

Original article

Açaí (*Euterpe oleracea* Martius) supplementation improves oxidative stress biomarkers in liver tissue of dams fed a high-fat diet and increases antioxidant enzymes' gene expression in offspring

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ABSTRACT

Lipids excess from an uterine environment can increase free radicals production of and thus induce oxidative status imbalance, a key factor for progression of non-alcoholic fatty liver disease (NAFLD) in offspring. Food antioxidant components in maternal diet may play an important role in preventing offspring metabolic disorders. The objective of the study was to evaluate the effects of açaí pulp supplementation on maternal high-fat diet, by assessing activity and expression of antioxidant enzymes and biomarkers of oxidative stress in the liver. Female Fisher rats were divided into four groups and fed a control diet (C), a high-fat diet (HF), a control diet supplemented with açaí (CA) and a high-fat diet supplemented with açaí (HFA) before mating, during gestation and lactation. The effects of açaí supplementation on oxidative stress biomarkers and antioxidant enzymes expression were evaluated in dams and male offspring after weaning. HFA diet increased body weight in dams, however reduced absolute and relative liver weight. There was a reduction in liver biomarkers of oxidative stress, malondialdehyde and carbonyl protein, as well as in catalase, glutathione peroxidase and superoxide dismutase activity. In offspring, HFA diet reduced liver weight and increased *Gpx1*, *Gpx4* and *Sod1* mRNA expression. These results suggest that açaí is able to restore redox status, preventing oxidative damage in dams by a direct mechanism and to promote beneficial effects on expression of antioxidant defences related genes in offspring.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is defined by an excessive accumulation of triacylglycerols (TAG) in the liver. This condition encompasses a spectrum of diseases ranging from simple hepatic steatosis to non-alcoholic steatohepatitis (NASH), characterised by an inflammatory and oxidative status, which may progress to cirrhosis or hepatic carcinoma [1]. The prevalence of NAFLD has increased exponentially in children with the dramatic growth of obesity, and is rapidly becoming the most common cause of liver disease in Western countries [2]. Several studies point to an association between NAFLD in the paediatric population and high prevalence of maternal obesity, mainly due to the excess of nutrients in western diets [3,4].

Lipids excess from an uterine environment can promote

mitochondrial dysfunction and reduce mitochondria oxidative capacity [5]. Mitochondrial abnormalities alter the balance between pro-oxidant and antioxidant mechanisms, leading to an increase in non-metabolised fatty acids in the cytosol and consequent induction of reactive oxygen species (ROS) production [6]. Dysfunction can occur through multiple mechanisms, ranging from mitochondrial DNA damage to sirtuin imbalance [7].

Sirtuin 3 (SIRT3) is an enzyme of the protein family currently classified as histone deacetylase class III (HDAC), located in the mitochondria [8]. SIRT3 regulates cellular antioxidant capacity through direct modulation of the main antioxidant enzymes that act to prevent oxidative damage [9]. The antioxidant effects of SIRT3 are mediated in part by its interaction with MnSOD (manganese-dependent superoxide dismutase or SOD2) [10]. The reduction of SIRT3 expression and/or

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activity is probably linked to reduced mitochondrial function and oxidative capacity, while the increase in ROS may, in turn, further alter the function of SIRT3.

Two antioxidant defence systems protect the body from ROS deleterious effects. One comprises of enzymes that catalyse reactions to neutralise free radicals; increased production of ROS promotes induction of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR), via the Keap1-Nrf2 [Kelch-like ECH-associated protein 1-nuclear factor (erythroid-derived 2)-like 2] pathway [11]. Nrf2 is normally present in the cytosol bound to the repressor protein Keap1. Under oxidative stress condition, ROS can oxidise cysteine residues of Keap1, allowing Nrf2 to dissociate from Keap1, translocate to the nucleus and bind to the antioxidant response element (ARE) promoter region of gene encoding several antioxidant enzymes such as SOD, CAT and GPx [11]. These enzymes play an important role: cytoplasm CuZnSOD or SOD1 and mitochondrial MnSOD or SOD2 catalyse a dismutation that degrades superoxide anions into H₂O₂, which is then converted to water and oxygen by the action of CAT or GPx. CAT is one of the most efficient enzyme in the antioxidant enzyme system, whereas GPx, with selenium as part of the active centre, catalyses the conversion of H₂O₂ from the oxidation of glutathione, which is constantly reused by glutathione reductase (GR) [12].

The other antioxidant defence system includes diet associated compounds such as polyphenols. These antioxidants can act directly through elimination of free radicals; however, sequestration of these radicals is not their unique biochemical action [13]. Antioxidants may exert their effects at different levels and their actions include hydrogen donor, electron donor, peroxide decomposer, singlet oxygen quencher, enzyme inhibitor, synergist, and metal ion-chelating activity [13]. In liver diseases, especially those involving oxidative stress processes, antioxidant substances such as vitamins E, C and polyphenols, may play an important role by inhibiting the spread of free radical damage and the progression of simple steatosis to steatohepatitis [14]. In this perspective, the search for food bioactive components with positive effects on liver redox metabolism has emerged as an attempt to better understand their effects on NAFLD.

Açaí (*Euterpe oleracea* Martius) is a fruit widely consumed in Brazil and its consumption has increased in several countries. In 2017, Brazil produced more than 2 thousand tons of açaí, exported largely to the United States and Japan [15]. Part of the interest in açaí is due to the potential health benefits attributed to its nutritional composition and presence of bioactive compounds [16–18]. Açaí has a concentration of polyunsaturated (PUFA) and monounsaturated (MUFA) fatty acids of approximately 60% that can regulate lipid metabolism, while polyphenols mainly from the anthocyanin class, can contribute to neutralise free radicals [19]. Studies have shown that introduction of açaí in a high-fat diet is able to prevent the development of NAFLD via adiponectin mediation [20] and avoid the progression of liver damage by controlling oxidative stress [21]. Recently, our group demonstrated that the addition of açaí in the maternal diet can also attenuate the accumulation of lipids in the liver, reducing the progression of NAFLD in dams and protecting the offspring against a more harmful metabolic outcome [22]. However, it is not yet clear how supplementation of açaí to the maternal diet may improve lipid accumulation in the liver of dams and offspring, and if modulation of redox status plays a role. The objective of this study is, therefore, to evaluate how açaí can contribute to the prevention of NAFLD, by assessing antioxidant defences and biomarkers of oxidative stress in the liver from dams and their offspring.

2. Material and methods

2.1. Açaí pulp

Açaí pulp was acquired as a single lot (07/2016) from Icefruit Comércio de Alimentos Ltda (Tatuí, São Paulo, Brazil) to guarantee

homogeneity of the pulp during the whole experiment. The açaí pulp was frozen, pasteurised and free of preservatives or colourants. The nutritional and phenolics content of the açaí pulp utilised in this study has been previously published [22].

2.2. Animals and diet

Twenty-eight primipara female *Fischer* rats (90 days of age, weighting 200 g approximately) were obtained from Laboratory of Experimental Nutrition at the School of Nutrition of the Federal University of Ouro Preto (Minas Gerais, Brazil). Animals were divided into four groups according to the diet received: control (C), high-fat diet (HF, 60% of total calories as fat, been 53% saturated fat, 6% soybean oil and 1% cholesterol), control diet supplemented with 2% of açaí pulp (CA) or high-fat diet supplemented with 2% of açaí pulp (HFA). The composition of these experimental diets was based on AING-93G proposed by Reeves et al., 1993 [23] and are presented in Table 1. The values of lard and cholesterol were defined from previously published studies [20,24]. Supplementation with 2% açaí pulp (2 g/kg diet) was based on previous experimental work [17]. All animals were maintained in a standard environment 23 °C ± 2 °C, 55% humidity and 12-h light/darkness cycle with food and water provided ad libitum.

Initially, female rats were allowed to acclimatise with the diets for two weeks. After this, the mating was performed with a male rat together with two females for one week. Females were then separated and housed in individual polypropylene cages to allow for the natural progression of gestation and continued to receive their diet during gestation and lactation (~6 weeks). At birth, some of the litters were euthanised by isoflurane anaesthesia and killed by decapitation, to keep up to 6 male pups per dam, in order to guarantee homogeneity of the litters. At weaning (21 days), the offspring (P21) respective to each group (namely C-P21, CA-P21, HF-P21 and HFA-P21) and the mothers were anaesthetised by isoflurane and killed by total blood collection from the brachial plexus. All procedures described were approved by the Ethics Committee in Animal Research of the Federal University of Ouro Preto (Protocol No. 2015/15).

2.3. Body and liver weight

Dams' body weight was measured at the beginning of study, before mating and at the end of experiment. Regarding offspring, body weight of P21 was measured before euthanasia.

After euthanasia, the liver of dams and offspring was removed,

Table 1
Composition of experimental diets.

Nutrients (g/kg)	Diets			
	C	HF	CA	HFA
Casein	200	260	200	260
Sucrose	100	100	100	100
Corn starch	530.7	170.7	510.7	150.7
Cellulose fibre	50	50	50	50
Soybean oil	70	40	70	40
Choline	2.5	2.5	2.5	2.5
Minerals mix ^a	10	10	10	10
Vitamins mix ^a	35	35	35	35
Methionine	1.8	1.8	1.8	1.8
Cholesterol	–	10	–	10
Lard	–	320	–	320
Açaí	–	–	20	20
Energy (kJ/kg)	16,965	22,468	16,234	22,133
Protein energy (%)	20.4	19.5	20.8	19.8
Fat energy (%)	15.9	60.3	16.2	61.2
Carbohydrate energy (%)	63.7	20.2	63.0	19.0

C, control diet; CA, control diet supplemented with açaí pulp (2% w/w); HF, high-fat diet; HFA, high-fat diet supplemented with açaí pulp (2% w/w).

^a Minerals and Vitamins mixture as recommended by the AIN-93G rodent diet.

washed with cold saline solution and weighed. One section of liver was submerged in liquid nitrogen and immediately stored at -80°C for gene expression analyses. The other section was stored for analysis of oxidative stress biomarkers.

2.4. Liver fat content

Liver fat was extracted using organic solvents and content measured as previously described by Folch [25]. The fat content is expressed in mg/g of liver tissue.

2.5. Preparation of liver extracts for oxidative stress assay

Liver extracts were prepared using an Ultra-Turrax T8 (IKA Lab-ottechnik; Staufen, Germany) homogeniser as previously described [26]. Approximately 100 mg of liver from each animal were quickly weighed and then homogenised in ice-cold 50 mmol l^{-1} potassium phosphate buffer and 0.5 mmol l^{-1} EDTA (pH 7.2), containing at the moment of homogenisation, 10 $\mu\text{mol l}^{-1}$ phenylmethylsulphonyl fluoride (PMSF). Tissues were homogenised for 1 min and rested on ice for 30 s. This step was repeated three times. Samples were then centrifuged at 10,000 \times g for 10 min at 4°C . Supernatants were collected and stored in freezer -80°C for further analyses.

2.6. SOD, CAT and GPx enzyme activity

CAT activity was evaluated by monitoring the decomposition of H_2O_2 at 240 nm during 2 min, based on the method proposed by Aebi [27]. SOD activity was assessed by using the method proposed by Madesh and Balasubramanian [28]. The methodology is based on the enzyme's ability to neutralise the superoxide anion ($\text{O}_2^{\cdot-}$) produced by the oxidation of pyrogallol. The dye MTT [3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyl tetrazolium bromide] is reduced to formazan crystals in $\text{O}_2^{\cdot-}$ dependent reaction yielding a purplish-coloured compound measured by spectrophotometry at 570 nm. The activity of GPx was assessed by measuring the oxidation rate of NADPH in the presence of reduced glutathione (GSH) and glutathione reductase (GR), according to the method described by Flohé and Gunzler [29]. All results for enzymes activity are expressed in U/mg of protein.

2.7. Carbonyl protein (CO)

Liver concentration of carbonyl protein (CO) groups were determined following the method previously described by Levine et al. [30]. The assay uses 2,4-dinitrophenylhydrazine (DNPH), which reacts with carbonyl groups to generate the corresponding hydrazone, 2,4-dinitrophenyl (DNP) and absorbance measured spectrophotometrically at 360 nm. The carbonylated protein content was calculated using the molar absorption coefficient of DNPH 22,000 $\text{M}^{-1}\text{cm}^{-1}$. The concentration of CO is expressed in nmols/mg of protein.

2.8. Malondialdehyde (MDA)

Lipid peroxidation was estimated by quantifying MDA in the liver, which are intermediate products generated by the oxidation of lipids, as proposed by Esterbauer & Cheeseman [31]. The results are expressed in nmols/mg of protein.

2.9. Protein measurements

Liver homogenate total proteins were quantified according to the method of Bradford (1976) [32]. Briefly, 10 μl of diluted sample (1:20, v/v) or series dilutions of standards and 190 μl of Coomassie Blue G-250 reagent (0.01%) were added to a 96 well microplate. The microplate was incubated for 3 min and then read at 595 nm. Serum bovine albumin (BSA) was used as standard.

2.10. Glutathione measurements (GSH)

One hundred mg of liver tissue was homogenised in 1 ml 5% sulphosalicylic acid. The content was centrifuged at 10,000 \times g for 10 min at 4°C . The supernatant was collected and stored at -80°C until use. Total glutathione content (GSH) was determined by a kinetic assay using a protocol adapted from the commercial Sigma kit (Sigma, catalogue #CS0260). Briefly, 10 μl of each sample (or standard curve) was added into 96-well plate with 150 μl of working solution (containing phosphate buffer 1x, 6 U/ml of diluted glutathione reductase and 1.5 mg/ml stock solution of DTNB). The plate was incubated for 5 min at room temperature. After that, 50 μl of NADPH were quickly added and absorbance read every 1 min over the following 5 min. A standard curve was calculated and used to determine the concentration in nmols of total glutathione in 10 μl of sample. This value was then converted to 1 ml of sample. The results are expressed in nmol/ml.

2.11. Gene expression of Gpx1, Gpx4, Sod1, Sod2 and Sirt3

Total RNA was extracted from frozen liver tissue using TRITM reagent (InvitrogenTM, USA) following the manufacturer's instructions. RNA purification was determined by ratio A260/A280, utilising a UV/VIS spectrophotometer (Thermo Spectronic, Helios γ). RNA quality and integrity were checked using an agarose gel before PCR. mRNA levels were evaluated by reverse transcriptase quantitative real-time PCR (RT-qPCR). The cDNA obtained by RT-PCR was used as template in the qPCR reactions, which used SYBER Green PCR Master Mix kit (Applied Biosystems, USA), according to the manufacturer's recommendations. The reactions were done in triplicate and each reaction had a negative control added with 2 μl of water instead of the template. Oligonucleotide primers' sequences: *Gpx1*: 5'-CACCGAAATGAATGATCTGC-3' (forward); 5'-TGTATCTGCGCACTGGAAC-3' (reverse) (product length 370 bp based on sequence NM_030826.4); *Gpx4*: 5'-ATGCACGAATTCG-CAGCC-3' (forward); 5'-CTAGAGATAGCACGGCAGGTC-3' (reverse) (product length 463 bp based on sequence NM_017165.3); *Sod1*: 5'-GCAGAAGGCAAGCGGTGAAC-3' (forward); 5'-TAGCAGGACAGCA-GATGAGT-3' (reverse) (product length 447 bp based on sequence NM_017150.1); *Sod2*: 5'-CTGAGGAGAGCAGCGGTGCT-3' (forward); 5'-CTTGCCAGCGCCTCGTGGT-3' (reverse) (product length 258 bp based on sequence NM_017051.2); *Sirt3*: 5'-GGCACTACAGGCCCAATGTC-3' (forward); 5'-TCTCTCAAGCCCGTCGATGT-3' (reverse) (product length 100 bp based on sequence XM_017601788.1); β_2 microglobulin.: 5'-TGACCGTGATCTTTCTGGTG-3' (forward); 5'-ATTTGAGGTGGGTG-GAAGT-3' (product length 151 bp based on sequence NM_012512.2).

The qPCR protocol had an initial denaturation temperature at 95°C for 1 min, then 40 cycles of 95°C for 15 s by combining annealing and elongation at 60°C for 1 min. The specificity of the products obtained was confirmed initially by gel electrophoresis on a 1% agarose gel containing GelRED, and also by analysis of the amplified product dissociation curves. The data obtained were analysed using the comparative Ct method. Target gene expression was determined relative to the expression of the endogenous β_2 microglobulin.

2.12. Statistical analysis

The sample size was calculated using the expected change in fat liver based on a previous study [33] using a 95% power at the 5% level of significance in BioEstat program, version 5.9. In that case, the condition was met with $n = 6$, and did not require further adjustment.

All data were tested for normality using the Shapiro-Wilk test and followed the Gaussian distribution. Data from the four groups were analysed by two-way ANOVA followed by Tukey test to detect differences between the groups and expressed as mean \pm standard deviation (SD). Data from two groups were compared by Student's *t*-test. Statistical analysis was performed using GraphPad Prism 6 for Windows (GraphPad Software, San Diego, CA) and were considered statistically

significant for p values < 0.05 .

3. Results

3.1. Phenotypic characteristic of dams and offspring

The experimental model used in this work involved NAFLD induced by a high-fat diet. The presence of steatosis was confirmed by histologic analyses, in addition to assess the effect of the dietary intervention on dams' body and liver weight (Fig. 1). Histological analysis revealed that the HF group had a higher degree of steatosis (moderate and severe), while the HFA group showed a reduction in steatosis when compared to HF (Fig. 1A and B). In addition, the accumulation of lipid droplets in the liver was accompanied by the presence of inflammatory cell infiltrate. In relation to pre-gestational body weight, we observed an effect of the diet ($p = 0.044$), however, when we performed post hoc analysis the difference disappeared. Significant differences were observed at the end of the experiment: there was an effect of açai pulp ($p = 0.011$, two-way ANOVA) on the body weight (Table 2). Tukey's post hoc test showed that dams fed with HFA presented 20% higher body weight compared to the C group ($p = 0.015$). During the study, food intake was measured over one week before the mating. Food intake and correspondent calorie intake in dams that received a diet rich in fat supplemented or not with açai pulp is presented in Table 2. An effect of açai ($p = 0.026$) was observed, however, after Tukey's post hoc test, the effect was not statistically different (Table 2).

Dams' absolute liver weight was measured and relative liver weight calculated. Regarding absolute liver weight, an overall effect of diet ($p < 0.0001$) and açai pulp ($p = 0.007$) was observed: the post hoc test pointed dams fed with HF diet had an increased liver weight when compared with C (42%, $p = 0.0007$) and CA (72%, $p < 0.0001$) groups. Açai pulp supplementation in high-fat diet increased liver weight by 42% when compared with CA group ($p = 0.0088$, two-way ANOVA) (Table 2). Considering relative liver weight, the HF diet ($p = 0.002$) and açai pulp ($p = 0.001$) also showed differences (Table 2). An increase of 36% ($p = 0.033$) and 80% ($p = 0.0002$) was confirmed by post hoc test in relative liver weight in dams from HF group compared to C and CA groups, respectively. Supplementation of açai pulp prevented the increase of this parameter by 36% ($p = 0.028$, Tukey's post hoc test), suggesting a lower accumulation of lipids in the liver of dams fed with

HFA diet. The fat content in the liver was checked to assess the extent of NAFLD; the results are shown in Table 2. There was a significant increase in total fat content in the HF group after post hoc test compared to C (117%, $p = 0.0006$), CA (278%, $p < 0.0001$) and HFA (82%, $p = 0.004$).

In order to investigate the effect of maternal diet on offspring's liver, liver sections were treated and stained as previously described [22]. Representative histological sections from each experimental group are shown in Fig. 2A and analysis of the degree of steatosis is reported in Fig. 2B. Through histology and the degree of steatosis present in P21 livers (Fig. 2A), it was possible to observe that offspring of dams fed with HF diet (HF-P21) had more lipid droplets compared to CA-P21 and HFA-P21 groups. Offspring from high-fat diet (HF-P21) fed dams showed a more pronounced degree of steatosis (Fig. 2B) than offspring from CA (CA-P21) and HFA (HFA-P21) (absent to mild). Offspring that received HFA diet (HFA-P21) had a lower degree of steatosis, suggesting a protective effect of açai in relation to liver lipid accumulation.

The effect of maternal diet on body weight of P21 is shown in Table 3. We observed an effect of HF diet ($p < 0.0001$) and interaction between the effect of the HF diet and açai ($p = 0.007$). After Tukey's post hoc test, CA-P21 presented lower values of body weight in comparison to HF-P21 (40%, $p = 0.0067$) and HFA-P21 (60%, $p < 0.0001$) groups. HFA-P21 had a greater body weight relative to C-P21 group (25%, $p = 0.034$). Evaluating the absolute liver weight, we also observed an effect of diet ($p < 0.0001$, two-way ANOVA). Tukey's post hoc test confirmed that absolute liver weight in HF-P21 was higher compared to C-P21 (49%, $p = 0.0002$) and CA-P21 (64%, $p < 0.0001$). We also observed that pups from HFA-P21 had increased liver weight in comparison to C-P21 (45%, $p = 0.002$) and CA-P21 (60%, $p = 0.0002$) groups. When assessing relative liver weight, a diet effect ($p = 0.014$) and an interaction between diet and açai effect ($p = 0.0008$) was also found after two-way ANOVA test. However, as it was found for dams, a lower relative liver weight in HFA-P21 versus HF-P21 (17%, $p = 0.026$, Tukey's post hoc test) was observed. HF-P21 group presented a 35% increase ($p = 0.0006$, Tukey's post hoc test) in comparison with C-P21 group. Levels of fat content were checked in P21 groups, in order to determine the effect of maternal diet on promoting early changes in liver dynamics (Table 3). No significant differences were found in liver fat values between groups.

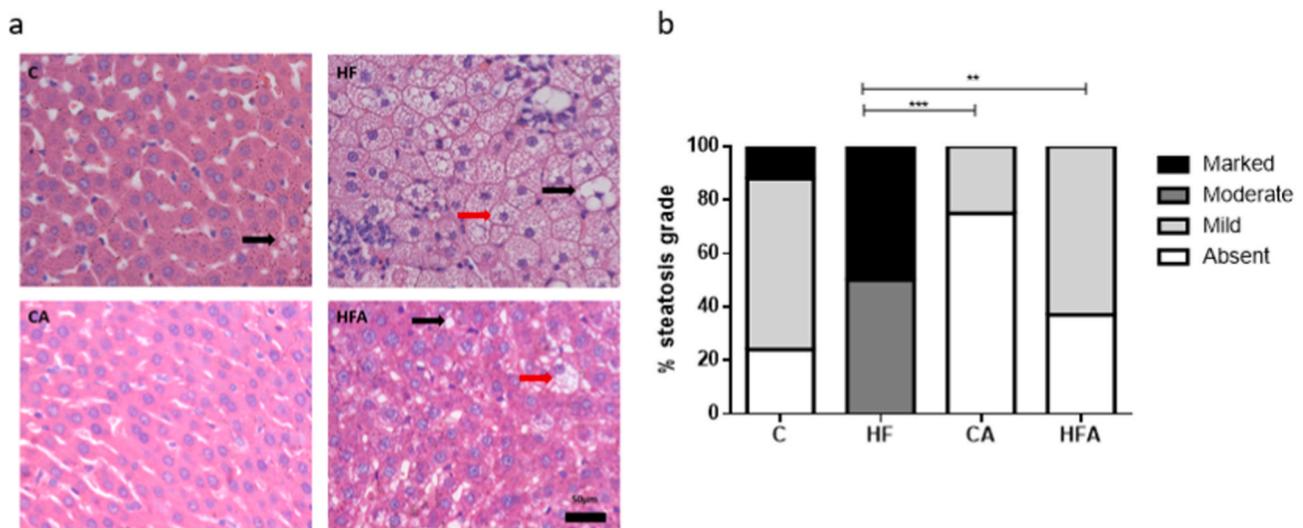


Fig. 1. A- Representative histological sections of the liver of dams fed with a control diet (C), high-fat diet (HF), açai diet (CA) and high-fat supplemented with açai (HFA), stained with hematoxylin and eosin. Black arrow shows macrosteatosis and red arrow shows microsteatosis. The images were photographed at a magnification of 400 \times . Bar Scale = 50 μ m; B- Grade of hepatic steatosis of dams ($n = 7$ dams per group). Value of $p < 0.05$ was considered statistically significant using Kruskal-Wallis test. ** 0.01, *** < 0.005 .

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Table 2
Body and liver weight of dams.

	C	CA	HF	HFA	Diet effect	Açaí effect	Interaction
Initial BW(g)	210.1 ± 8.83	205.4 ± 8.35	210.4 ± 10.48	218.9 ± 12.84	0.088	0.625	0.102
Pre-Gestational BW (g)	215.4 ± 11.16	212.1 ± 8.49	220.7 ± 13.62	226.5 ± 15.06	0.044	0.789	0.339
Final BW (g)	213.1 ± 20.78	237.7 ± 27.63	230.9 ± 12.61	255.6 ± 29.68 ^a	0.994	0.011	0.057
Liver weight (g)	7.0 ± 1.17	5.9 ± 0.52	10.2 ± 2.89 ^{a,b}	8.4 ± 0.99 ^b	<0.0001	0.007	0.468
Relative liver weight (%)	3.3 ± 0.73	2.5 ± 0.55	4.5 ± 1.03 ^{a,b}	3.3 ± 0.34 ^c	0.002	0.001	0.514
Liver fat (mg/g)	98.6 ± 37.46	56.8 ± 18.16	214.9 ± 71.8 ^{a,b}	117.5 ± 45.62 ^c	<0.0001	0.0007	0.1338
Calorie intake (kj/g)	229.8 ± 22.59	240.03 ± 18.06	217.85 ± 26.08	242.38 ± 14.95	0.524	0.0266	0.3438

BW, body weight; C, control diet; CA, control diet supplemented with açaí pulp; HF, high-fat diet; HFA, high-fat diet supplemented with açaí pulp. The results are shown as mean ± SD (n = 7 dams per group). Two-way ANOVA followed by Tukey post hoc test. p value < 0.05 for Tukey post hoc test comparing ^aHF or HFA versus C; ^bHF or HFA versus CA; ^cHFA versus HF.

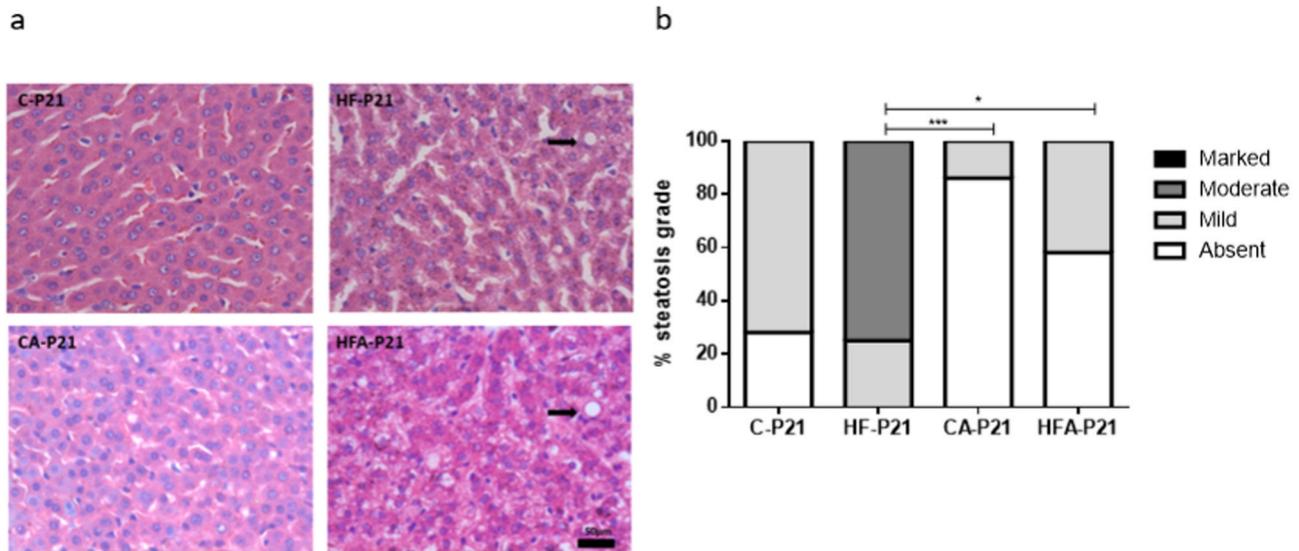


Fig. 2. A- Representative histological sections of the liver of offspring P21 fed with a control diet (C), high-fat diet (HF), açaí diet (CA) and high-fat supplemented with açaí (HFA), stained with hematoxylin and eosin. Black arrow shows macrosteatosis and red arrow shows microsteatosis. The images were photographed at a magnification of 400×. Bar Scale = 50 μm; B- Grade of hepatic steatosis of dams (n = 7 pups per group). Value of p < 0.05 was considered statistically significant using Kruskal-Wallis test. * < 0.05, *** < 0.005.

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Table 3
Body and liver weight of P21 offspring.

	C-P21	CA-P21	HF-P21	HFA-P21	Diet effect	Açaí effect	Interaction
BW (g)	30.5 ± 3.0	24.0 ± 4.4	33.7 ± 5.8 ^a	38.4 ± 7.5 ^{a,b}	<0.0001	0.637	0.007
Liver weight (g)	1.1 ± 0.1	1.0 ± 0.2	1.6 ± 0.3 ^{a,b}	1.6 ± 0.2 ^{a,b}	<0.0001	0.307	0.992
Relative liver weight (g)	3.7 ± 0.5	4.3 ± 0.4	5.0 ± 0.9 ^b	4.0 ± 0.3 ^c	0.014	0.605	0.001
Fat liver (mg/g)	63.9 ± 37.7	71.5 ± 7.3	88.4 ± 28.6	81.9 ± 18.9	0.0748	0.954	0.4611

BW, body weight; C-P21, offspring post-weaning from maternal control diet; CA-P21, offspring post-weaning from maternal control diet supplemented with açaí pulp; HF-P21, offspring post-weaning from maternal high-fat diet; HFA-P21, offspring post-weaning from maternal high-fat diet supplemented with açaí pulp. The results are shown as mean ± SD (n = 7 pups per group). Two-way ANOVA followed by Tukey post hoc test. p value < 0.05 for Tukey post hoc test comparing ^aHF-P21 or HFA-P21 versus CA-P21; ^bHF-P21 or HFA-P21 versus C-P21; ^cHFA-P21 versus HF-P21.

3.2. Biomarkers of oxidative stress

Antioxidant status of dams was assessed by measuring CAT, GPx and SOD enzymes activity in the liver (Table 4). In relation to CAT activity, an effect of diet (p < 0.0001), açaí pulp (p = 0.034) and interaction between the two (p = 0.003) was found. Tukey's post hoc test identified differences in the dams fed with HF diet as presented higher CAT activity compared to C (60%, p < 0.0001) and CA (52%, p < 0.0001) group; whereas the supplementation with açaí pulp in high-fat diet reduced the increased CAT activity by 30% in comparison to the HF group (p = 0.004). Dams receiving HFA diet showed an increase in CAT activity only in relation to C group (78%, p = 0.009). In relation to GPx,

we found an overall effect of diet (p = 0.0002) and açaí pulp (p = 0.0004), by two-way ANOVA analysis. Similar results to CAT were observed after the post hoc test: an increase in GPx activity was found in HF group when compared to C (31%, p = 0.003) and CA (45%, p < 0.0001) groups; whereas the addition of açaí pulp to the high-fat diet prevented this increase in GPx activity, dams fed with HFA presented a 29% reduction when compared to HF group (p = 0.004). Regarding SOD activity, we observed only an effect of diet (p < 0.0001, two-way ANOVA). The differences between groups were observed when Tukey's post hoc test was carried out: HF group presented an increase in SOD activity, 90% versus C (p < 0.0001) and 91% versus CA (p < 0.0001) groups. Dams fed with HFA diet showed an increased in

Table 4
Antioxidants enzymes activity and biomarkers of oxidative stress of dams.

	C	CA	HF	HFA	Diet effect	Açaí effect	Interaction
CAT (U/mg of protein)	0.23 ± 0.06	0.26 ± 0.04	0.51 ± 0.15 ^{a,b}	0.41 ± 0.11 ^{a,c}	<0.0001	0.034	0.003
GPx (U/mg of protein)	0.60 ± 0.06	0.47 ± 0.09	0.86 ± 0.21 ^{a,b}	0.61 ± 0.13 ^c	0.0002	0.0004	0.202
SOD (U/mg of protein)	1.39 ± 0.12	1.39 ± 0.20	2.65 ± 0.53 ^{a,b}	2.28 ± 0.41 ^{a,b,c}	<0.0001	0.056	0.060
MDA (µM/mg of protein)	0.93 ± 0.49	0.80 ± 0.28	2.81 ± 1.12 ^{a,b}	1.33 ± 0.59 ^c	0.0001	0.005	0.016
CO (nmols/mg of protein)	0.72 ± 0.28	0.80 ± 0.12	1.21 ± 0.18 ^{a,b}	0.86 ± 0.29 ^c	0.003	0.117	0.013
GSH total (nmol/ml)	2.71 ± 0.45	2.14 ± 0.87	12.26 ± 4.76 ^{a,b}	3.47 ± 3.64 ^c	<0.0001	0.0002	0.0007

C, control diet; CA, control diet supplemented with açaí pulp; CAT, catalase; CO, protein carbonyl; GPx, glutathione peroxidase; GSH, glutathione; HF, high-fat diet; HFA, high-fat diet supplemented with açaí pulp; MDA, malondialdehyde; SOD, superoxide dismutase.

The results are shown as mean ± SD (n = 7 dams per group). Two-way ANOVA followed by Tukey post hoc test. p value < 0.05 for Tukey post hoc test comparing ^aHF or HFA versus C; ^bHF or HFA versus CA; ^cHFA versus HF.

SOD activity (57%) when compared to the C and CA groups (p = 0.0002, for both). However, the HFA group presented a reduction in SOD activity (18%, p = 0.0432) in relation to the HF group.

In order to better understand how maternal diet and açaí supplementation may affect oxidative status, some biomarkers of oxidative stress were also assessed (Table 4). In relation to lipid damage, measured by MDA concentration, we observed an overall effect of diet (p = 0.0001), açaí pulp (p = 0.005) and interaction between the two (p = 0.016) by two-way ANOVA analysis. A greater MDA concentration was observed in the HF group compared to C (138%, p = 0.0003) and CA (253%, p < 0.0001) groups after Tukey's post hoc test; whereas the addition of açaí pulp in high-fat diet prevented lipid damage (52%, p = 0.001, Tukey's post hoc test) when compared to HF group (Table 4). Regarding protein damage, assessed by CO concentration, two-way ANOVA analysis showed an overall effect of the diet (p = 0.003) and interaction between diet and açaí supplementation (p = 0.013) was found. A similar trend was observed with the HF group presenting higher hepatic protein oxidation levels when compared to C (41%, p = 0.001), CA (34%, p = 0.009) and HFA (30%, p = 0.032) groups after performing Tukey's post hoc test. GSH is the main nonprotein thiol present in the body and an important component of antioxidant defence system: an effect of diet (p < 0.0001), açaí pulp (p = 0.0002) and interaction between the two (p = 0.0007) was found in relation to GSH levels by two-way ANOVA. GSH concentration was higher in the HF group when compared to C (352%, p < 0.0001) and CA (472%, p < 0.0001) groups, but supplementation of açaí to HF diet reduced GSH levels in the HFA (253%, p < 0.0001) group in comparison with HF after Tukey's post hoc test.

Redox balance was also evaluated in offspring P21. The antioxidant status of offspring was assessed by measuring CAT, GPx and SOD enzyme activity in liver (Table 5). Although no significant differences were observed in the antioxidant enzymes activities among the different groups, the same trend of lower hepatic GPx activity was observed in dams for HF-P21 and HFA-P21. Regarding biomarkers of oxidative stress, we only found an effect of açaí (p = 0.030) in GSH levels, however, when we performed post hoc test the difference disappeared (Table 5). No other differences were observed among groups. However, it is interesting to notice that hepatic levels of protein oxidation and GSH

Table 5
Antioxidants enzymes activity and biomarkers of oxidative stress in P21 offspring.

	C-P21	CA-P21	HF-P21	HFA-P21	Diet effect	Açaí effect	Interaction
CAT (U/mg of protein)	0.22 ± 0.03	0.25 ± 0.13	0.23 ± 0.06	0.23 ± 0.04	0.857	0.662	0.529
GPx (U/mg of protein)	0.64 ± 0.21	0.57 ± 0.12	0.67 ± 0.18	0.54 ± 0.08	0.975	0.103	0.619
SOD (U/mg of protein)	1.47 ± 0.2	1.68 ± 0.47	1.68 ± 0.42	1.79 ± 0.25	0.208	0.201	0.709
MDA (µM/mg of protein)	1.43 ± 0.81	1.44 ± 0.90	0.89 ± 0.45	0.94 ± 0.58	0.907	0.700	0.810
CO (nmols/mg of protein)	0.84 ± 0.1	1.18 ± 0.98	0.97 ± 0.26	0.86 ± 0.12	0.237	0.37	0.515
GSH total (nmol/ml)	4.10 ± 3.88	2.3 ± 1.13	7.64 ± 8.46	1.14 ± 1.79	0.515	0.030	0.205

C-P21, offspring post-weaning from maternal control diet; CA-P21, offspring post-weaning from maternal control diet supplemented with açaí pulp; CO, carbonyl protein; GPx, glutathione peroxidase; GSH, glutathione; HF-P21, offspring post-weaning from maternal high-fat diet; HFA-P21, offspring post-weaning from maternal high-fat diet supplemented with açaí pulp; MDA, malondialdehyde; SOD, superoxide dismutase.

The results are shown as mean ± SD (n = 7 pups per group). Two-way ANOVA followed by Tukey post hoc test.

in HF-P21 and HFA-P21 showed similar results to what found in dams: lower hepatic levels of oxidative stress biomarkers in the offspring.

3.3. Gene expression

Considering the antioxidant properties of açaí, the effect of supplementing maternal diet with açaí during gestation and lactation on gene expression of *Gpx1*, *Gpx4*, *Sod1* and *Sod2* enzymes was assessed. However, no significant differences were found for the genes studied in dams (Fig. 3A). In contrast, in offspring, a significant increase in mRNA levels for *Gpx1* (5-fold change, p < 0.0001), *Gpx4* (2-fold change, p = 0.021) and *Sod1* (4.5-fold change, p = 0.032) were observed in the HFA-P21 group when compared to HF-P21 group, indicating a possible trans-generational effect of açaí against oxidative damage (Fig. 3B).

Recognising the involvement of SIRT3 in redox metabolism and mitochondrial biogenesis, mRNA expression for *Sirt3* was also assessed; however, no significant differences were observed between the HF and HFA fed dams and in their P21 offspring (Fig. 4).

4. Discussion

NAFLD is known to induce redox imbalance in the body and to stimulate higher ROS production, which has been suggested as one of the causes for progression of simple steatosis to NASH [6]. Mitigating the damage caused by ROS in the liver appears to be an effective approach in the treatment of NAFLD. In fact, studies have shown that dietary antioxidant components, such as vitamin E, resveratrol and silybin, may reduce markers of oxidative stress, activation of hepatic stellate cells, and fibrosis development in mice [34–36].

Açaí is a fruit rich in polyphenols and anthocyanins extensively described in the literature [16,19]. In our previous work we determined the content of phytochemicals present in the açaí pulp used in this study and found to contain 549.5 mg GAE/100 g of pulp for polyphenols and 6.5 mg/L of pulp for anthocyanins [22]. We believed that the content of phytochemicals in the açaí pulp may be effective in regulating ROS production alleviating accumulation of fat droplets in the liver, with an improvement in liver fat content and degree of steatosis. Therefore, we evaluated the effect of supplementing maternal diet with açaí on

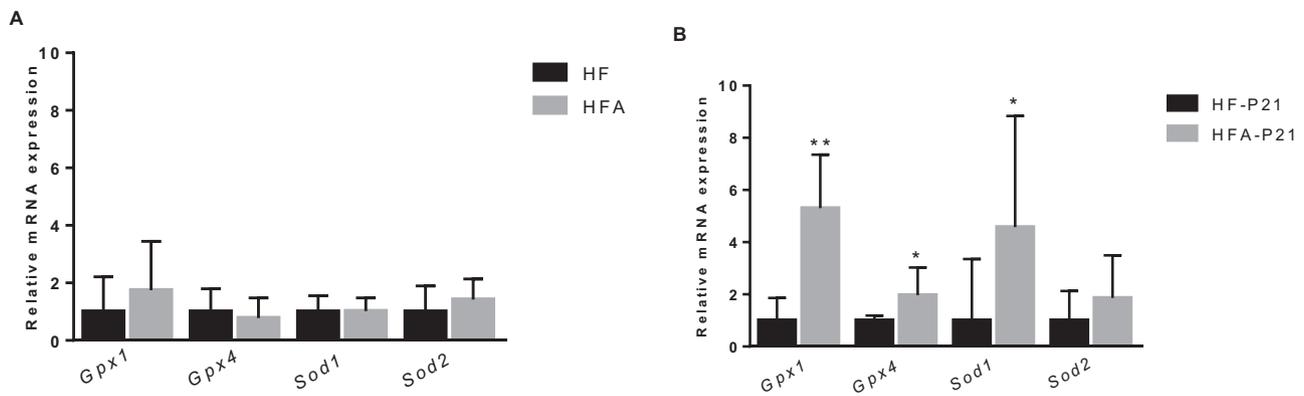


Fig. 3. Relative mRNA abundance for genes related to redox metabolism in the liver of dams (A) and P21 (B) relative to beta-2-microglobulin. HF: high-fat diet; HF-P21: offspring post-weaning from maternal high-fat diet; HFA: high-fat diet supplemented with açai pulp; HFA-P21: offspring post-weaning from maternal high-fat diet supplemented with açai pulp; Gpx1: glutathione peroxidase 1; Gpx4: glutathione peroxidase 4; Sod1: superoxide dismutase 1; Sod2: superoxide dismutase 2. The results are shown as mean \pm SD ($n = 7$ dams/pups per group). Analyses by Student's *t*-test. * $p < 0.05$; ** $p < 0.01$.

biomarkers of oxidative stress, activity and gene expression of antioxidant enzymes, in addition to have already shown its effect on lipid metabolism [22]. A study in mice showed that addition of polyphenols to HF diet was able to increase Nrf2 activity and normalise expression of genes that codify antioxidants enzymes and their activities. [37]. Our results show that addition of açai to a high-fat diet fed in dams was able to modulate the activity of antioxidant enzymes at cellular level and reduce biomarkers of oxidative stress altered by the HF diet: MDA, protein carbonyl and total GSH. We believe that açai is able to improve oxidative balance in dams and, therefore, may promote protection against metabolic outcomes in their offspring, as suggested by the increase in gene expression of *Gpx1*, *Gpx4* and *Sod1*.

The results found in our study show that supplementation with açai pulp to maternal HF diet reduced the activity of SOD, CAT and GPX antioxidant enzymes in dams fed with HFA diet compared to HF group. These data suggest that compounds present in the açai pulp acted to reduce ROS, maintaining redox balance, without the physiological need to increase antioxidant enzymes' activity. Similarly to our work, a study evaluating the effect of green tea consumption during gestation and lactation found a reduction in CAT activity in dams and SOD in offspring (28 days) [38]. Like açai, green tea has high levels of antioxidant compounds, with the ability to neutralise ROS and produce more stable radicals. The possibility of an indirect effect of antioxidants cannot be ruled out. Several dietary antioxidants, such as polyphenols, have been listed as playing an important role in inducing gene expression of detoxifying molecules [39]. These molecules, in turn, could also be related to the expression of transcription and growth factors of the Nrf2 pathway. Although in our study we did not evaluate Nrf2 pathway, studies have shown that antioxidant compounds increase gene and protein expression of Nrf2 [40,41].

MDA is a product of lipid peroxidation which can react with proteins, causing irreversible modifications, and with DNA molecules making it highly mutagenic [42]. In the present study, reductions in hepatic levels of MDA in HFA fed dams were observed, suggesting that açai prevents lipid oxidation. Jensen et al. (2008) observed a reduction in thiobarbituric acid reactive substances (TBARS) serum concentration 2 h post consumption of 120 ml of açai juice, reaffirming the antioxidant effect of açai on lipid peroxidation [43]. A study using a model of diabetic rats showed that introduction of açai extract in the diet was also able to reduce the hepatic levels of TBARS [18]. MDA is a non-specific method to assess some lipid peroxidation products, such as TBARS, because MDA is not derived only from lipid peroxidation but also from aldehydes. However, it is a commonly biomarker found in the literature and we have used it in conjunction with other biomarkers of oxidative stress to improve our understanding about oxidative damage in this study.

Another biomarker of oxidative stress includes proteins that may have carbonyl groups ($C = O$) added to their amino acid chains [44]. In our study, we observed a significant increase in CO from dams fed with HF diet. In the same way, previous work showed that maternal cafeteria diet can induce changes in oxidant/antioxidant status increasing CO [45]. Interestingly, we found a reduction in hepatic protein carbonyl concentration in the group of dams fed with a HFA diet compared to the HF group, with values similar to those found for group C and CA. These data emphasise the importance of neutralising ROS as a way to combat oxidative damage. Although the offspring did not present a statistical significance, we observed the same trend in reducing protein oxidation in HFA-P21 compared to HF-P21, suggesting that maternal diet can act on antioxidant protection mechanisms of the offspring.

GSH is an important low molecular weight antioxidant synthesised in cells and is involved in reduction reactions, which provide the means for free radicals balance [46]. An increase in total GSH content in the HF dams' group and a reduction in the HFA group was observed in our study. It is important to recognise that total GSH dosage includes the predominant forms: reduced GSH and oxidised GSH (GSSG). The use of GSH in the antioxidant defence of cells involves the oxidation of GSH to neutralise H_2O_2 . It is possible that the increase in total glutathione occurs in an omnipresent response to oxidative stress, with an increase in the GSSG fraction; however, a limitation of our study is that we did not measure reduced and oxidised glutathione fractions. In a recent study evaluating rats receiving a high fructose diet supplemented with 2% açai, the addition of this fruit increased the ratio of total/oxidised glutathione (GSH/GSSG), reaffirming its beneficial action on the main antioxidant system [47]. Even though we did not observe a significance difference in the offspring, the levels of GSH in the liver of HFA-P21 presented the same tendency in lower levels compared to HF-P21. Together, our findings suggest a positive role of açai in maternal diet to confer resistance to oxidative stress in both mother and offspring.

The control of oxidative stress is an important factor in the progression of NAFLD, as a vicious cycle between mitochondrial oxidative capacity and increased oxidative stress may form the basis for the development of steatohepatitis. Considering the antioxidant capacity of açai, we investigated the effects of maternal supplementation with açai on the expression of different isoforms of SOD (SOD1 and SOD2) and GPx (GPx1 and GPx4) enzymes. In the present study a significant increase in the expression of *Gpx1* and *Gpx4* mRNA in HFA-P21 group compared to HF-P21 was observed, supporting findings from a study carried out in rats in which the maternal diet was able to reduce the antioxidant defence in the offspring by reducing the expression of the *Gpx1* and *Sod1* enzymes [48]. Our data suggest that the addition of açai pulp in the maternal diet may induce improvement of metabolic disorders in the offspring, at a molecular level before biochemical alterations

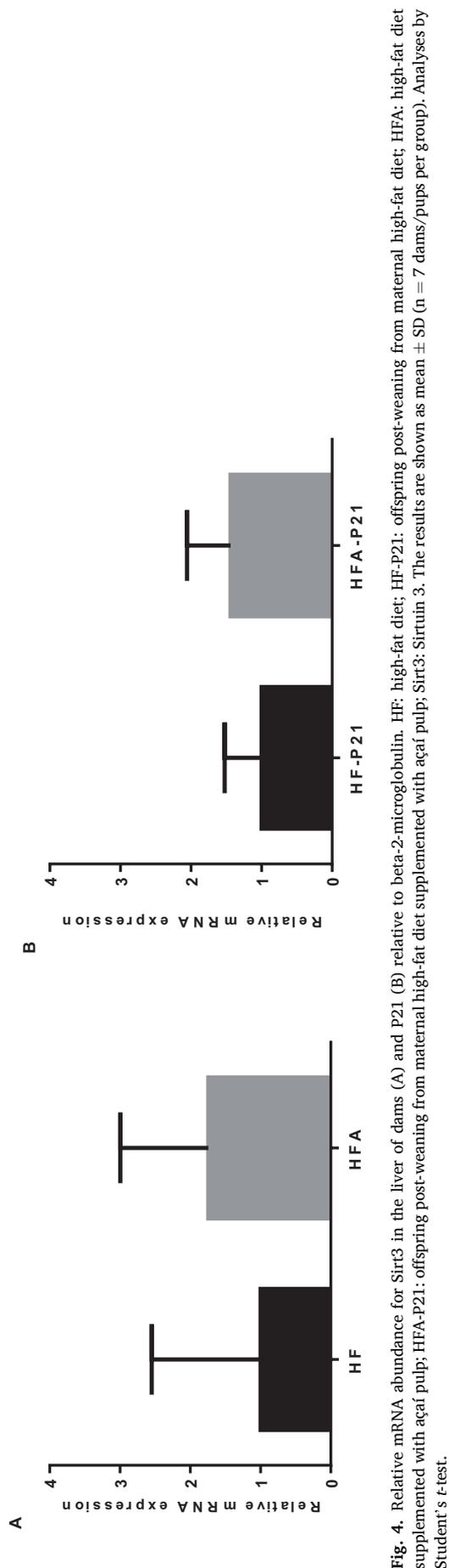


Fig. 4. Relative mRNA abundance for Sirt3 in the liver of dams (A) and P21 (B) relative to beta-2-microglobulin. HF: high-fat diet; HF-P21: offspring post-weaning from maternal high-fat diet; HFA: high-fat diet supplemented with açai pulp; HFA-P21: offspring post-weaning from maternal high-fat diet supplemented with açai pulp; Sirt3: Sirtuin 3. The results are shown as mean \pm SD (n = 7 dams/pups per group). Analyses by Student's *t*-test.

occur. Although the improvement observed at gene expression level was not observed at cellular levels with an increase in enzyme activity in offspring, this discrepancy can be explained by the assay used to measure both SOD and GPx activity not being specific for the different enzyme isoforms, and by mRNA post-translational regulations.

SIRT3 may be related to the activation of antioxidant enzymes and may be a target for their activation by phenolic compounds. We evaluated the effects of açai on the expression of *Sirt3* in the groups of animals fed HF and HFA diet and no significant differences were observed in dams and P21. Studies evaluating the activation of SIRT3 as way to protect the offspring against oxidative stress are still scarce [49,50] and, to the best of our knowledge, this is the first study to verify the effect of maternal diet supplemented with açai in the expression of *Sirt3* in offspring.

Oxidative stress resulting from mitochondrial oxidation of fatty acids, and the expression of inflammatory cytokines have been identified as secondary causal factors, which lead to liver damage, fibrosis and inflammation [51]. Although the inflammatory profile of our study has not been evaluated, we have not ruled out the hypothesis of modulation of the inflammatory response through phenolic compounds and of the lipid profile. In a recent review by Valenzuela and Videla (2020) [52], it was reported that *n*-3 fatty acids and polyphenols are able to smooth the progression from steatosis to steatohepatitis in conjunction with an HF diet. The high proportion of unsaturated fatty acids (>70%) present in the açai pulp may also have had a positive effect on lipid metabolism. Interestingly, in a previous study carried out by our research group, we observed an improvement in the lipid profile in dams fed with açai pulp [22]. Subsequently, the role of açai on inflammatory and intestinal modulation may be the objective of a study to better understand the metabolic effects of the fruit on the maternal diet and their respective offspring.

The aetiology/pathogenesis of NAFLD in the paediatric population is likely to be multifactorial, including adverse events in the uterus, which may affect the development and progression of this disease [53]. As a result, several studies have been carried out to understand how the maternal high-fat diet is able to induce metabolic outcomes in the foetal liver and predispose the body to early disorders. However, studies reporting the effects of combining a high-fat diet with bioactive foods or compounds on the molecular pathways related to the development of NAFLD are still limited. The maternal obesogenic environment can promote several epigenetic changes that will be passed on to offspring. Epigenetic studies are fundamental in models of maternal diet and effects in their offspring, since epigenetics plays a key role in genomic imprinting, programming and reprogramming in early life, and consequently with increased susceptibility to disease in adult life [54]. It is noteworthy that in this study the offspring was not followed up beyond early adulthood. Thus, other changes promoted by the maternal diet and/or açai supplementation could have been seen in a longer experimental design.

In summary, from the results presented here and from our previous study [22], we suggest that açai may have a beneficial effect on health, through a synergism between its nutritional and bioactive components, improving the oxidative state of dams and perhaps protecting offspring against lipids excess via increasing gene expression of antioxidants enzymes and lipid metabolism as shown previously.

Authorship

R.N.F. designed the research (project conception, development of overall research plan, and study oversight); P.O.B., M.O.S., M.P.S.S., and M.E.S. conducted research (hands-on conduct of the experiments and data collection); P.O.B., M.O.S., M. P. S. S., G.T.S. and G. B. analysed data and performed statistical analysis; P.O.B. and G. B. wrote the paper. All authors read and approved the final manuscript.

Declarations of interest

None.

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