, TAC, and MDA were measured using colorimetric analysis. Different letters indicate significant differences by Newman-Keuls test at 5% probability. AIN-93M group: Animals fed with standard diet; HFHF group: high-fat high-fructose diet; millet: HFHF diet added with germinated millet flour.

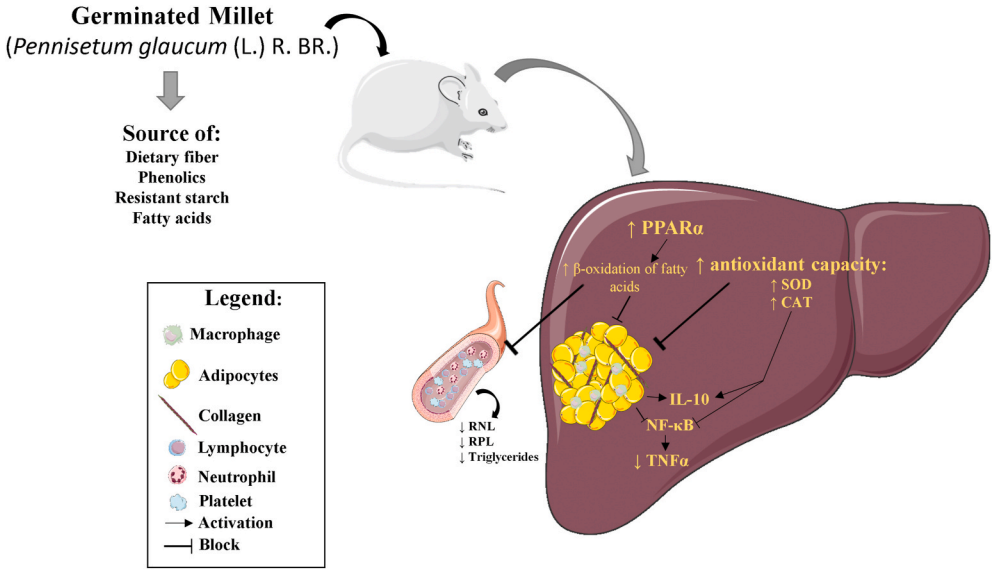

Fig. 4. Schematic figure of the effects of millet germinating on metabolic changes caused by a diet rich in saturated fat and fructose. The germinated millet increased the expression of PPARα, which induced the β-oxidation of fatty acids. Therefore, less fat deposition occurred in the liver, reducing hepatic steatosis, which reduced the expression of NF-κB and the production of pro-inflammatory factors, such as TNF-α, in addition to stimulating a better blood cellular immune response. In addition, the germinated millet increased the production of antioxidant enzymes, such as catalase and superoxide dismutase, which reduced oxidative stress and helped reduce inflammation. PPARα: Peroxisome Proliferator Activated Receptor Alpha; SOD: Superoxide Dismutase; CAT: catalase; IL-10: Interleukin-10; NF-κB: Nuclear fator kappa B; TNF-α: Tumor Necrosis Factor Alpha; RNL: Neutrophil/lymphocyte ratio; RPL: Platelet/lymphocyte ratio.

curation, Formal analysis, Writing – original draft, Software. **Oscar David Medina Martinez:** Data curation, Formal analysis. **Mariana Grancieri:** Formal analysis, Visualization, Writing – review & editing. **Renata Celi Lopes Toledo:** Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – review & editing. **Amanda M. Dias Martins:** Conceptualization, Investigation, Resources. **Desirrê Morais Dias:** Conceptualization, Investigation, Resources. **Carlos Wanderlei Piler Carvalho:** Conceptualization, Resources, Supervision, Funding acquisition, Investigation, Methodology, Writing – review & editing. **Hércia Stampini Duarte Martino:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcs.2021.103207.

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