Journal of Functional Foods 111 (2023) 105889

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

Effects of coffee with different roasting degrees on obesity and related metabolic disorders

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ARTICLE INFO

Keywords:
Coffee roasting
Obesity
Metabolic disorders
Body weight
Insulin resistance
Liver steatosis

ABSTRACT

This study aimed to assess the effect of unroasted, dark and very dark roasted coffee on obesity and metabolic disorders in obese rats. All coffee samples significantly reduced weight gain (~17%) compared to obese control. Coffee reduced glucose levels (~17%) upon a glucose tolerance test in all cases compared to the control, while fasting glucose only decreased (~26%) with very dark coffee. Insulin levels and insulin resistance significantly decreased (~77% and 65% respectively) with all coffee samples compared to the control. Unroasted and dark roasted coffee decreased triglycerides (~21% and ~ 11%, respectively), and unroasted coffee also reduced free fatty acids (~43%) and adipocyte size. Coffee decreased liver steatosis (~55%) and Caspase-3 levels (~27%), regardless of the roasting degree. Overall, coffee plays a positive role in restraining obesity and related metabolic disorders but, depending on the metabolic pathway and relevant marker, an effect of roasting could be either found or not.

1. Introduction

In recent decades, the worldwide prevalence of obesity and related metabolic disorders have reached very high levels, posing a significant public health challenge. Obesity is characterized by the excessive storage of fats in subcutaneous and visceral adipose tissue (AT) and in unusual sites called ectopic depots (Meex et al., 2019), following excessive energy intake. The latter can also lead to a pathological hypertrophy of adipocytes disrupting their proliferation and differentiation abilities. These changes play a key role in triggering inflammatory responses within adipocytes, mediated by several signalling pathways. Because of these alterations, an increase in insulin resistance occurs in adipocytes as well as an enhanced release of free fatty acids (FFAs) and their accumulations in ectopic sites (Cusi, 2010) such as hepatocytes.

The deposition of fat, including diacylglycerols, ceramides, FFAs, and their metabolites possessing potential bioactivity, in hepatocytes may interfere with the function of these cells, especially in relation to their response to changes in insulin levels. The impaired response of hepatocytes to insulin contributes to the development of glucose intolerance and fasting hyperglycaemia (Farese et al., 2012).

Under normal conditions, insulin interacts with liver cells through receptors on the cell surface. This interaction inhibits glycogenolysis and gluconeogenesis but at the same time promotes glycogen synthesis and lipogenesis. However, when hepatic insulin resistance emerges, insulin ability to suppress hepatic glucose production diminishes. The failure of the insulin system in such circumstances cannot be attributed to a decrease in hormone levels because obesity is often associated with high levels of insulin. Conversely, it may depend on compromised insulin signalling within insulin-resistant hepatocytes (Leavens et al., 2011).

The accumulations of fat into the liver can also potentially lead to liver damage, resulting in the condition called hepatocyte lipoapoptosis (Wang, 2014). Growing evidence in now suggesting that dysregulation

Abbreviations: TG, Triglycerides; FFA, Free fatty acids; OGTT, Oral glucose tolerance tests; AT, adipose tissue.

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https://doi.org/10.1016/j.jff.2023.105889
Received 9 September 2023; Received in revised form 26 October 2023; Accepted 3 November 2023
Available online 8 November 2023

of hepatocyte apoptosis is a crucial factor in liver damage and the progression of steatohepatitis (Civera et al., 2010). In this context, the activation of specific intracellular proteases, known as caspases, undertakes critical importance as mediators of programmed cell death, particularly apoptosis. Among the several caspases that participate in both extrinsic and intrinsic apoptotic pathways, caspases 8, 9, and 3 hold the highest impact. For the extrinsic apoptotic pathway, caspase 8 is the first to be activated by death receptors. This event set off the activation of caspase 3. In the same way in the intrinsic pathway, caspase 9 becomes activated as a result of the release of cytochrome-C from the mitochondria, ultimately leading to the activation of caspase 3. Notably, caspase 9 has also been identified as a substrate of caspase 3 during the process of apoptosis (Srinivasula et al., 1996).

In recent years, there has been an increasing interest in exploring natural alternatives for treating obesity and associated metabolic disorders. This surge of interest is fuelled by the recognized limitations and potential adverse effects associated with current interventions, including surgery and pharmaceutical treatments (Konstantinidi and Koutelidakis, 2019; Ruban et al., 2019). In this context, several studies have emphasised the potential of coffee consumption to restrain the onset of obesity and metabolic disorders (Lee et al., 2019; Nordestgaard et al., 2015; Pimentel et al., 2019). For instance, Shokouh et al., (2019) report the efficacy of both varieties of coffee, *arabica* and *robusta* to reduce body weight gain in a rat model of type-2 diabetes. In human studies, Larsen et al., (2018) report that increased coffee consumption was associated with a decreased concurrent gain in body weight, fat mass and waist circumference, but the associations were weak. Even though there is substantial evidence supporting the anti-obesity potential of coffee, little attention has been given to the impact of the coffee roasting degree on this effect. *In vitro* assessment (Duangjai et al., 2021) suggests that coffee roasting may augment anti-hyperglycaemic and anti-hyperlipidaemic activities. *In vivo* evaluations (Choi et al., 2018) have exposed that roasting degree can modify the antioxidant and anti-inflammatory effects of coffee extracts as seen in a septic shock model. Notably, our working group recently exhibited that the anti-inflammatory and antioxidant effects of coffee vary according to the roasting degree as demonstrated in a study on rats fed with high fructose and saturated fats (Anese et al., 2023).

Coffee contains several bioactive components, such as phenolic compounds, melanoidins and caffeine. However, the biological potential of coffee could hardly be attributed to specific compounds, given that coffee composition is rather complex and changes as a function of processing and digestion (Alongi & Anese, 2018; Alongi et al., 2020). Considering these findings, we hypothesize that the roasting degree could potentially also influence obesity and related metabolic disorders such as hyperglycemia, insulin resistance, and liver steatosis. Therefore, the objective of our study was to assess the effect of coffee with different roasting degrees on obesity and related metabolic disorders in diet-induced obese rats.

2. Materials and methods

2.1. Coffee sample preparation

The coffee samples used in this study (*Coffea canephora* var. robusta Pierre ex Froehn from Vietnam) were produced and comprehensively characterized in previous studies (Anese et al., 2023; Alongi et al., 2020). Since in these works roasting was carried out on a lab scale, the time–temperature combinations differed from those applied during industrial processing. Nevertheless, the conditions applied (*i.e.*, hot air circulation at 200 °C for 45 min or 60 min, Alongi & Anese, 2018) allowed to obtain dark and very dark roasted coffee, classified according to the weight loss (Clarke, 1987). Samples presented a homogeneous particle size of ~ 500 μm obtained by sieving through a 35-Mesh sieve.

Coffee brews were obtained by solid–liquid extraction as previously reported by Alongi & Anese (2018), Alongi et al. (2020), and Anese et al. (2023). Briefly, 1 g of ground coffee samples were boiled in 8 mL milli-Q deionized water for 5 min under stirring at 300 rpm (Line Magnetic Labnet, International Inc., New Jersey, USA). The samples were cooled and filtered using a filter with a pore size of 0.5 mm. Coffee brews were prepared daily.

2.2. Experimental animals

Animal studies were carried out in compliance with both the Norma Oficial Mexicana (NOM-062-ZOO-1999) and the recommendations from The National Institutes of Health (NIH, 2002). The protocol also meets the terms of The Mexican Social Security Institute Research Committee (R-2023–785-067).

Male Wistar rats (N = 32), twelve weeks of age and weighing 220 ± 20 g (Universidad Autónoma de México, Campus Juriquilla, Querétaro, México) were kept in a room with regulated light and temperature (12–12 h light–dark cycle, 25 ± 1° C).

Prior to initiating the experiments, the rats underwent an adaptation phase involving a minor adjustment to their circadian rhythms. This adjustment consisted of shifting their primary sleep period (initially at the beginning of the light phase) by a couple of hours. This alteration was implemented to align with the operational schedule of the animal research facility. Additionally, during the acclimatization period (one week) the rats were fed with rodent powder diet (Rodent Lab Chow 5001, Purina®, Québec, Canada). The diet caloric content was ~ 372.5 kcal/100 g, consisting of 4.5% lipids, 60% carbohydrates, and 23% proteins.

To induce obesity, we followed a modified version of the methodology described by Gamboa-Gómez et al. (2017). All rats were given an obesogenic diet containing 15.1% of lipids (of which 27% corresponded to saturated fat from lard), 70.9% of carbohydrates (of which 45.6% corresponded to corn crystalline fructose), and 13.9% of proteins, with a caloric content of ~ 511.1 kcal/100 g.

Rats were divided in 4 groups of 8 rats each, *i.e.*, 3 treatment groups and 1 control group. In conjunction with the obesogenic diet, the coffee brew treatments were administered early in the morning (during the final hour of the dark phase). We selected this time window with the intention of achieving peak caffeine concentration at the end of the dark phase when the animals are still awake. This approach allowed for the least possible additional wakefulness, considering the caffeine's half-life, which is ~ 1 h (Olini et al., 2013). Our goal was to minimize the presence of caffeine in the circulatory system once the primary sleep period commenced.

The coffee brew dose was calculated according to Reagan-Shaw et al., 2008, based on equation (1):

$$AD(mL/kg) = [HED(mL/kg)]/[Km_{(animal)}/Km_{(human)}] \quad (1)$$

where AD is the animal dose, HED represents the human equivalent dose for an adult human weighing 60 kg, corresponding to 75 mL equivalent to three standard 25 mL espresso cups (Nzekoue et al., 2021; Olechno et al., 2021). Note that the K_m conversion factor values vary between different animal species and humans. Specifically, the K_m values for adult humans and rats are 37 and 6, respectively. The results were expressed in mL/kg of body weight. Based on this calculation, the administered coffee brew doses were 7.4 mL/kg of body weight. For each rat, dosages were adjusted based on their weight gain throughout the experiment, that was recorded on a weekly basis.

To warrant consistency, the control group received an inert vehicle (*i.e.*, drinking water) instead of coffee brew.

The rats were allowed unrestricted access to their respective diets and water throughout the sixteen-week experimental period and intakes were recorded daily.

2.3. Oral glucose tolerance test (OGTT)

In the fifteenth week of the experimentation period, an OGTT was performed according to the method described by Udia et al. (2013) with some modifications. The rats were kept in a fasted state before administering a glucose load equivalent to 2 g/Kg of body weight. Blood samples were collected from the tail vein at intervals of 0, 30, 60, and 120 min, and glucose levels were measured using a glucometer (Stat Strip® Glucose, Nova Biomedical, Waltham, MA, US).

2.4. Euthanasia, blood, and organs collection

At the end of the sixteenth week of experimentation, euthanasia was carried out. The rats were first anesthetized using sodium phenobarbital dosed at 50 mg/kg of body weight and then a thoracotomy was performed. Blood samples were collected from the left ventricle and then centrifuged at 3000 × *g* for 15 min at 4 °C to get serum (An et al., 2021).

The liver and adipose tissue (AT) were promptly extracted and washed with a cold commercial and sterile saline solution (~2 °C, 0.9% NaCl, pH 7) (Pisa®, Jalisco, Mexico) to maintain adequate pH levels. Subsequently, the organs were dried, weighed, portioned, and frozen for future analysis (Galisteo et al., 2004).

A segment of both the liver and AT was immersed in a 10% formalin solution for further histological examination (Galisteo et al., 2004).

2.5. Tissue homogenate preparations

Segments of the liver and AT that had been previously frozen were homogenized according to the methodology described by Gamboa-Gómez et al. (2014). The tissue samples were first pulverized with liquid nitrogen and then homogenized in phosphate buffer (50 mM, pH 7) containing EDTA (0.5 mM). After determining the protein concentration using the Bradford method, the homogenates were stored at −80 °C for subsequent analysis.

2.6. Protein concentration

Following the determination of the protein concentration by the Bradford method, the serum was stored at −80 °C for subsequent analysis. To accomplish this, a calibration curve with albumin as the standard was constructed. The binding of protein molecules to Coomassie dye under acidic conditions caused a color change, which was then measured by a spectrophotometer at 595 nm (Bradford, 1976). The protein concentration was expressed in mg of protein per mL.

2.7. Serum measurements

The levels of fasting glucose, serum triglyceride (TG), high-density lipoprotein cholesterol (HDL-c), total cholesterol (TC), and very-low-density lipoprotein cholesterol (VDL-c), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were assessed using a commercial assays kit (Biosystem Laboratories, Barcelona, Spain) and an automated A15 spectrophotometer.

The concentration of low-density lipoprotein cholesterol (LDL-c) was estimated using Equation (2), which was previously reported by Friedewald et al. (1972).

$$LDL-c = [(TC) - HDL - (TG/5)] \quad (2)$$

The serum FFAs concentration was determined following the methodology reported by Falholt et al. (1973). This approach involves the extraction of lipids from serum with a mixture of chloroform-heptane-methanol (1:1:1), and phosphate buffer (50 mM, pH 7). To prepare a calibration curve, a standard solution of palmitic acid was diluted (0–25 μg/mL) (SIGMA Co., St. Louis, USA). The results were expressed in μg of palmitic acid equivalents/mL.

2.8. Serum insulin concentration and insulin resistance assessment

Following the manufacturer's instructions, fasting insulin levels were measured using a rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Millipore, USA) and a spectrophotometer (Spectronic® 20 GenesysTM, Spectronic Instruments, USA).

Insulin resistance was estimated using mathematical models based on fasting insulin and fasting glucose levels. The homeostasis model assessment for insulin resistance (HOMA-IR) was determined using the following equation (Equation (3):

$$HOMA - IR = [F_I \times F_G/22.5] \quad (3)$$

where F_I correspond to fasting insulin (μUI/mL) and F_G to fasting glucose (mmol/L). The 22.5 is a scaling factor that was determined to normalize the results of the HOMA formula.

2.9. Evaluation of adipose hypertrophy

Adipose tissue hypertrophy in obese rats receiving coffee brews of different roast degrees was assessed through cell-size evaluation (Morris et al., 2008). The AT samples preserved in a 10% formalin solution were embedded in paraffin, from which 4–5 μm-thick tissue sections were prepared for histological analysis. Tissue sections stained with hematoxylin and eosin were observed and photographed 40X magnification. Five images were captured for each animal and ten fields were evaluated in each section. A pathologist (J.L.G.) performed the histological evaluation. Adipocytes were examined by systematic random sampling, and the diameter of each cell was manually measured using a micrometre. The cell volume was calculated using Equation (4).

$$Volume = 1/4(3\pi r^3) \quad (4)$$

where r is radius, and the results were expressed as picolitres.

2.10. Evaluation of liver steatosis infiltrates inflammatory cells

Liver formalin-preserved samples were embedded in paraffin, and 4–5 μm-thick tissue sections were prepared and stained with hematoxylin and eosin. Five images were taken from each section, and ten fields were assessed per image. A pathologist (J.L.G.) performed a histological assessment of liver tissue. The severity of liver steatosis and inflammatory cell infiltration was evaluated using a semi-quantitative scoring system reported by Harb et al. (2019). Macrovascular liver steatosis was expressed as the proportion of hepatocytes exhibiting fat droplets that were the same size or larger than the nucleus and frequently displaced the nucleus. The number of focal inflammatory cell infiltrates was also calculated.

2.11. Homogenate TG and FFAs content

The homogenate TG content was determined using the methodology described by Folch et al. (1957) with some modifications. Briefly, liver and AT homogenates (1 mL) were mixed with a solution of chloroform–methanol (1.5 mL, 2:1). Afterwards, 500 μL of saline solution (0.9%) were added and samples were centrifugated at 4000 × *g* for 10 min. The lower phase was recovered and incubated overnight in the refrigerator (~4° C). Subsequently, the TG content was measured using commercial assay kits from Biosystem (Biosystem Laboratories, Barcelona, Spain), following the manufacturer's instructions.

The homogenate FFAs concentration was determined following the methodology reported by Falholt et al. (1973). This approach involves the extraction of lipids from tissue homogenates of liver and AT with a mixture of chloroform-heptane-methanol (1:1:1), and phosphate buffer (50 mM, pH 7). To prepare a calibration curve, a standard solution of palmitic acid was diluted (0–25 μg/mL) (SIGMA Co., St. Louis, USA). The results were expressed in μg of palmitic acid equivalents per mg of

protein.

2.12. Liver apoptosis markers

Liver apoptosis markers were evaluated by a Western Blot assay (Cai et al., 2019) determining the expression levels of caspases 3, 8, and 9. The homogenates were subjected to 15% denaturing SDS-PAGE. After that samples were transferred to nitrocellulose membranes for 1 h at 100 V within a blocking solution (8% skimmed milk in TBS-T: 20 mM Tris/HCl,100 mM NaCl, 0.2% Tween-20, and pH 7.6). The blots were then rinsed 3 times with 10 mL of TBS-T and incubated overnight with the first antibody, including β-actin (1:1000), Caspase 3 (1:1000), Caspase 8 (1:1000) and Caspase 9 (1:1000), at dilution made in 10 mL of TBS-T. Both primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). After three additional TBS-T washes 10 mL each the nitrocellulose membranes were incubated, for two hours, with a secondary antibody, the goat anti-rabbit IgG with horseradish peroxidase (1:1000) in 1 mL TBS-T. The immunoreactivity was detected using the ClarityTM Western ECL Substrate (Bio-Rad, CA, USA) and observed with a photo documentation system (ChemiDocTM MP Imaging System, Bio-Rad, CA, USA).

Densitometric scanning was performed to quantify band densities using the ImageJ software.

2.13. Statistical analysis

The data are presented as the mean ± standard error (SE). To compare the study groups, we executed a one-way analysis of variance (ANOVA), followed by a Tukey post-hoc test. Any p-value$<$0.05 was considered statistically significant. All data were analysed using IBM SPSS Statistics for Windows, version 20 (IBM Corp., Armonk, N.Y., USA).

3. Results

3.1. Body weight gain

Fig. 1 displays the results of the body weight measurements. After sixteen weeks of an obesogenic diet, the obese control group reached an average body weight of approximately 641.7 g, whereas the treated groups exhibited a reduced body weight compared to the control, regardless of the coffee roasting degree (18% for unroasted, 15% for dark, and 17% for very dark groups, respectively).

Regarding food consumption, no differences were observed between the obese control and treated groups (Figure S1).

3.2. OGTT, fasting glucose, insulin levels, and HOMA-IR values

Fig. 2 illustrates the findings from the OGTT, fasting glucose, insulin levels, and HOMA-IR values. After performing the OGTT all treatment groups showed a reduction in serum glucose concentration of about 17% compared with the obese control group. Regarding fasting glucose results, only those rats that received very dark coffee brew showed a significant reduction compared with the control (~26%).

Our findings also indicate that insulin levels and HOMA-IR values significantly decreased in all treated groups by around 77 and 65% respectively when compared with the control group, with no differences among the treatments.

3.3. Lipid Profile, FFAs, and AST/ALT as liver damage markers

The results of the lipid profile, serum FFAs, AST, and ALT enzymes are presented in Table 1. A decrease in the TG levels of about 21 and 11% was observed in rats given unroasted and dark coffee brew respectively when compared to the obese control group. Nevertheless, total cholesterol, HDL, LDL, VLDL, AST, and ALT levels in the serum showed no significant variation between the control and treated groups. In terms of serum FFAs levels, a significant decrease of 46% was only seen in rats treated with unroasted coffee brew, compared to the control group.

3.4. Adipose tissue hypertrophy, liver steatosis, and lipid content assessment

The results of adipose hypertrophy are depicted in Fig. 3. Only rats

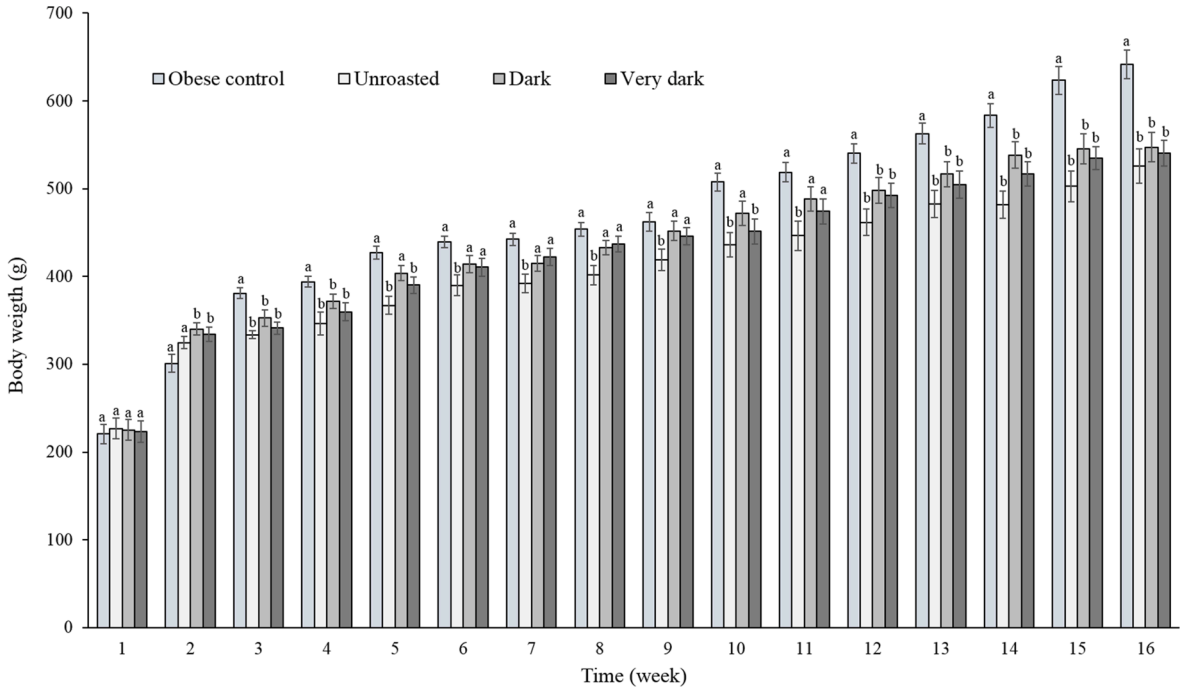

Fig. 1. Body weight gain of obese rats who received coffee brews with different roasting degree: unroasted, dark, and very dark. Values are means ± standard deviation. Different letters (a-c) between columns per week indicate a significant difference between study groups ($p < 0.05$) by Tukey's test.

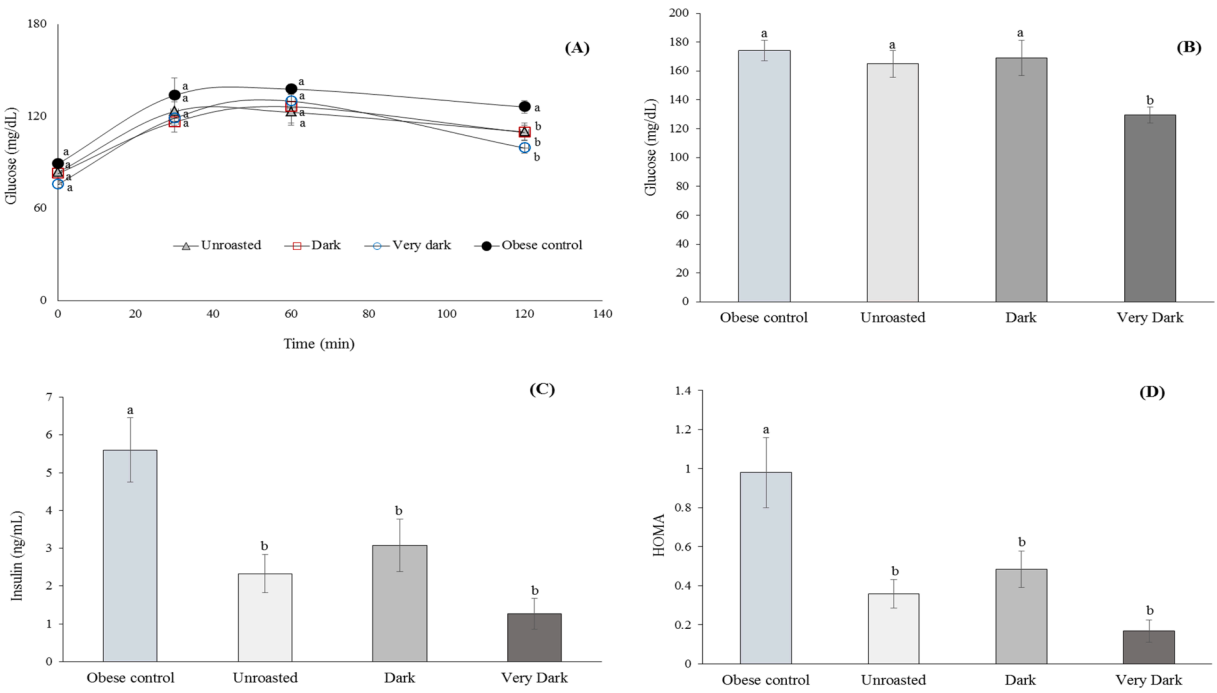

Fig. 2. Oral glucose tolerance test (A), Fasting glucose (B), Insulin concentration (C), and Homeostatic Model Assessment for Insulin Resistance (HOMA) values (D) of obese rats who received coffee brews with different roasting degree: unroasted, dark, and very dark. Values are means ± standard error. Different letters (a-c) between each point on the curve or by columns per figure indicate a significant difference between study groups ($p < 0.05$) by Tukey's test.

Table 1
Lipid metabolic parameters in obese rats administered coffee brews of varying roasting degree: unroasted, dark, and very dark.

	Obese control	Unroasted	Dark	Very dark
TG (mg/dL)	160.0 ± 6.1^{a}	126.7 ± 7.2^{c}	142.9 ± 5.1^{b}	164.5 ± 8.4^{a}
FFAs (μg eq. of palmitic acid/mg of protein)	1.35 ± 0.1^{a}	0.72 ± 0.1^{b}	1.35 ± 0.1^{a}	1.38 ± 0.2^{a}
TC (mg/dL)	80.6 ± 2.3^{a}	77.5 ± 5.2^{a}	80.7 ± 2.5^{a}	65.9 ± 3.8^{a}
HDL-c (mg/dL)	28.8 ± 6.0^{a}	29.1 ± 5.7^{a}	31.9 ± 1.1^{a}	32.2 ± 5.4^{a}
LDL-c (mg/dL)	23.2 ± 4.8^{a}	29.9 ± 5.9^{a}	25.5 ± 3.3^{a}	21.4 ± 1.4^{a}
VLDL-c (mg/dL)	30.9 ± 1.1^{a}	28.5 ± 3.4^{a}	36.7 ± 5.3^{a}	35.0 ± 2.5^{a}
AST (U/L)	84.4 ± 3.4^{a}	72.3 ± 7.1^{a}	82.8 ± 8.0^{a}	82.3 ± 8.0^{a}
ALT (U/L)	33.5 ± 2.0^{a}	29.5 ± 2.4^{a}	33.0 ± 4.6^{a}	28.3 ± 3.6^{a}

Values are means of duplicated determinations ± standard error, otherwise is indicated.
Different letters (a-c) between rows indicate a significant difference between study groups ($p < 0.05$) by Tukey's test.
Triglycerides (TG), Free fatty acids (FFAs), Total cholesterol (TC), High-density lipoprotein (HDL-c); Low-density lipoprotein (LDL-c); Very-low-density lipoprotein (VLDL-c), Aspartate aminotransferase (AST); and alanine aminotransferase (ALT).

treated with unroasted coffee showed a significant decrease in adipocyte volume, exhibiting a 42% reduction compared to the obese control.

Regarding the concentration of lipids in AT (Table 2), all treated groups exhibited a decreased concentration of FFAs, with an approximate reduction of 82%. Significant reductions in TG levels were only observed in rats administered with dark and very dark coffee brews, showing decreases of 82 and 52% respectively when compared to the obese control (Table 2).

The results of liver steatosis are shown in Fig. 4. All groups revealed<30% of droplets in the hepatic area, indicating grade 1 steatosis. This suggests that fatty hepatocytes occupied<33% of the hepatic parenchyma. All treated groups exhibited a smaller steatosis area compared to the obese control, with reductions of 54, 64 and 47% for unroasted, dark and very dark treated groups, respectively. All treated groups also showed fewer inflammatory foci per focus compared to the obese control, with a reduction of around 85% (Fig. 4).

Regarding the lipid concentration in the liver (Table 2), all treated groups showed a decreased concentration of FFAs, with reductions of 73, 47 and 79% for unroasted, dark and very dark coffee brew groups, respectively, compared to the obese control. Also, rats given dark and very dark coffee decreased TG in the liver by approximately 55 and 39%, respectively, when compared with the obese control (Table 2).

3.5. *Apoptosis liver markers*

Fig. 5 depicts the results of liver apoptosis markers, *i.e.*, Caspase-3, Caspase-8, and Caspase-9. A significant decrease in Caspase-3 was observed in all treated groups when compared to the control group, with reductions of 42, 22 and 17% for the unroasted, dark and very dark coffee brew groups, respectively. The expression of Caspase-8 was markedly reduced in rats treated with unroasted coffee brew in contrast to the obese control and was even nullified in rats treated with dark and very dark coffee brews. Conversely, the expression of Caspase-9 was absent in both the obese control and the unroasted coffee brew treated group, becoming evident instead in the groups treated with dark and very dark coffee brews.

4. Discussion

Obesity is defined as the pathological accumulation of body fat, leading to comorbidities accompanied by metabolic and physiological disturbances (Sarma et al., 2021). Animal models of diet-induced obesity have considerable face validity with human obesity and are widely used to investigate the metabolic and physiological disturbances

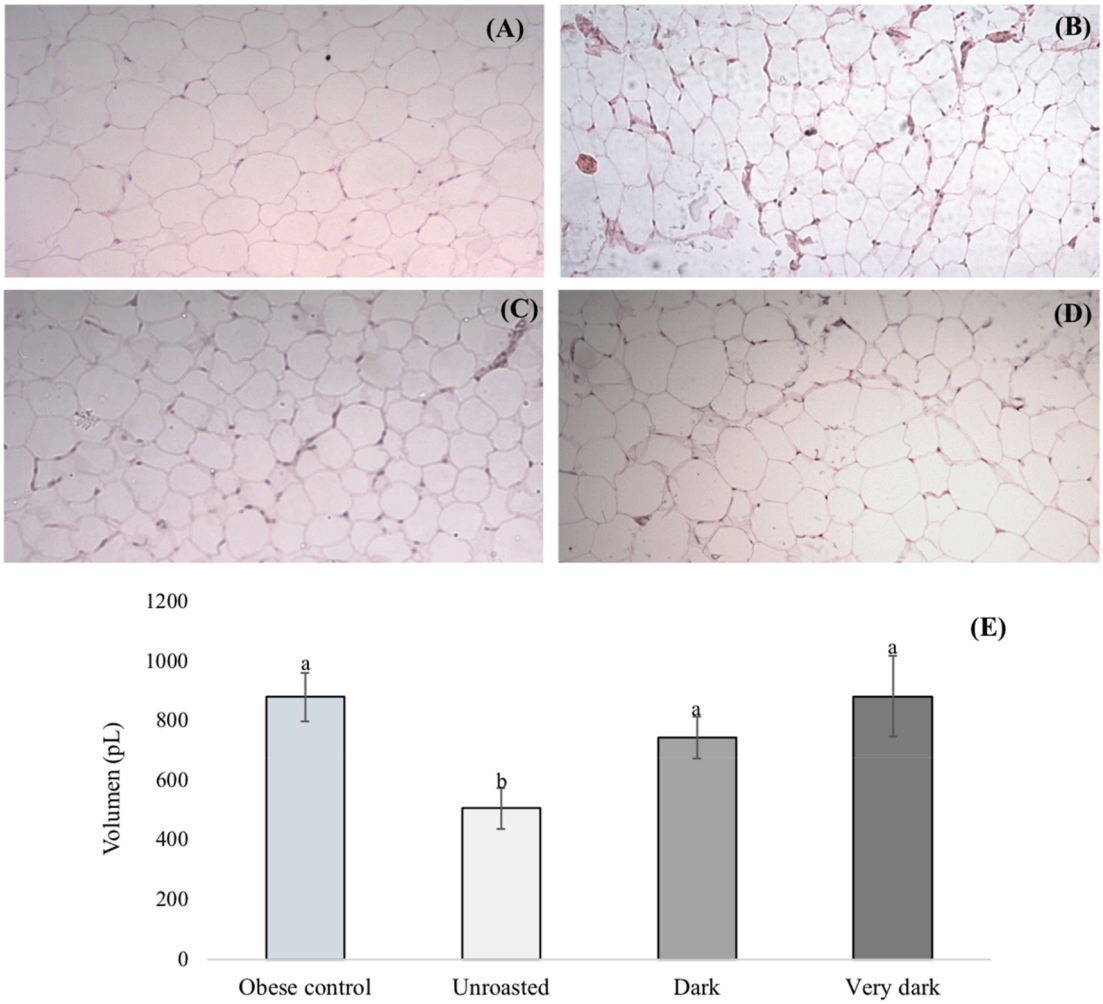

Fig. 3. Representative photographs of Hematoxylin-eosin–stained adipose tissue sections (100X): of obese control (A), unroasted (B), dark (C), and very dark (D) group, and adipocytes volume values (E) of obese rats who received coffee brews with different roasting degree. Values are means ± standard error. Different letters (a-c) between each point on the curve or by columns per figure indicate a significant difference between study groups ($p < 0.05$) by Tukey's test.

Table 2
Free fatty acids (FFAs) and triglycerides (TG) concentration in hepatic and adipose tissue of obese rats who received coffee brews with different roasting degree: unroasted, dark, and very dark.

Sample	Liver		Adipose tissue	
	FFAs (μg/mg of protein)	TG (μg/ mg of protein)	FFAs (μg/mg of protein)	TG (μg/ mg of protein)
Obese control	1.9 ± 0.1^{a}	10.7 ± 1.1^{a}	23.5 ± 0.1^{a}	362.2 ± 34.7^{a}
Unroasted	0.5 ± 0.1^{b}	11.5 ± 2.1^{a}	4.4 ± 0.1^{b}	286.8 ± 45.5^{a}
Dark	1.0 ± 0.1^{b}	4.8 ± 0.6^{b}	3.7 ± 0.1^{b}	64.8 ± 7.2^{b}
Very dark	0.4 ± 0.1^{b}	6.5 ± 0.3^{b}	3.7 ± 0.1^{b}	173.7 ± 8.8^{c}

Values are means of duplicated determinations ± standard error.
Different letters (a-c) between columns indicate a significant difference between study groups ($p < 0.05$) by Tukey's test.

associated with obesity, including insulin resistance and liver steatosis (Bakhti et al., 2018). Considering these factors, we employed a high fructose and saturated fat diet to induce obesity and examine the effect of coffee roasting degree on metabolic and physiological disturbances associated with obesity. Our findings revealed that the obesogenic diet administered for a period of sixteen weeks resulted in a body weight gain of ~ 640 g in obese control rats, corresponding to a 62% increase compared to reported values for healthy male Wistar rats of the same age (Nistiar et al., 2012). On the other hand, we demonstrated that coffee brews restrained body weight gain in obese rats (~16%), regardless of the roasting degree. It must be pointed out that this effect cannot be attributed to food or water intake, as these were comparable among groups throughout the entire intervention period (supplementary material). In concordance with our results, previous studies have also reported that both unroasted and roasted coffee can decrease body weight gain in rats induced to be obese (Feyisa et al., 2019; Ilmiawati et al., 2020).

Anti-obesity treatments are considered successful if they induce a decrease of 5 to 10% in body weight gain and allow long-term maintenance of the weight loss once achieved (Campfield et al., 1998). Additionally, a 5–10% loss in body weight, regardless of the method used to achieve it, is associated with improvements in cardiovascular risk profiles and a reduced incidence of type 2 diabetes (Christensen et al., 2007). Given the above-mentioned considerations all coffee brews could be considered effective as natural anti-obesogenic alternatives. Several mechanisms through which coffee may prevent weight gain have been reported, including increased lipolytic activity, cellular thermogenesis, norepinephrine release, elevated resting metabolic rate, and accelerated energy expenditure, among others (Baspinar et al., 2017). It is important to mention that some bioactive compounds in coffee, which have been attributed to anti-obesogenic potential, are either unaffected by the roasting process or are in fact created during this process. For instance, on one hand, caffeine is known to remain stable after roasting and has

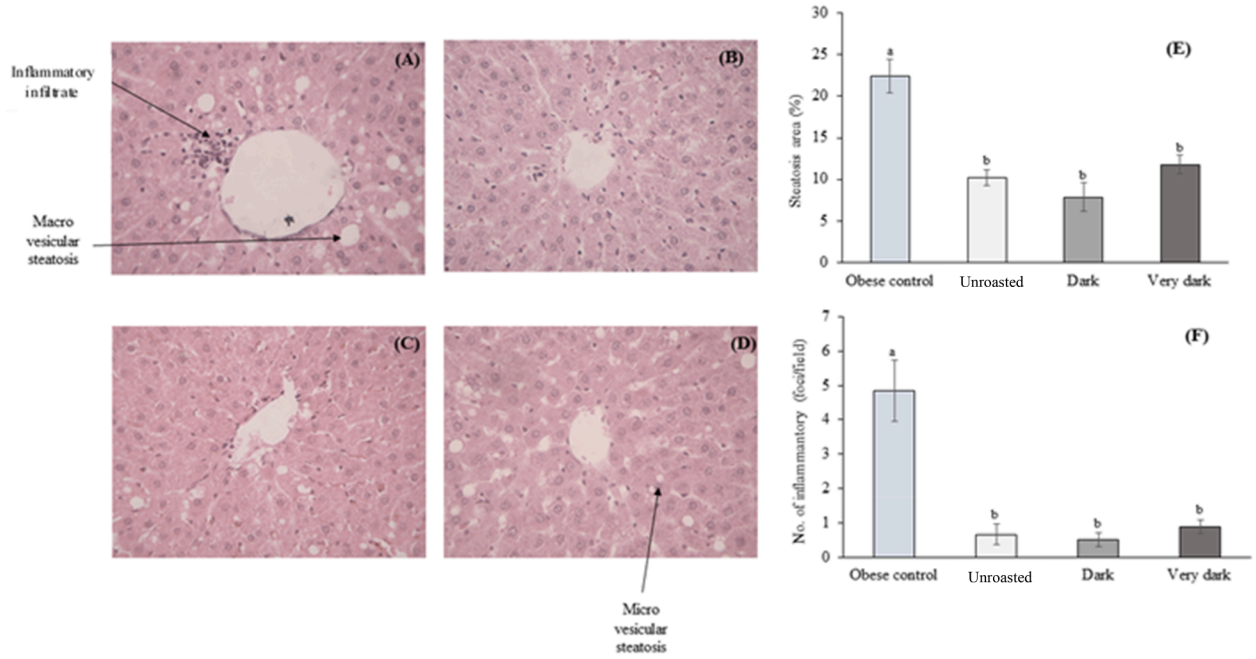

Fig. 4. Representative photographs of Hematoxylin-eosin–stained hepatic tissue sections (100X): obese control (A), unroasted (B), dark (C), and very dark (D) group, steatosis percentage area (E), and inflammatory infiltrate (F) of obese rats who received coffee brews with different roasting degree. In figure (E) and (F) values are means ± standard error. Different letters (a-b) by columns per figure indicate a significant difference between study groups ($p < 0.05$) by Tukey's test.

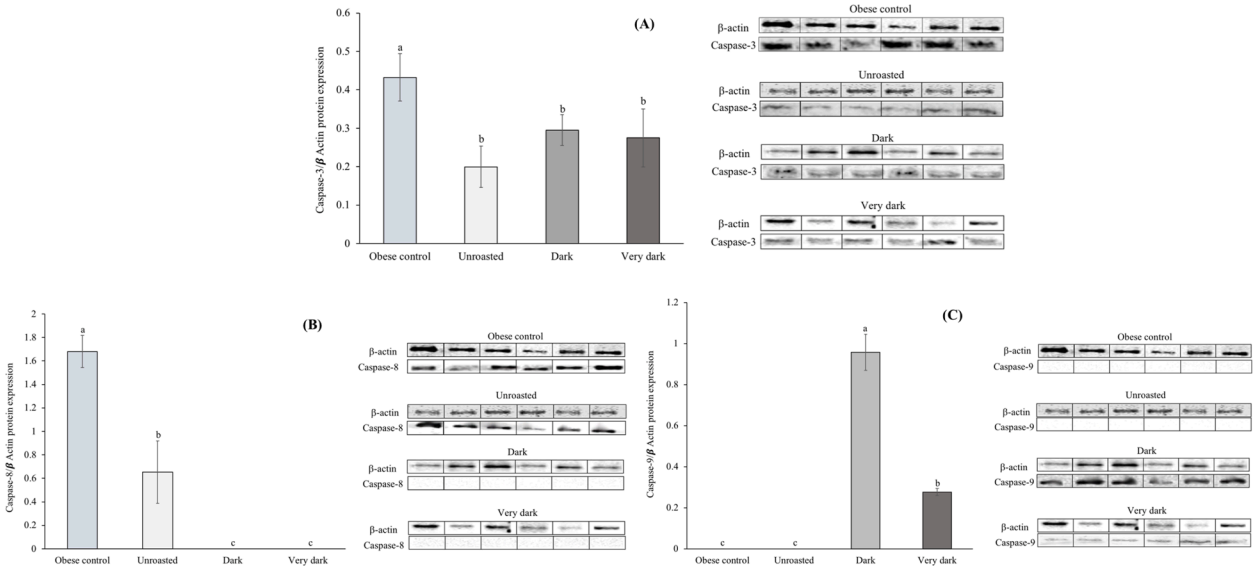

Fig. 5. Hepatic apoptosis markers: Caspase-3 (A), Caspase-8 (B), and Caspase-9 (C) of obese rats who received coffee brews with different roasting degree. Values are means ± standard error. Different letters (a-c) between each point on the curve or by columns per figure indicate a significant difference between study groups ($p < 0.05$) by Tukey's test.

been reported to increase energy expenditure and resting metabolic rate by approximately 13% (Bangsbo et al., 1992; Cambell et al., 2016; Sualeh et al., 2020). On other hand, chlorogenic acids, which are the primary polyphenols in coffee, undergo changes during the roasting process. Some of them degrade to various extents, and experience acyl migration, trans–cis isomerization, dehydration, epimerization, and condensation. They can also interact with proteins and polysaccharides and are further transformed during roasting into low-molecular-weight Maillard reaction products (MRPs) and, eventually, into melanoidins (Coelho et al., 2014; Moreira et al., 2017). Previous *in vitro* and *in vivo* studies have reported that melanoidins exhibit antioxidant activity and may contribute to cancer prevention by inhibiting oxidative stress (Budryn et al., 2017). Additionally, it has been reported that melanoidins reduce daily energy intake and modulate postprandial glycemia (Hernández et al., 2007; Walker et al., 2020). The melanoidin content of the coffee brews used in the present study and measured in previous work actually increased by 54 and 60% in the dark and very dark roasted samples compared to the unroasted one (Alongi et al., 2020).

It must be pointed out that the coffee anti-obesity mechanisms due to the restrained body weight gain in treated obese rats (~16%) could have also been attributed the effect of caffein on sleep, since epidemiological studies have established a link between short or disrupted sleep and an elevated risk of developing obesity (Barf et al., 2012). To avoid this bias, we administered treatments early in the morning (during the final hour

of the dark phase) to minimize the impact of caffeine and its potential disruption of sleep, with consequent effects on reduced weight gain. This approach reasonably allows us to infer that the observed weight loss truly reflects the direct impact of coffee on obesity. Nevertheless, we did not directly measure caffeine's effect on the rats' sleep patterns and further studies in this area would reinforce our hypothesis.

After sixteen weeks of feeding with a high fructose and saturated fat diet, obese rats had fasting glucose levels of approximately 165 mg/dL and serum glucose values still above 120 mg/dL after 120 min of a glucose load. In contrast, healthy Wistar male rats of the same age had reported fasting glucose and 120-minute load glucose levels of 94 mg/dL (Kawasaki et al., 2005). Overall, coffee administration improved glucose homeostasis. Even though only the group treated with dark coffee exhibited a decrease in fasting glucose, all treated groups showed a decrease in serum glucose values after 120 min of load (~17%). The effect of coffee on glucose homeostasis in obesity has been controversially reported in the literature. For example, Ramos et al. (2022) report that the intervention with roasted coffee on obese rats induced with a high-fat diet neither improved glucose homeostasis nor increased insulin secretion. This lack of effect was attributed to caffeine which raises the plasma levels of epinephrine, reducing insulin sensitivity, and leading to an increase in blood glucose levels (Keijzers et al., 2002). However, coffee contains other bioactive compounds, including phenolic compounds with chlorogenic acids and their derivatives being the most abundant classes. The single compounds have been thoroughly characterized in previous work (Alongi et al., 2020), and the concentration of the major classes (*i.e.*, chlorogenic acids, chlorogenic acid lactones, di-chlorogenic acids and other derivatives) is reported in the supplementary material (Table S1). In the unroasted coffee brew, phenolic compounds overall accounted for around 470 mg/g_{dw}, with almost 80% being chlorogenic acids and 20% di-chlorogenic acids. Lactones and other derivatives accounted together for<1% of the total phenolic compounds quantified. In the brews obtained from dark and very dark roasted coffee, the phenolic concentration overall decreased by 20% compared to the unroasted one, with differences among different classes. Chlorogenic acids decreased by 20%, dimers by around 30%, and other derivatives by 70% due to thermal degradation, leading to the formation of other compounds, including lactones. In fact, their concentration dramatically increased (up to 8-fold) upon roasting.

The phenolic compounds contained in coffee brews were demonstrated to counteract the negative effects of caffeine on glucose metabolism by reducing fasting plasma glucose levels (Lukitasari et al., 2017), inhibiting glucose absorption in the gut (Johnston et al., 2003), and hindering the action of α-glucosidase (Alongi & Anese, 2018), GLUT4, and glucose-6-phosphatase in the liver, eventually leading to a decrease in plasma glucose levels (Akash et al., 2014).

In addition to the observed hyperglycaemia in the obese control group, insulin levels were elevated to 5 ng/mL, compared to healthy Wistar male rats of the same age exhibited insulin levels of 1 ng/mL (Kawasaki et al., 2005). On the other hand, rats that received any of the coffee brews experienced a reduction in insulin levels by approximately 60% and subsequently decreased HOMA-IR values (60%) compared to the obese control group. Consistently with our findings, previous studies have reported that both unroasted and roasted coffee improve insulin sensitivity (Loopstra-Masters et al., 2011; Lukitasari et al., 2017; Nikpayam et al., 2019).

One of the potential mechanisms to consider, by which coffee consumption may improve insulin resistance, is related to alterations in the gut microbiota. It has been reported that gut microbiota can influence insulin resistance (Gesta et al., 2007). The gut microbiota plays a role in regulating the host's dietary intake, energy metabolism, and energy expenditure (Lee and Lee, 2020). Changes in the composition of intestinal bacteria could potentially impact energy metabolism and have diverse effects on critical metabolic organs, such as skeletal muscle, the liver, and AT (Lee and Lee, 2020). In this regard, it has been reported that coffee's bioactive compounds could serve as a source of carbon and nitrogen, potentially promoting the growth of colonic bacteria such as *Bifidobacteria* and sulfate-reducing bacteria (Jiménez-Zamora et al., 2015). Furthermore, a synergistic effect has been reported because the presence of probiotic bacteria contributes to the significant release of chlorogenic acids from coffee dietary fiber, including melanoidins, in the 'large intestine' after 4 h of digestion (Grzelczyk et al., 2022), potentially prolonging the positive effects of coffee. However, additional studies on this topic are required to confirm whether this could be the mechanism underlying the observed effects in this work.

Besides its crucial role in regulating glucose uptake, insulin also enhanced AT lipolysis (Kovacs & Stumvoll, 2005), leading to a sustained increase in FFAs delivery to several organs, such as the liver. This increased delivery of FFAs, combined with decreased fatty acid oxidation contributes to the accumulation of TG in abnormal locations, a condition known as ectopic TG storage. Based on the aforementioned considerations, we evaluated both free fatty acids (FFAs) and triglycerides (TG) in serum, AT, and liver. Only rats treated with unroasted coffee brew showed a significant decrease in serum FFAs levels compared to the obese control, confirming an improved insulin sensitivity. Considering AT and liver, a decrease in FFAs was observed in all treated groups, regardless of the roast degree, when compared to the obese control group. However, only the groups treated with roasted coffee brews experienced a reduction in TG concentration in both AT and liver. Although it can be hypothesized that both unroasted and roasted coffee contain bioactive compounds capable of modifying insulin resistance and lipotoxicity, thereby reducing lipolysis, the compounds formed during roasting appear to specifically impact other metabolic pathways. Vitaglione et al. (2010) reported that coffee melanoidins reduce liver fat accumulation by promoting fatty acid β-oxidation. This mechanism promotes a decrease in the accumulation of TG in tissues.

Another noteworthy finding from our study was that rats treated with dark coffee brew had lower levels of TG in their serum, liver, and TA compared to those that received very dark coffee brew. Therefore, it can be inferred that the compounds responsible for the observed effect are those formed early in the coffee roasting process and that suffer partial oxidation and degradation as roasting advances (Perrone et al., 2012). These findings suggest that the changes in composition and concentration of bioactive compounds triggered by roasting considerably impact TG levels.

Although all coffee brews effectively lowered lipid levels in AT, only the unroasted coffee reduced adipocyte size compared to the obese control. This effect could be attributed to the presence of chlorogenic acid, the most abundant and active compound in unroasted coffee, known for its capability to reduce lipid accumulation in adipocytes (Pimpley et al., 2020). However, it is important to note that dark and very dark roasting determined a reduction of chlorogenic acid content by 80% compared to unroasted coffee (Table S1). This reduction in chlorogenic acid content during roasting may potentially explain the differences we observed.

When examining liver steatosis, all treated groups displayed reduced steatosis area (~56%) and a fewer number of inflammatory cells (~83%) compared with the obese control, independently from the roasting degree. In accordance with our findings, previous studies have reported the positive impact of coffee on liver steatosis (Shokouh et al., 2019; Yamauchi et al., 2010), and this effect has been attributed to compounds unaffected by roasting degree, like caffeine, which is known to enhance hepatic steatosis via its lipolytic activity (Sinha et al., 2014) and through activating the cAMP/CREB/SIRT3/AMPK/ACC pathway (Zhang et al., 2015).

With the aim to evaluate markers of apoptosis as indicative of liver damage, we assessed Caspases-3, −8, and −9. The obese control group exhibited increased expression of Caspase-3, suggesting greater activation of the apoptosis process. Caspase-3 a cysteine–aspartic acid protease activated by Caspases-8 or −9 plays a central role in the execution phase of cell apoptosis (Granado-Serrano et al., 2006). In the obese

control rats, Caspase-8 was highly expressed, while no expression of Caspase-9 was detected. Coffee intake, irrespective of roasting degree, decreased Caspase-3 expression compared to the obese control. However, it seems that the mechanism of action varied with the degree of roast. Specifically, unroasted coffee did not alter Caspase-9 expression compared to the obese control, but it decreased Caspase-8 expression. Conversely, roasted coffee completely suppressed Caspase-8 expression. Even though caspase-9 expression was detected in rats given roasted coffee - contrary to the obese control - the level was insufficient to elevate Caspase-3 expression to that seen in the obese control group. Our results are consistent with previous studies suggesting that coffee offers protection against liver injury, induced by thioacetamide in male Wistar rats, by reducing cleaved Caspase-3 indexes (Furtado et al., 2012), regardless of the roasting degree (Abdelaal et al., 2019). However, it is important to acknowledge that the roasting degree could influence the specific pathway leading to the expression of enzymes involved in apoptosis mechanisms.

5. Limitations of the study

This study has certain limitations that should be acknowledged. Given that the key parameters we measured have previously been reported for healthy animals of the same strain and age (Kawasaki et al., 2005; Nistiar et al., 2012), and considering that our animal model did not receive drug administration, we opted not to include a normal control group with a regular diet as a reported strategy to minimize the sample size (Kramer et al., 2017).

6. Conclusion

Our study shows that coffee brews regardless of roasting degree, can diminish body weight gain, enhance insulin sensitivity, and decrease hepatic steatosis. However, adipose hypertrophy was only improved with the use of unroasted coffee.

In addition, coffee brews led to a reduction in the expression of programmed cell death indicators through apoptosis. No significant differences were found among the treatments, suggesting that the degree of coffee roasting does not significantly influence these markers of liver damage. However, the specific biological pathways activated by each treatment appear to differ.

Overall, it can be concluded that depending on the metabolic pathway and marker under study, an effect of roasting could be either found or not. Given that roasting affects the composition of coffee, different compounds can be claimed responsible for the effects observed on different metabolic pathways. The outcomes of this study can be applied to steer coffee roasting in order to pursue a specific effect targeting obesity and related metabolic disorders. Indeed, future clinical trials are needed to validate our findings.

Ethical Statement

Animal studies were carried out in compliance with both the Norma Oficial Mexicana (NOM-062-ZOO-1999) and the recommendations from The National Institutes of Health (NIH, 2002). The protocol also meets the terms of The Mexican Social Security Institute Research Committee (R-2023-785-067).

CRediT authorship contribution statement

Claudia I. Gamboa-Gómez: . **Laura J. Barragán-Zúñiga:** . **Fernando Guerrero-Romero:** . **Gerardo Martínez-Aguilar:** . **José Luis Gónzalez:** . **Almendra A. Valenzuela-Ramírez:** Methodology, Formal analysis. **Juan A. Rojas-Contreras:** Investigation, Formal analysis. **Monica Anese:** Writing – review & editing, Conceptualization. **Maribel Cervantes Flores:** Visualization, Methodology, Formal analysis, Data curation. **Marilisa Alongi:** .

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.

Data availability

Data will be made available on request.

Acknowledgements

This project was supported by COCYTED/DG-202/2020 and with the scholarship of the Mexican National System of Researchers (SNI, No. 209339). Author AAVR. thanks the Mexican Council of Science and Technology (CONACYT) for the graduate scholarship (CVU 1042204). The authors appreciate the technical support of Víctor Iván Sayago-Monreal and Julio C. Ramírez-España.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2023.105889.

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