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Kombucha tea improves glucose tolerance and reduces hepatic steatosis in obese mice

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ABSTRACT

Nonalcoholic fatty liver disease (NAFLD), often associated with obesity, is becoming one of the most common liver diseases worldwide. It is estimated to affect one billion individuals and may be present in approximately 25% of the population globally. NAFLD is viewed as a hepatic manifestation of metabolic syndrome, with humans and animal models presenting dyslipidemia, hypertension, and diabetes. The gut-liver axis has been considered the main pathogenesis branch for NAFLD development. Considering that foods or beverages could modulate the gastrointestinal tract, immune system, energy homeostasis regulation, and even the gut-liver axis, we conducted an exploratory study to analyze the effects of kombucha probiotic on hepatic steatosis, glucose tolerance, and hepatic enzymes involved in carbohydrate and fat metabolism using a pre-clinical model. The diet-induced obese mice presented glucose intolerance, hyperinsulinemia, hepatic steatosis, increased collagen fiber deposition in liver vascular spaces, and upregulated TNF-alpha and SREBP-1 gene expression. Mice receiving the kombucha supplement displayed improved glucose tolerance, reduced hyperinsulinemia, decreased citrate synthase and phosphofructokinase-1 enzyme activities, downregulated G-protein-coupled bile acid receptor, also known as TGR5, and farnesol X receptor gene expression, and attenuated steatosis and hepatic collagen fiber deposition. The improvement in glucose tolerance was accompanied by the recovery of acute insulin-induced liver AKT serine phosphorylation. Thus, it is possible to conclude that this probiotic drink has a beneficial effect in reducing the metabolic alterations associated with diet-induced obesity. This probiotic beverage deserves an extension of studies to confirm or refute its potentially beneficial effects.

1. Introduction

Nonalcoholic Fatty Liver Disease (NAFLD) is a spectrum of liver diseases characterized by the ectopic accumulation of fat in the liver in people without a history of excessive alcohol consumption. It is diagnosed by liver biopsy when hepatocytes contain at least 5% lipid infiltrate [6,51]. NAFLD has a prevalence of approximately 30% of the general population in developed countries like the United States, but the prevalence reaches 98% in the obese population [47]. If left untreated, the initial stage of NAFLD (i.e., hepatic steatosis) can progress to nonalcoholic steatohepatitis (NASH) and later to cirrhosis or hepatocellular carcinoma [42].

The onset and progression of NAFLD are influenced by diet and gut microbiota [29,35]. Previous studies have demonstrated that high-fat diets (HFDs) promote microbiota changes and increase the intestinal barrier permeability, promoting the absorption of bacterial metabolites [3,33].

Metagenomic analysis of obese individuals with steatosis revealed an enrichment of genes related to lipid metabolism, endotoxin biosynthesis and aromatic and branched-chain amino acid metabolism dysregulation [24]. Moreover, bacterial metabolites can trigger inflammatory processes in tissues due to recognition by toll-like receptors (TLRs), potentially leading to inflammation in the hepatocytes and the progression of steatosis to a hepatitis state [41].

The relationship between intestinal microbiota and its contribution to the development and homeostasis of physiological systems is an area that stands out for its emerging relevance [22]. In 2006, it was recognized that intestinal microbiota is a significant environmental factor responsible for metabolic changes in the host organism [46]. Due to the intestinal microbiota's great potential for producing bioactive

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compounds that can influence host signaling pathways and cell receptors, it has been linked to the development of chronic diseases such as diabetes, obesity, heart disease, asthma, inflammatory bowel diseases and NAFLD. Indeed, many studies investigating the microbiota have attempted to unravel its interactions of commensalism and/or mutualism [8,45].

Notably, the link between intestinal microbiota and disease development has aroused the interest of researchers worldwide in finding pharmacological treatments or natural substances to combat NAFLD. For example, a previous study showed that prebiotics and probiotics modulate the intestinal microbiota in several diseases, including NAFLD [43]. Additionally, Wong et al. [49] showed that prebiotics and probiotics improved the intestinal microbiota composition, which was associated with reducing liver inflammation, lipopolysaccharides (LPS) levels, and aminotransferase activity. Others have shown that probiotic supplementation reduced hypercholesterolemia and improved glucose tolerance and liver steatosis [9,34,40].

A natural, nonalcoholic fermented drink with a probiotic characteristic known as kombucha is considered a functional beverage [27]. It is produced by a symbiotic culture of bacteria and yeast, and its nutritional properties are derived from bioactive products that the live microorganisms produce. Experimental evidence shows that kombucha produces an antioxidant effect in rats consuming a hypercholesterolemic diet [5]. The same study also demonstrated that kombucha could modulate the microbiota of mice with NAFLD. It has been proposed that the generation of vitamins, minerals, polyphenols, acetic acid, and glucuronic acid are possibilities for these reported beneficial effects [18,26,30].

In the present study, we administered kombucha tea to a pre-clinical mouse model (i.e., pre-diabetic diet-induced obese mice with compensatory hyperinsulinemia and features of NAFLD) and evaluated the metabolic profile to determine the effect of this supplement on the NAFLD course.

2. Materials and methods

2.1. Preparation of Kombucha tea

Six grams of green tea (*Camellia sinensis*) were added to 1000 mL of boiling distilled water, left to infuse for 5 min, and then strained. Sixty grams of sucrose were added and stirred to dissolve in the green tea. After reaching room temperature, the tea was poured into a glass container previously sterilized at 120 ºC for 20 min. Finally, the freshly prepared tea was inoculated with a symbiotic kombucha culture with 100 mL of previously fermented tea. The container was covered with cheesecloth to prevent external contamination by insects and to allow aerobic fermentation. Fermentation (25 ± 3 ºC) continued for 10 days, until reaching pH 3.1–3.2 and Brix 4–5%. The kombucha colony was obtained commercially from Kombucha Kamp© (Gardena, CA, USA).

Kombucha tea was collected and centrifuged at 7,000 × *g* for 20 min, and the supernatant was passed through a 0.22 μm syringe filter (Millipore®, Burlington, Massachusetts, USA). The daily dose administrated to each mouse corresponded to 0.2 mL containing 10^7–10^8 microorganisms/mL of kombucha, which was determined spectrophotometrically by measuring the absorbance at 600 nm. This dose was based on a previous study that administered probiotics [48].

2.2. Animals

Male C57BL/6 mice, weighing 20–25 g, were obtained from the animal facility at the University of São Paulo (USP) Medical School and were maintained in a temperature-controlled room at 22 ± 2ºC with free access to food and tap water and a 12-hour light-dark cycle (lights on from 6 am to 6 pm). The mice were divided into two main groups. One received regular rodent chow (RC) (CR-1, Nuvilab®, Colombo, Parana, Brazil), containing 19% protein, 56% carbohydrate, 3.5% lipid, 5% cellulose and 4.5% vitamins and minerals, providing 3.2 kcal/kg. The second group received a pelletized high-fat diet (HFD) containing 15.8% protein, 27% carbohydrate, 57.2% lipid and 4.5% vitamins and minerals, providing 5.5 kcal/kg (PragSoluções Biosciences, Jau, São Paulo, Brazil). Both groups consumed their respective diet for 12 consecutive weeks.

At the end of the 10th week of the diet protocol, the RC and HFD mice were administered kombucha tea or an equal volume of tap water daily by gavage. This procedure was performed for nine consecutive days. At this point, the body weight and food intake were measured daily. The final experimental groups consisted of the control group + tap water (RC), control group + kombucha (RC+K), HFD + tap water (HFD), and HFD + kombucha (HFD+K). According to a previous review article, nine days of kombucha supplementation is equivalent to one year of continuous use in humans [19].

Food intake, caloric intake and feeding efficiency were estimated using the following formulas:

Food intake = initial weight of food provided – final weight of food recovered (g)

Caloric intake = mean food intake (g) × percentage of dietary metabolizable energy

Feed efficiency = body weight gain (g) / mean food intake (g) [36].

The collected data from each box of five mice were used in these calculations.

All experimental procedures were performed following the Guidelines for the ethical use of animals in applied etiology studies and were previously approved by the Ethics Committee on the use of animals at the ICB-USP (protocol number at CEUA 9618010218).

2.3. Glucose tolerance test

Awake mice were submitted to an intraperitoneal glucose tolerance test (ipGTT). After six hours of fasting, each mouse received an intraperitoneal injection of a solution of 10% glucose (1 mg/g body weight). The blood samples were collected from a superficial cut in the tail at 0, 15, 30, 60, 90 and 120 min to determine serum glucose and insulin concentrations using a commercially available mouse insulin ELISA kit (Mercodia®, Winston Salem, NC, USA).

2.4. Immunoblotting

The animals were anesthetized with thiopental (50 mg/kg b.w.), and after the loss of corneal reflexes, the abdominal wall was opened to visualize the liver and portal vein. Some mice received a bolus saline infusion through the portal vein with or without regular insulin (1 U) (Humulin R, Eli Lilly of Brazil, SP, Brazil) to analyze the intracellular insulin action. Thirty seconds after the insulin injection, the liver was removed and homogenized in lysis buffer (100 mM Tris, 10% SDS, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate, pH 7.4). The tissue extracts were centrifuged at 15,294 × *g*, at 4 °C, for 40 min. The protein content of the supernatants was measured by the Bradford method.

Aliquots of the supernatant containing 50 μg total protein were treated with Laemmli buffer supplemented with 200 mM dithiothreitol, loaded onto 8% and 10% polyacrylamide gels, and subjected to SDS-PAGE. The proteins were then transferred from the gels to nitrocellulose membranes using a Bio-Rad mini trans blot Apparatus (USA). The membranes were incubated in TBST-B blocking buffer (10 mM Tris, 150 mM NaCl, 0.05% tween 20%, and 5% skim milk) for 2 h at room temperature (RT) to prevent any nonspecific binding to the membrane. The nitrocellulose membranes were incubated with the specific primary antibodies overnight at 4 °C and subsequently incubated with a secondary antibody conjugated to horseradish peroxidase for 1 h at RT. The

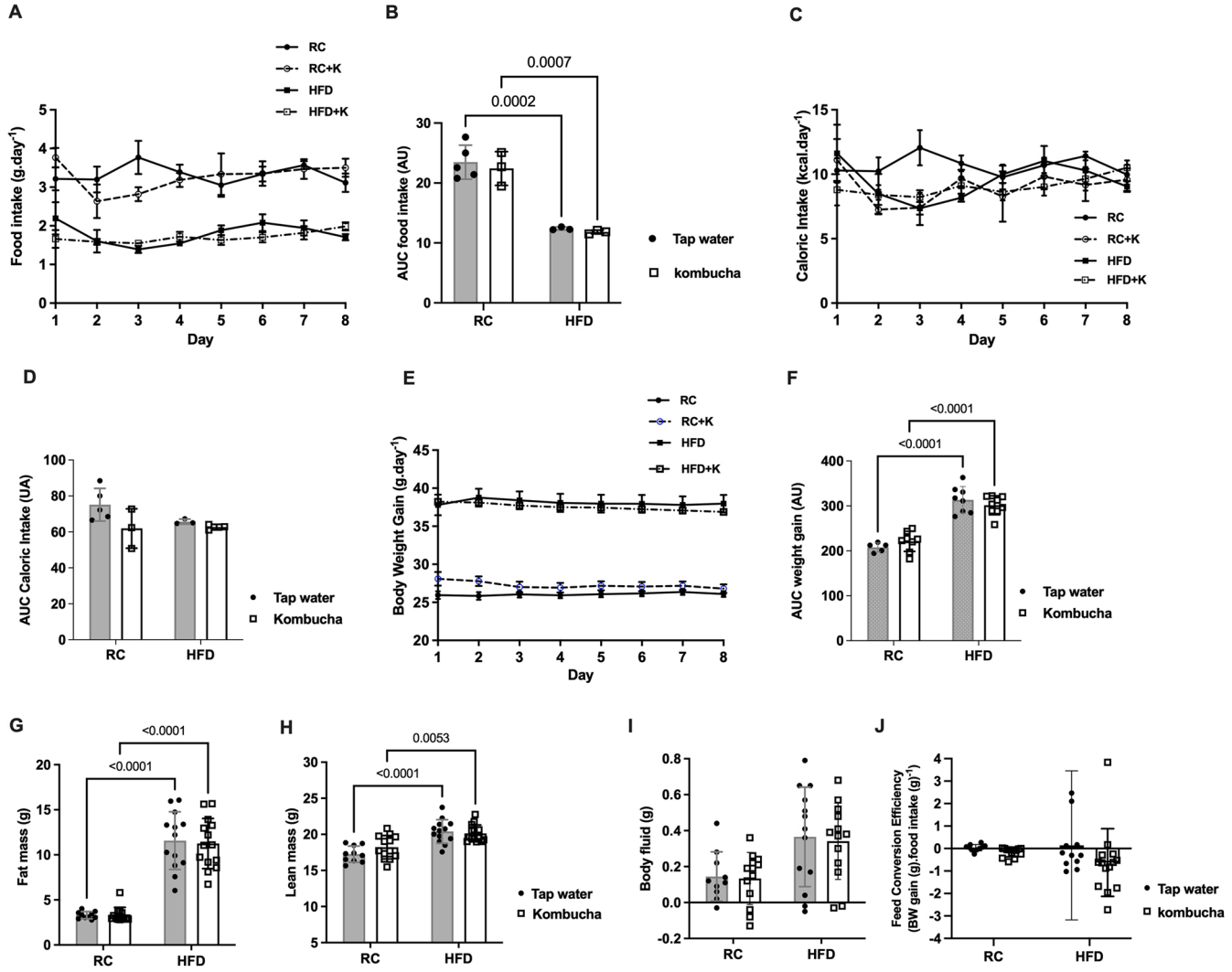

Fig. 1. Effect of kombucha administration in diet-induced obese mice on food and caloric intake, body mass and composition, and feed conversion efficiency. C57BL/6 J male mice were fed with either a high-fat diet (HFD) for ten weeks or regular chow (RC) and received through oral gavage once a day kombucha tea (RC+K; HFD+K) or tap water (RC; HFD) for nine days. (A, B) Food intake and AUC food intake; (C, D) Caloric intake and AUC Caloric intake; (E, F) body weight gain and AUC weight gain; (G) Fat mass; (H) Lean mass; (I) Body fluid; (J) Feed Efficiency during nine days of kombucha administration. Data are represented as mean ± SD; each isolated symbol, either circle or square, represents one sample from one mouse. Two-way ANOVA indicated statistical differences.

immunoblots were developed using the SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, Massachusetts, USA). The immunoblots were visualized using an Amersham TM Imager 600 and quantified using the open-access ImageJ software (NIH, Bethesda, MD, USA). The primary antibodies employed included IR (catalog number 3025 S, Cell Signaling, MA, USA) and pSer473AKT (catalog number 7985-R, Santa Cruz, USA). Protein loading into SDS-PAGE was normalized by staining the nitrocellulose membranes with Ponceau S.

2.5. Real-time polymerase chain reaction

Total RNA from the liver was extracted with Trizol reagent (Thermo Scientific) and reverse transcribed into cDNA using the High-Capacity cDNA kit (Applied Biosystems, Waltham, Massachusetts, USA). Gene expression was evaluated by real-time polymerase chain reaction (RT-PCR) using a Rotor-Gene Q (Qiagen, USA) and SYBR Green as the fluorescent dye (Platinum® SYBR ® Green qPCR Supermix UDG, Invitrogen, USA).

2.6. Enzymatic activity

Approximately 25 mg of the liver was homogenized in 500 μL of 50 mM Tris-HCl, 1 mM EDTA (pH 7.4), a protease inhibitor cocktail and 50 μL of 1% Triton X-100 (TX-100), and centrifuged at 17,925 × *g* at 4 °C for 10 min to separate the supernatant from the homogenate. Assays were performed from the homogenate diluted 10 × with homogenization buffer.

The assay for maximum PK activity [52] used buffer containing 100 mM KH_2PO_4, 10 mM $MgCl_2$, 80 mM KCl, 0.05% TX-100, 9 U LDH, 0.17 mM NADH and 5 mM ADP, added to 2 μL of homogenate. The reaction was started with 2 mM PEP, and absorbance readings were taken at 340 nm in a 96-well UV plate.

The assay for maximum GK activity [15] used buffer containing 75 mM Tris-HCl, 7.5 mM $MgCl_2$, 0.8 mM EDTA, 1.5 mM KCl, 4 mM β-mercaptoethanol, 0.05% TX-100, 0.4 mM NADP+ , 2.5 mM ATP and 1.4 U glucose-6-phosphate dehydrogenase (G6PDH), added to 20 μL homogenate. Next, 1 mM of glucose was added to start the reaction, and the absorbance was read at 340 nm.

The assay for maximum PFK activity [39] used assay buffer containing 50 mM Tris-HCl, 2 mM $MgSO_4$, 5 mM KCl, 0.05% TX-100, 2 U

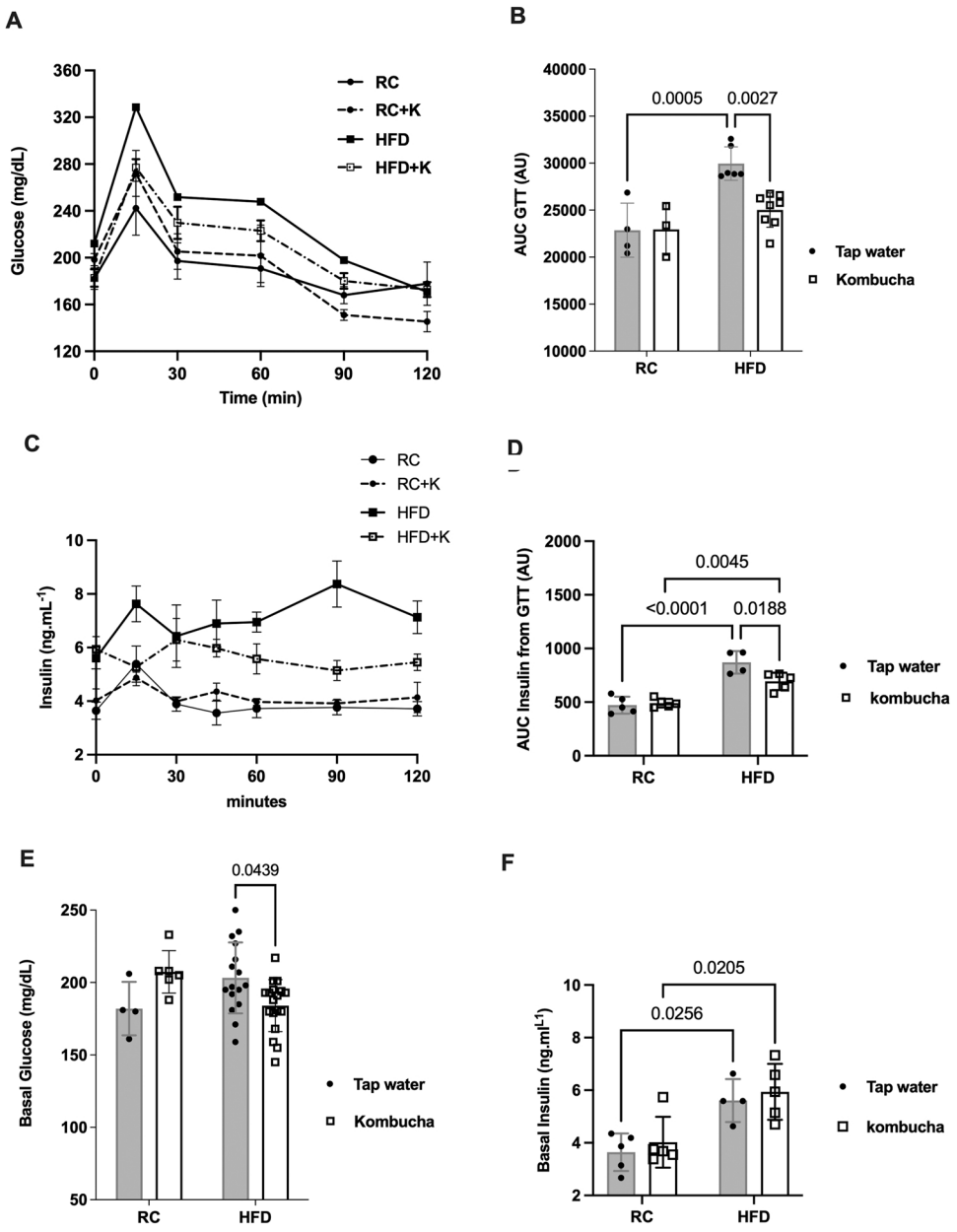

Fig. 2. Kombucha administration can improve glucose tolerance and insulin sensitivity in diet-induced obese mice. C57BL/6 J male mice were fed with HFD for ten weeks or RC and received through oral gavage once a day kombucha tea (RC+K; HFD+K) or tap water (RC; HFD) for nine days. (A) Six-hour fasted glucose blood levels, (B) time course of plasma glucose levels during intraperitoneal GTT; (C) Area Under the curve (AUC) of glucose during the GTT; (D) Insulinemia after six hours; (E) time course of plasma insulin levels during the GTT; (F) AUC of insulin during the GTT. Data are represented as mean ± SD. Each circle or square symbol within the scatter plots represents one sample from one mouse. Two-way ANOVA indicated statistical differences.

LDH, 4 U PK, 1 mM ATP, 0.2 mM NADH and 2 mM PEP, added to 10 μL of homogenate. The reaction was started with the addition of 3 mM fructose-6-phosphate (F6P), and the absorbance was read at 340 nm.

The citrate synthase activity was measured as described by Alp et al. (1976) in a reaction mixture containing 100 mM Tris-HCl, 1 mM MgCl2, 1 mM EDTA, 0.2 mM dithiobis (2-nitrobenzoic acid), 0.3 mM acetyl-CoA, and 0. 5 mM oxaloacetate (omitted in control), pH 8.1. The absorbance was read at 412 nm ($\varepsilon = 13.6\ \mu mol.mL^{-1}.cm^{-1}$).

The carnitine palmitoyl transferase (CPT-1) activity was measured as described by Bieber et. al. (1972) in a reaction mixture containing 60 mM Tris-HCl, 1.5 mM EDTA, 0.25 mM DTNB, 0.05% TX-100 (v/v), 0.035 mM palmitoyl-CoA and the homogenate (pH 8.1). The reaction was started with the addition of 1.25 mM L-carnitine, and absorbance was read at 412 nm.

The assay for maximum PEPCK activity [39] used a buffer containing 66 mM Tris-HCl, 1 mM $MnCl_2$, 1 mM phosphoenolpyruvate, 0.16 mM NADH, 1.54 mM IDP, 17 mM $NaHCO_3$, added to 20 μL of homogenate. The reaction was started with the addition of 12 U of malate dehydrogenase, and the absorbance was read at 340 nm.

The HCDH (3-hydroxy acyl CoA dehydrogenase) activity was measured as described by Lynen & Wieland (1955) in a reaction mixture containing 100 mM PBS, 0.45 mM β-NADH and 0.1 mM S-Acetoacetyl Coenzyme A (SAAC). The reaction was started with the addition of SAAC, and the absorbance was read at 340 nm.

The assay for BHADH activity used a buffer containing 91.1 mM Tris-HCl, 1 mM ATP, 0.262 mM NADH, 0.728 mM PEP, 10 mM Creatine, 5 mM KCl, 2 mM $MgSO_4$, 10 U LDH, and 0.4–0.06 U pyruvate kinase.

The enzyme activity results are expressed as μmol/min per gram of tissue.

2.7. Liver histology

2.7.1. Hematoxylin & eosin and Picrosirius staining

The liver was fixed in 10% formaldehyde solution for 8 h to evaluate liver histological parameters. The fixed samples were kept overnight in 70% alcohol. The following day they were dehydrated through a series of baths in 95% alcohol, 100% alcohol and xylene. After dehydration, the tissue samples were embedded in paraffin at 60 ºC. A microtome

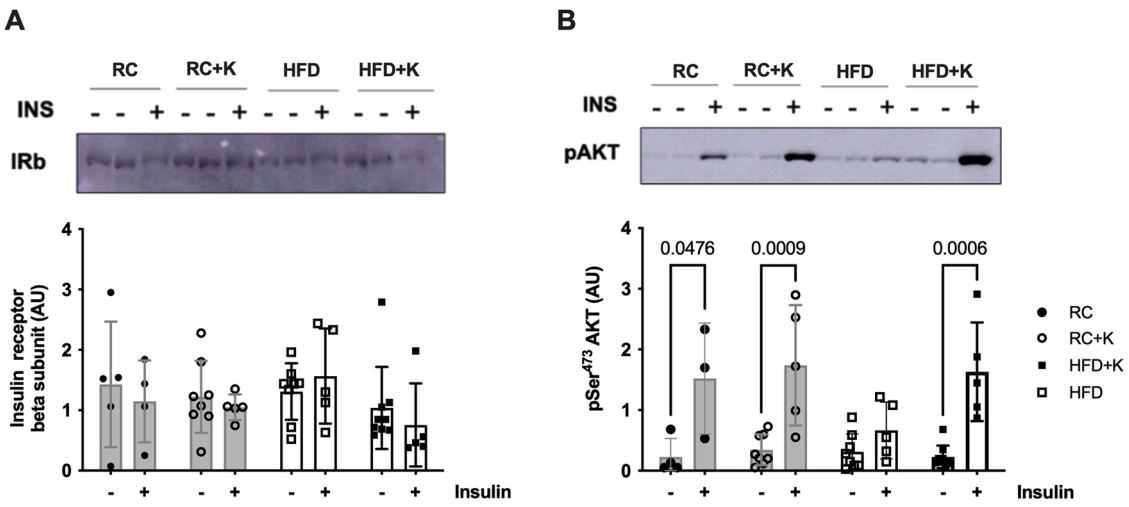

Fig. 3. Kombucha administration can recuperate the serine phosphorylation of AKT stimulated by acute insulin infusion in the livers of diet-induced obese mice. C57BL/6 J male mice were fed with HFD for ten weeks or RC and received through oral gavage once a day kombucha tea (RC+K; HFD+K) or tap water (RC; HFD) for nine days. (A) Typical immunoblotting with anti-insulin receptor beta subunit (IRb) antibody; (B) Typical immunoblotting with anti-phosphoserine 473 AKT antibody in liver samples from mice that received a bolus infusion of insulin (+) or not (-) 30 s previously the liver extraction (for details see the Methods section). Data are represented as mean ± SD. Each circle or square symbol within the scatter plots represents one sample from one mouse. Normalization was done by Ponceau staining. Two-way ANOVA indicated statistical differences.

(Zeiss, Jena, Germany) cut the samples into six-micron slices, and the slices were stained with hematoxylin and eosin (H&E). Hepatocyte morphology was evaluated, and the intracellular vacuoles were quantified. Twenty images were obtained from each animal with a Nikon DS-R1 digital camera coupled to a Nikon Eclipse Ti-U microscope (20 × magnification). The images were visualized using the NIS-Elements BR 3.1 software. The slices were also submitted to Picrosirius staining to identify collagen fibers.

2.7.2. Oil red O staining

The liver samples were embedded in tissue-tek (Thermo Scientific), placed in isopropanol alcohol, and frozen in liquid nitrogen. Twelve-micron slices were prepared using a cryostat (Micron H560, Thermo Scientific). Three slices from different parts of the samples were placed onto each slide, and two slides were prepared for each animal. Next, the slides were stained with Oil Red O (ORO) and Mayer's hematoxylin. Ten images from each animal were obtained on a microscope with 20 × objective magnification. The ImageJ program (NIH) was utilized to identify the ORO-stained area.

2.8. Statistical analysis

The results were analyzed using the GraphPad Prism version 9.3.1 (350) ® program (GraphPad Software, La Jolla, CA, USA). The minimum sample size per group for each parameter, defined as enough to analyze the distribution of samples, was determined using the D'Agostino and Pearson omnibus normality test recommended by the same software. All samples were evaluated for normal distribution and subjected to a two-way ANOVA followed by the post-hoc Tukey's multiple comparisons test. The results are expressed as the mean ± standard deviation (mean ± SD), and scatter plots were used to display all the samples used in each analysis (each sample corresponds to one mouse). We considered the "Rs" for animal usage in experimental sciences. Some analyses achieved reduction by using liver fragments from the same animals. Refinement was conducted under optimal bioterium conditions.

3. Results

3.1. Kombucha improves glucose tolerance without changes in body fat mass or caloric intake

The body weight and food consumption were monitored daily during the nine days of kombucha treatment following ten weeks of the feeding protocols. Despite the reduced HFD intake compared to the RC group (Fig. 1A, B), the caloric intake was similar between the groups (Fig. 1C, D) [for food intake, diet factor (F1,10) = 81.07, $p < 0.001$]. As expected, the HFD consumption increased total body mass (Fig. 1E, F) [diet factor, F (1,26) = 118.2, $p < 0.0001$], which was accompanied by increases in body fat to 300% and lean mass to almost 20%, with no change in fluid mass (Fig. 1G-I). Furthermore, the feed conversion efficiency during kombucha administration was not different between groups (Fig. 1J). Overall, kombucha supplementation had no impact on any of these factors.

Despite similar basal blood glucose levels in both RC and HFD mice (Fig. 2A) (interaction F (1,40) = 8.925, p = 0.0048), there was a clear glucose intolerance detected in awake 6-hour fasted HFD mice (Fig. 2B, C) [F (1,17) interaction factor = 6.229, p = 0.0231; diet factor = 20.64, p = 0.0003; kombucha factor = 5.830, p = 0.0273] with hyperinsulinemia (Fig. 2D, E) [AUC F(1,18) interaction factor = 3.260, p = 0.0877; diet factor = 57.13, $p < 0.0001$]. The kombucha treatment induced a 10% reduction in the glucose blood levels in the HFD group (Fig. 2A) (HFD: 203 ± 6 vs. HFD+K: 184 ± 4 g/dL, p = 0.0439), improved glucose tolerance, accompanied by reduced glucose-induced insulin secretion in the HFD mice (Fig. 2B, C, E, F), despite no alterations in basal insulinemia (Fig. 2D).

3.2. Kombucha improves insulin-induced AKT phosphorylation

Anesthetized mice received an acute insulin injection via the portal vein to evaluate insulin-induced AKT serine phosphorylation (Fig. 3). Despite no effect on insulin receptor protein expression (Fig. 3A), the HFD impaired the insulin-induced AKT serine phosphorylation. Kombucha restored this hepatic insulin action to a similar 7.5–8-fold increase in the insulin-induced AKT phosphorylation under basal conditions (without acute insulin infusion) compared to the RC groups (Fig. 3B) [F (3,39) interaction factor = 2.836, p = 0.0505; groups factor = 2.572,

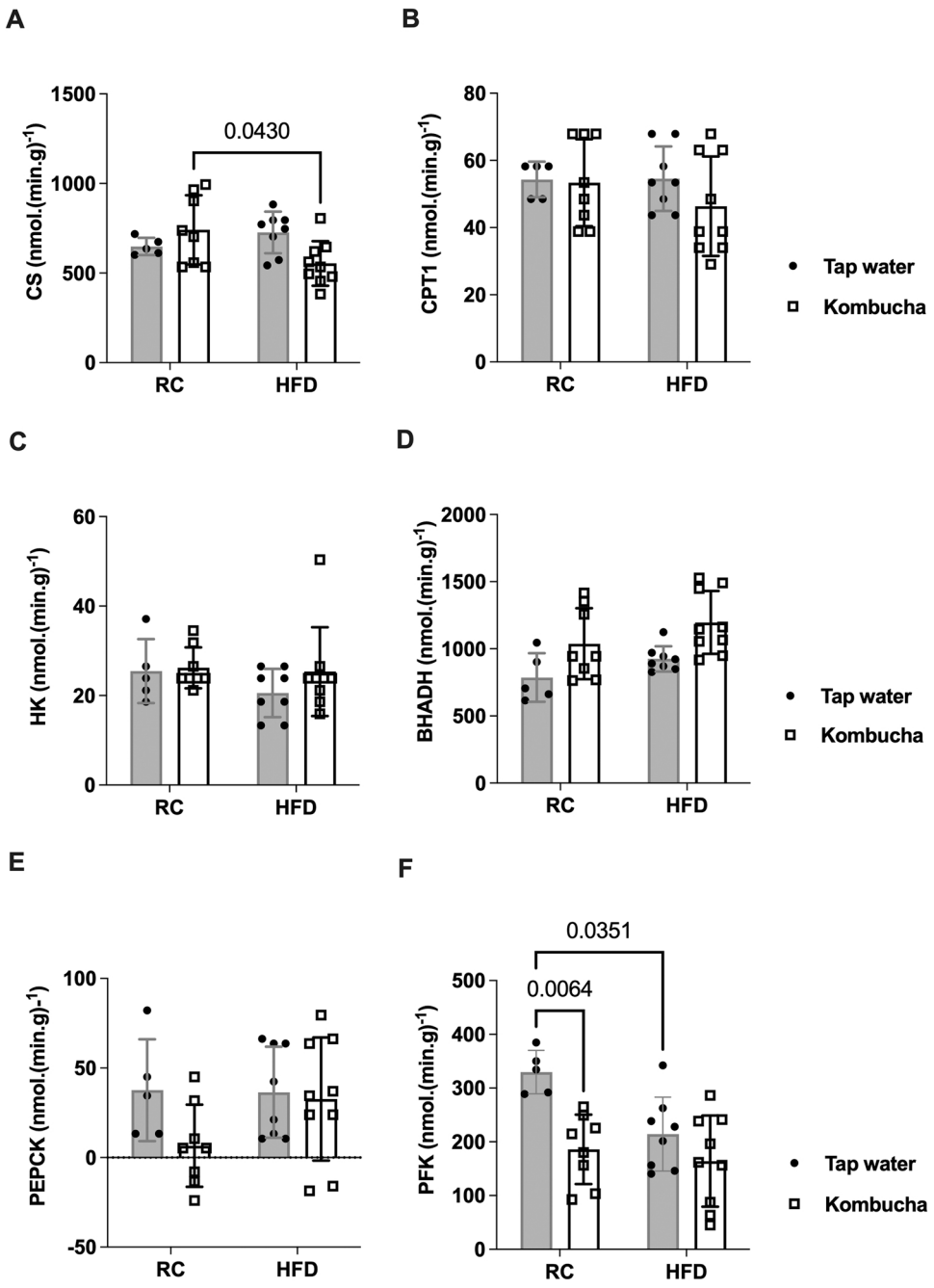

Fig. 4. Liver enzyme activity of the fatty acid and glycolytic pathway. C57BL/6 J male mice were fed with HFD for ten weeks or RC and received through oral gavage once a day kombucha tea (RC+K; HFD+K) or tap water (RC; HFD) for nine days. (A, B, D) Fatty acid enzymes pathway; (C, E, F) Glycolysis enzymes pathway. Data are represented as mean ± SD. Each circle or square symbol within the scatter plots represents one sample from one mouse. Two-way ANOVA indicated statistical differences.

$p = 0.0679$; F(1,39) insulin injection factor = 46.28, $p < 0.0001$].

3.3. Enzymatic activity

Neither the HFD nor kombucha affected in vitro hexokinase (HK), citrate synthase (CS), phosphoenolpyruvate carboxykinase (PEPCK), carnitine O-palmitoyl transferase 1 (CPT1), or 3-hydroxyacylCoA dehydrogenase (BHADH) enzymatic activities (Fig. 4A-E). In contrast, phosphofructokinase-1 (PFK) activity was reduced by almost half in the HFD mice compared to the RC group. The kombucha tea induced a similar reduction in the PFK activity in control mice but had no other impact on the HFD group (Fig. 4F) [F(1,6) diet factor = 8.806, $p = 0.0142$; kombucha factor = 13.80, $p = 0.0010$].

3.4. Inflammatory cytokine, lipogenesis enzyme, and bile acid pathway gene expression

The IL-1 and F4/80 gene expression levels were similar between the groups (Fig. 5A, C). As expected, TNF-alpha gene expression was upregulated nearly three-fold compared to RC mice (Fig. 5B) (RC = 1.5 ± 0.7 vs. HFD = 4.3 ± 0.7 arbitrary units, $p < 0.05$) [F (1,22) diet effect = 14.64, $p = 0.0009$; kombucha factor = 2.974, $p = 0.0986$]. There was also a 2.6-fold enhancement in the SREBP1 in the HFD mice compared to RC (C = 1.1 ± 0.2 vs. H = 3.0 ± 0.5, $p < 0.05$) [F (1,25) diet factor = 33.10, $p < 0.0001$]. The kombucha supplementation did not impact the expression of any of these genes.

On the other hand, the G-protein-coupled bile acid receptor (Gpbar1), also known as TGR5, gene expression was downregulated by nearly 50% in the HFD+K mice compared to RC+K (Fig. 5E) [F (1,26) diet factor = 7.518, $p = 0.0109$]. Additionally, the HFD+K group's

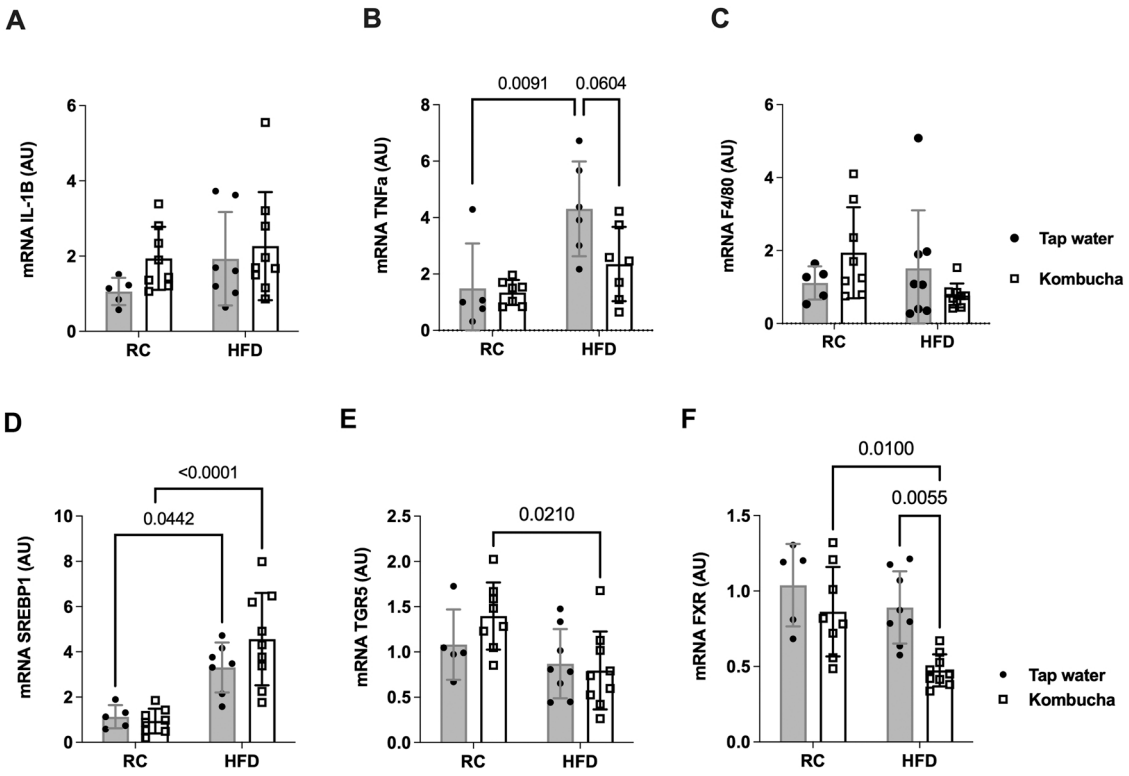

Fig. 5. Analysis of the expression of genes involved in proinflammatory, lipogenesis and bile acid pathways. C57BL/6 J male mice were fed with HFD for ten weeks or RC and received through oral gavage once a day kombucha tea (RC+K; HFD+K) or tap water (RC; HFD) for nine days. (A, B, C) Proinflammatory pathway; (D) Lipogenesis pathway; (E, F) Bile acids pathway. Data are represented as mean ± SD. Each circle or square symbol within the scatter plots represents one sample from one mouse. Two-way ANOVA indicated statistical differences.

farnesol X receptor (FXR) expression levels were also reduced by half compared to the RC+K and HFD groups (Fig. 5F) [F (1,26) diet factor = 9.467, p = 0.0099; kombucha factor = 11.54, p = 0.0022].

3.5. Kombucha reduces features of NAFLD related to obesity

The histological pattern of NAFLD in the HFD mice is shown in Fig. 6A. The H&E and ORO staining revealed hepatocyte vacuolization in the HFD mice (Fig. 6A). This observation was further confirmed by a 20-fold increase in neutral fat accumulation in the HFD groups compared to RC mice (Fig. 6B; 39 ± 1% vs. 2 ± 0.4%, p < 0.0001). Interestingly, hepatocyte fat accumulation was reduced by 25% in the HFD+K mice (Fig. 6B; 29 ± 3%, p = 0.0010) [F (1,16) interaction = 8.806, p = 0.0001; diet factor = 489.0, p < 0.0001; kombucha factor = 14.54, p = 0.0015]. Furthermore, there was a 1.7-fold increase in the perivascular area of collagen deposition in the HFD mice compared to the RC group (Fig. 6C; 1.2 ± 0.09% vs. 0.7 ± 0.06%, p = 0.0003). Notably, the HFD+K group had collagen deposition levels similar to the RC group (0.7 ± 0.02%) [F (1,19) interaction = 7.017, p = 0.0158; diet = 23.00, p = 0.0001; kombucha = 21.81, p = 0.0002]. The four groups had similar blood AST and ALT activities (data not shown).

4. Discussion

In the present study, we evaluated the effect of kombucha on glucose tolerance, insulin sensitivity, body mass composition, and NAFLD in male mice fed an HFD. The results indicated improved glucose tolerance after nine days of kombucha tea supplementation in diet-induced obese mice without any detectable alterations in body composition. Furthermore, kombucha reduced the presence of intra hepatocyte lipid droplets, collagen deposition in the liver's perivascular spaces, and hepatic FXR gene expression.

As expected, the HFD promoted glucose intolerance associated with hyperinsulinemia and impaired AKT serine phosphorylation in the liver following acute insulin administration, thus decreasing the activation of the intracellular insulin pathway and confirming insulin resistance in the livers of HFD mice. [1] recently demonstrated that a prebiotic treatment could reduce adiposity but not body mass in an obese animal model. Moreover, it has been reported that probiotics attenuate adipose tissue lipid deposition by improving insulin sensitivity and reducing tissue inflammation, thereby ameliorating fat body mass and associated metabolic disorders [50].

Studies have shown that an HFD promotes intestinal dysbiosis, at least partially, by shifting the bacterial phylotype [16]. For example, some bacteria release lipopolysaccharides (LPS), a membrane endotoxin, into the intestinal lumen, triggering gut inflammation. LPS can penetrate intestinal barriers and enter the bloodstream, promoting systemic tissue inflammation and insulin resistance [7,38]. Other studies have demonstrated that prebiotics and probiotics reduce fasting blood glucose and improve insulin sensitivity in obese animal models and humans with type 2 diabetes [14,23]. Furthermore, prebiotics and probiotics were reported to reduce intestinal gram-negative bacteria, attenuating intestinal and systemic inflammation [44]. Furthermore, a previous study revealed a relationship between NAFLD and metabolic changes and inflammatory processes, with the detection of proinflammatory cytokines, which is consistent with our results showing upregulated SREBP-1 and TNF-alpha gene expression levels in the livers of the HFD mice [32].

Increased collagen deposition in the perivascular spaces of the liver, a characteristic feature of the progression from NAFLD to NASH, was observed in the HFD group. Interestingly, the probiotic kombucha effectively reduced lipid and collagen deposition in hepatocytes and downregulated the gene expression of the proinflammatory cytokine TNF-alpha. Other studies evaluating the anti-inflammatory properties of

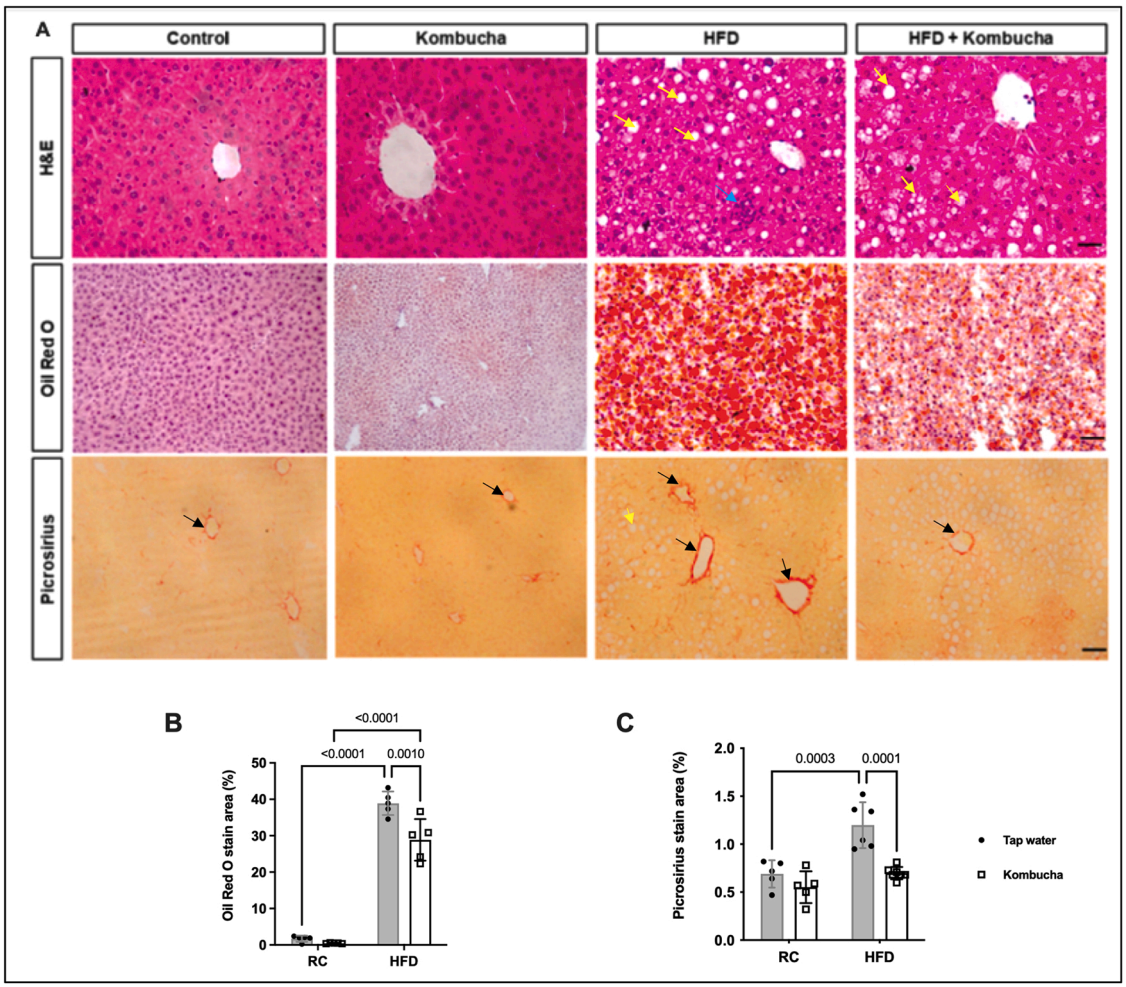

Fig. 6. Kombucha administration reduces intra hepatocyte lipid deposition and liver perivascular collagen deposition in diet-induced obese mice C57BL/6 J male mice were fed with HFD for ten weeks or RC and received through oral gavage once a day kombucha tea (RC+K; HFD+K) or tap water (RC; HFD) for nine days. (A) Representative photomicrographs of liver samples stained with Hematoxylin & Eosin (HE), Oil Red O (ORO), and Picrosirius red from all groups. All imagens correspond a 40X magnification. (Yellow arrows indicate cellular inflammatory infiltration, black arrows indicate lipid droplets, and blue arrows indicate collagen deposition in the perivascular areas). (B) Percentage of ORO area, and (C) Proportion of collagen deposition staining in perivascular areas. Each circle or square symbol within the scatter plots represents the mean data of five analyzed fields from one mouse. Two-way ANOVA indicated statistical differences.

probiotics also reported downregulated proinflammatory cytokine expression (e.g., TNF-alpha, IL1-B and IL-6) and a reduction in hepatic triglycerides, which was associated with attenuated hepatic steatosis [28,50].

[4] showed that prebiotics and probiotics promoted a reduction in the activity of liver enzymes such as ALT and AST in NAFLD patients, improving liver damage and serum triglyceride concentrations. The positive effects of kombucha were also demonstrated in a db/db mouse model, reducing steatosis and improving hepatic lipid metabolism [25, 26].

The reduced PFK activity in the livers of the HFD mice indicates reduced glycolysis mediated by the upregulation of SREBP-1 gene expression. SREBP is also responsible for the transcriptional activation of genes involved in fatty acid and triacylglycerol formation; processes collectively called lipogenesis [21]. This glycolytic enzyme catalyzes the phosphorylation of fructose-6-phosphate to fructose 1,6 bisphosphate, a central step in glycolysis, and its reduction increases the G6-P/F1, 6-bisphosphate ratio and inhibits the hepatic glycolytic pathway. Consequently, reduced PFK activity leads to the conversion of F6P to F 1.6 P, directing the G6P (F6P) towards glycogen and pentose synthesis. Notably, the glucokinase isoform in hepatocytes is not inhibited by its product, unlike the hexokinase in other tissues.

It is important to point out that the pentose pathway has essentially two products, one generating NADPH and ribose and the other involving the interconversion of pentose-phosphates to glycolysis intermediates (e.g., glyceraldehyde-3-phosphate) [31]. Thus, we can speculate that attenuated PFK activity could increase de novo lipogenesis and hepatic cholesterol and triglyceride production, contributing to the initial step of NAFLD development, hepatic steatosis. Interestingly, kombucha tea supplementation alone reduced PFK activity; however, it did not induce liver steatosis and reduced NAFLD in the HFD group. In this sense, it is plausible that the kombucha establishes a balance among reduced glycolysis, enhanced lipogenesis, and increased liver lipolysis. This possibility requires further investigation.

Concerning bile acids, these steroid acids are involved in regulating liver homeostasis, which can contribute to liver disease. Several bile acid receptors, including farnesol X receptor (FXR), Takeda G-coupled protein receptor 5 (TGR5; GPBAR1, M-BAR), pregnane X receptor (PXR), sphingosine-1-phosphate receptor 2 (S1PR2) have been identified. Notably, it has been shown that TGR5 and FXR receptor agonists can reduce hepatic steatosis [10]. Previous studies reported that FXR activation improves liver lipid and glucose metabolism, energy metabolism, and inflammation [2,12,13,33]. Herein, kombucha tea supplementation downregulated FXR and TGR5 gene expression in HFD mice but improved the analyzed NAFLD parameters. Thus, there must be other intracellular pathways involved in this response.

Table 1
Primer sequences.

TNF-alfa	sense 5′TCTTCTCATTCCTGCTTGTGGC3′
	anti-sense 5′CACTTGGTGGTTTGCTACGAGG3′
B2M	sense 5′CCCCACTGAGACTGATACATACG3’
	anti-sense 5′CGATCCCAGTAGACGGTCTTG3′
SREBP-1	sense 5′CAGTCACCAGCTTCAGTCCAGG3′
	anti-sense 5′CTGCTCAGGTCATGTTGGAAACC3′
IL 1-β	sense 5′GGCAGCTACCTGTGTCTTTCCC3′
	anti-sense 5′ATATGGGTCCGACAGCACGAG
F4/80	sense 5′CCTGAACATGCAACCTGCCAC3′
	anti-sense 5′GGGCATGAGCAGCTGTAGGATC3’
TGR5	sense 5′CAGTCTTGGCCTATGAGCGT3’
	anti-sense 5′CTGCCCAATGAGATGAGCGA3′
FXR	sense 5′GGCTGCAAAGGTTTCTTCCG3′
	anti-sense 5′CAGCCAACATCCCCATCTCT3′

In conclusion, the present study demonstrated that kombucha tea could attenuate NAFLD and its glycemic parameters in HFD-induced obese mice. It is important to point out that alterations were not accompanied by body weight changes. Thus, the results are likely related to reduced liver and systemic inflammation and improved glucose tolerance. Since our study provides evidence demonstrating kombucha tea’s ability to modulate metabolic parameters, further investigation is required to investigate the potential link between this fermented beverage, the glucose pathway, lipolysis, and gut microbiota dysbiosis.(Table 1).

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Conflict of interest statement

The authors declare no competing interests.

Data Availability

Data will be made available on request.

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Author Contributions

The author(s) have made the following declarations about their contributions. GVM and CROC conceived and designed the experiments. GVM, LCCA, GM, and SLM performed the experiments. GVM and CROC analyzed the data. CROC contributed reagents/materials/analysis tools. GVM, LCCA and CROC wrote the paper.

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