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A flavonoid-rich extract of bergamot juice improves high-fat diet-induced intestinal permeability and associated hepatic damage in mice

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Abbreviations: BJe, flavonoid-rich extract of *Citrus bergamia* (bergamot) juice; BJ, bergamot juice; LPS, lipopolysaccharides; HFD, high-fat diet; NAFLD, non-alcoholic fatty liver disease; TLR-4, toll-like receptor 4; FITC, fluorescein isothiocyanate; GI, gastrointestinal tract; BW, body weight; ALT, alanine transaminase; TG, triglycerides; MCP-1, monocyte chemotactic protein-1; JNK2, c-Jun N-terminal kinases 2; MyD88, myeloid differentiation factor 88; IKKα, inhibitor of Kappa-B kinase α; TNF-α tumor necrosis factor α; F4/80, mucin-like hormone receptor-like 1; HSC-70, heat shock cognate protein 70; JNK1/2, c-Jun N-terminal kinases; IL-6, interleukin-6; TGF-β1, transforming growth factor-beta 1; COL 1A1, collagen type I alpha 1; ZO-1, zonula occludens-1; iNOS, inducible nitric oxide synthase; MAPK, mitogen activated kinases; H&E, hematoxylin and eosin; NAS, NAFLD activity score.

Abstract

Consumption of high-fat diets (HFD) is a contributing factor to obesity, insulin resistance and non-alcoholic fatty liver disease (NAFLD). Several studies suggested a protective role of bioactives present in *Citrus* fruits against the above mentioned chronic metabolic conditions. In this study, we evaluated if a flavonoid-rich extract of *Citrus bergamia* (bergamot) juice (BJe) could inhibit HFD-induced intestinal permeability and endotoxemia, and through this mechanism, mitigate the associated hepatic damages in C57BL/6J mice. After 12 weeks on the treatments, HFD consumption caused high body weight (BW) gain, hyperinsulinemia, hyperglycemia, and dyslipidemia, which were mitigated by BJe (50 mg/kg BW) supplementation. Furthermore, supplementation with BJe prevented HFD-induced liver alterations, including increased plasma alanine aminotransferase (ALT) activity, -increased hepatic lipid deposition, high NAS score, and fibrosis. Mice fed the HFD for 12 weeks showed: i) a decrease in small intestine tight junction proteins levels (ZO-1, occludin, and claudin-1); ii) increased intestinal permeability; and iii) endotoxemia. All these adverse events were mitigated by BJe supplementation. Linking the capacity of BJe to prevent HFD-associated endotoxemia, supplementation with this extract decreased the HFD-induced overexpression of hepatic TLR-4, downstream signaling pathways (MyD88, NF-κB and MAPK), and the associated inflammation, evidenced by increased MCP-1, TNF-α, IL-6, iNOS, and F4/80 levels. Overall, we suggest that BJe could mitigate the harmful consequences of western style diet consumption on liver physiology by protecting the gastrointestinal tract from permeabilization and associated metabolic endotoxemia.

Keywords: Bergamot, *Citrus bergamia,* intestinal permeability, endotoxemia, high-fat diet, liver inflammation.

1. Introduction

The gastrointestinal tract (GI) is essential to sustain human health. One of the GI tract main functions is to act as a barrier that prevents the passage of food and bacterial toxins into the circulation. In fact, intestinal permeabilization is considered as a major event in the development of Western-style diets- and obesity-associated diseases [1]. A dynamic protein complex, the tight junction (TJ), regulates the permeability of the gut barrier. TJs regulate the paracellular transport of water and ions and prevent the passage of luminal toxins and unwanted food components into the circulation [2]. Disruption of TJ proteins and damage to the intestinal barrier caused by the consumption of high-fat diets (HFD), lead to the paracellular transport of lipopolysaccharides (LPS) to the circulation, which results in metabolic endotoxemia and systemic inflammation [3, 4]. LPS transported to the circulation target different tissues, including the liver (gut-liver axis), leading to the development of metabolic diseases, including non-alcoholic fatty liver disease (NAFLD) [5]. NAFLD is the most common cause of chronic liver illness with a global prevalence of about 25% in the general population, while it is estimated to be more than 90% in obese individuals [6]. Clinically, NAFLD is characterized by lipid deposition in hepatocytes (≥ 5%), especially triglycerides (TG), and an involving a cluster of other conditions that range from steatosis, non-alcoholic fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH) that can cause hepatic cirrhosis and development of liver inflammation and ultimately hepatocarcinoma [7-9] .

In term of mechanisms, LPS binds to Toll-like receptor 4 (TLR-4), initiating the activation of signaling pathways, e.g. mitogen activated kinases (MAPK) and NF-κB leading to the production of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). Hepatic upregulation of TLR-4 consequent to exposure to high concentration of LPS, and the activation of the downstream signaling cascades, can trigger liver inflammation and fibrosis, leading to hepatic damage and NAFLD [10].

Although the switch from HFD to a normo-caloric diet is the first step to counteract obesity and associated comorbidities [11], the consumption of fruits and vegetables has been shown to mitigate obesity-associated diseases [12-15]. Thus, dietary strategies that can prevent and control metabolic endotoxemia could help mitigate HFD-associated hepatic inflammation and related diseases. *Citrus* fruits are among the most widely cultivated fruits worldwide, which, together with their juices, represent a major source of dietary flavonoids. Their health properties have been extensively studied, especially for their anti-inflammatory [16, 17], antidiabetic [18], neuroprotective [19,20], anti-cancer [21], cardio-protective [22] , and anti-aging [23] properties. Among *Citrus* fruits, *Citrus bergamia* Risso (Bergamot) is a small tree from the Rutaceae family, mostly cultivated in the Calabria region (Italy) for the extraction of its essential oil, largely employed in the fragrance industry. Bergamot juice (BJ), considered just a by-product until last decade, has been recently re-evaluated for its anti-microbial [24] and anti-proliferative[25] properties. Furthermore, its flavonoid-rich extract (BJe) displayed a wide range of biological activities, including anticancer [26], anti-inflammatory [23, 27], hypolipemic [28], along with a protective effect against metabolic syndrome [29, 30]. However, the potential effects and the mechanisms of action of BJe on HFD-induced alteration of the gut-liver axis, with a focus on the beneficial effects of BJe on HFD-induced intestinal permeability and associated hepatic damage has not been investigated. Thus, considering the above evidence, and the major role of metabolic endotoxemia in the development of liver diseases, this work investigates if BJe consumption could mitigate HFD-induced endotoxemia and associated hepatic inflammation and NAFLD in mice.

2. Material and methods

2.1 Materials

Plasma and liver cholesterol and TG concentrations were determined using kits purchased from Wiener Lab Group (Rosario, SF, Argentina). Plasma insulin levels were measured using kits

purchased from Crystal Chem Inc. (Downers Grove, IL, USA). Endotoxin levels were investigated using a kit from Abbexa (Cambridge, UK). Antibodies for β-actin (#12620), monocyte chemotactic protein-1 (MCP-1; #2029), c-Jun N-terminal kinases 2 (JNK2; #9258), myeloid differentiation factor 88 (MyD88; #4283), inhibitor of Kappa-B kinase α (IKKα; #2682), phospho (Ser176/180)-IKKα/β (p-IKKα/β; #2697), were obtained from Cell Signaling Technology, (Danvers, MA, USA). Antibodies for tumor necrosis factor α (TNFα; sc-8301), mucin-like hormone receptor-like 1 (F4/80; sc-25830), heat shock cognate protein 70 (HSC-70; sc-1059), inducible nitric oxide synthase (iNOS; sc-7271), phospho c-Jun N-terminal kinases (Thr183/Tyr185)-JNK1/2 (pJNK1/2; sc-6254), interleukin-6 (IL-6; sc-32296), toll-like receptor 4 (TLR4 sc-293072), transforming growth factor beta 1 (TGF-β1; sc-130348), collagen type I alpha 1 (COL 1A1; sc-293182) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sc-25778) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for zonula occludens-1 (ZO-1; 33-9100), occludin (71-1500) and claudin-1 (71-7800) were purchased from Invitrogen (Carlsbad, CA, USA). PVDF membranes were obtained from Bio-Rad (Hercules, CA, USA). The enhanced chemiluminescence (ECL) Western blotting system was from Thermo Fisher Scientific Inc. (Piscataway, NJ; USA). Hematoxylin and Eosin solutions were purchased from Thermo Fisher Scientific Inc. (Piscataway, NJ). Plasma glucose and ALT kits, fluorescein isothiocyanate (FITC)-dextran (4 kDa), and all other chemicals were purchased from Sigma-Aldrich Co (St. Louis, MO, USA).

BJe was a generous gift from the Herbal & Antioxidant Derivatives Srl (Bianco, Reggio Calabria, Italy), which was obtained as previously described [31].

2.2 Chemical characterization of BJe

The chemical composition of BJe was investigated through HPLC analysis, performed by a Fast 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA). Two-solvent gradient of water and acetonitrile were employed to elute the sample, which was injected in a volume

of 2 µl. The flow rate was 3 ml/min, and the column ZORBAX Eclipse XDB-C18 column (50 mm) was maintained at 35 °C. The Diode Array Detector (DAD) detector was monitored at 280 nm. Flavonoid pure standards were purchased from Sigma-Aldrich (Burlington, MA, USA).

2.3 Animals and animal care

All experiments were performed in accordance with the guidelines for the care of laboratory animals as outlined in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. All procedures were administered under the auspices of the Animal Resource Services of the University of California, Davis. Experimental protocols were approved before implementation by the University of California, Davis Animal Use and Care Administrative Advisory Committee. Mice were housed under controlled conditions of temperature (21°C ± 1°C) and light (12 h light /12 h dark cycle) with free access to water and food. As shown in Fig. 1 A, forty C57BL/6J mice (male; 20–25 g) were acclimated for one week to the control diet and subsequently randomly divided in four different dietary groups (10 mice/group) that were fed for 12 weeks: i) a control diet (C) (containing approximately 10% total calories from fat) (Supplementary Table 1); ii) the C diet supplemented with BJe (50 mg/kg body weight, BW) (BJe); iii) a HFD (HF) (containing approximately 60% total calories from fat (lard) (Supplementary Table 1); and iii) the HFD supplemented with BJe (50 mg/ kg BW) (HF-BJe). The dose of 50 mg of BJe/kg BW was chosen based on a previous study, which demonstrated beneficial effects of bergamot polyphenol fraction on NAFLD outcomes in rats fed a cafeteria diet [32]. Considering the allometric scaling to translate the dose to mice to human [33, 34], the amount of BJe consumed by the mice in the current study, corresponds to a human equivalent intake of 284 mg bergamot extract, for a 70 kg adult.

BJe was added to the C diet and HFD to achieve the concentration of 50 mg BJe/BW. In order to prevent any potential BJe degradation and fat oxidation, all diets were prepared every week,

adjusting the quantity of extract according to changes in mice BW and food intake. The pellets were made, dried, and stored at -20°C until use. BW and food intake were recorded weekly. After 12 weeks on the dietary treatments, mice were euthanized by cervical dislocation, blood was collected from the submandibular vein into EDTA tubes, and plasma isolated by centrifugation at 3.000×g for 10 min at room temperature. Dissected tissues from liver and small intestine (ileum) were flash frozen in liquid nitrogen and then stored at -80 °C until further analysis. A consistent lobe of the liver was collected for histological analysis.

2.4 Determination of liver TG content

Liver TG content was evaluated as previously described by Daveri et al [35]. Briefly, a 100 µl aliquot of 10% (w/v) liver homogenate was mixed with 300 µl of a KOH (30% w/v)/ethanol (1:2, v/v) solution and evaporated overnight at 55 °C. The following day, 1 ml of 50% (v/v) ethanol was added, and samples centrifuged at 10.000×g for 5 minutes at room temperature. Then, 200 µl of supernatant were added to 215 µl of 1 M $MgCl_2$ and left in ice for 10 minutes. After centrifugation at 10.000×g for 5 minutes at room temperature, 10 µl of the supernatant were employed to quantify liver TG amount using the TG Color GPO/PAP AA enzymatic triglyceride kit (Wiener Lab).

2.5 Histology

Liver samples were fixed in neutralized paraformaldehyde solution (4% w/v) overnight. Samples were subsequently washed in phosphate buffer saline (PBS) solution, dehydrated, and embedded in paraffin. Hematoxylin and Eosin (H&E) staining was carried out in tissue sections of 5 µm thickness positioned on glass slides, following standard procedures. Sections were examined using an Olympus BX51 microscope (Olympus America Inc., Center Valley, PA). The researcher was blinded to identify all the samples. To ensure blind quantification of all

samples, after the staining with H&E, the name of each slide was covered, and randomly numbered before acquiring the pictures. After the quantification was completed, the slides were unblinded and the statistical analysis was performed. The hepatic histological analyses were performed using the NAS, as described by Liang et al [36]. Four fields per animal, selected randomly, were analyzed using NIH Image J software.

2.6 Western blot analysis

Both liver and ileum samples were homogenized, and protein concentration was measured using the Bradford method. Aliquots of total homogenates containing 15–30 μg of proteins were denatured with Laemmli buffer, separated through 7.5-12.5% SDS-polyacrylamide gel electrophoresis and electrotransferred to PVDF membranes. These were blocked in 5% (w/v) non-fat milk for 1 h and subsequently incubated overnight at 4 °C in the presence of the corresponding primary antibody (1:1000 dilution). After incubation for 90 minutes at room temperature with the HRP-conjugated secondary antibodies (1:10.000 dilution), the immune complexes were detected by enhanced chemiluminescence through a Phosphoimager 840 (Amersham Pharmacia Biotech. Inc., Piscataway, NJ, USA).

2.7 Intestinal permeability

Intestinal permeability was evaluated after 8 weeks on the different diets as previously described by Cremonini et al [37]. Briefly, after 4 hours of fasting, mice were gavaged with FITC-dextran (4 kDa; 200 mg/kg BW). One hour and a half later, blood was collected from the submandibular vein and serum was isolated by centrifugation at 3.000×g for 10 minutes at room temperature. Aliquots of serum (20 μl) along with a standard curve of FITC-dextran were plated in 96-well plates and diluted to 200 μl with NaCl 0.9% (w/v). Fluorescence was detected at λ_{ex}: 485 nm and λ_{em}: 520 nm employing a microplate spectro-fluorometer (Wallac 1420 VICTOR2™, PerkinElmer Life Science, Waltman, MA, USA).

2.8 Statistical analysis

Data were tested for normal bell-shaped curve distribution using the Shapiro-Wilk test. Subsequently, data were analyzed using a one-way ANOVA or repeated measures ANOVA to calculate the significant differences among the groups' treatments. In detail, data from the Western blots were analyzed by one-way analysis of variance (ANOVA) using Statview 5.0 (SAS Institute Inc., Cary, NC, USA). The Fisher Least Significance Difference (LSD) test was used to examine differences between group means. Regarding the BW results, data were analyzed using repeated measures ANOVA. A p value <0.05 was considered statistically significant. Data are shown as mean ± standard error of the mean (SEM).

3. Results

3.1 Quali-quantitative composition of BJe

The quali-quantitative composition of BJe was determined as described in Methods and is shown in Table 1. The analysis of the main flavonoids present in BJe used in this study confirmed similar chemical composition compared to the previously reported by Ferlazzo et al [38]. The principal flavonoids present in the BJe utilized in this study are: naringin (114 mg/g), neoeriocitrin (109 mg/g), neohesperidin (95 mg/g), brutieridin (46 mg/g), and melitidin (24 mg/g).

3.2 Animal outcomes and effects of BJe supplementation on HFD-altered metabolic parameters

The daily food intake of mice in the HF and HF-BJe groups was significantly lower compared to the C and BJe mice. Throughout the study, the caloric intake for animals fed the control diet and the HFD was similar (14.4 and 14.8 kcal/g/d, respectively). Starting at week 2 and throughout the following weeks, HF mice showed a significantly increased BW gain, compared

to all the other dietary groups (Figure 1B). After 12 weeks on the treatments, HF mice showed an 80% higher final BW compared to the control group, while the final BW of HF-BJe mice was 14% lower than for HF mice (Table 2). Furthermore, BJe supplementation decreased HFD-induced subcutaneous and visceral fat accumulation (Table 2).

12-week HFD consumption caused alterations in glucose homeostasis and lipid metabolism. Plasma glucose and insulin levels were 32% and 183% higher in the HF group compared to C mice, respectively (Table 2). Supplementation of HFD-fed mice with BJe caused a 14% and 33% reduction of glucose and insulin plasma levels compared to HF mice (Table 2). Mice fed the HFD showed increased plasma total cholesterol and TG (44% and 85%, respectively) compared to the C group (Table 2), which were mitigated by supplementation with BJe (reduction of 14% and 15%, respectively) (Table 2). These outcomes highlight the capability of BJe to attenuate the BW gain, hyperglycemia, hyperinsulinemia, and hyperlipemia caused by chronic HFD consumption.

3.3 BJe supplementation mitigates HFD-induced liver steatosis and fibrosis

One of the main factors contributing to the development of fatty liver disease is the consumption of HFDs[39]. Thus, we next investigated the effects of BJe supplementation on HFD-induced steatosis and hepatic damage. Liver weight was about 20% lower in mice fed the HFD supplemented with BJe compared to HF mice (Table 2). In addition, plasma alanine aminotransferase (ALT) activity, measured as a marker of liver damage, was 33% higher in the HF group compared to the C group, and 55% lower in HF-BJe mice (Table 2). Hepatic damage derived from hepatic lipid accumulation can lead to the development of NAFLD. Thus, we next assessed hepatic TG accumulation and hepatic structure alterations using H&E staining. After 12 weeks on the diets, liver TG content was 97% higher in the HF compared to the C group. Supplementation with BJe prevented HFD-induced hepatic TG accumulation (Fig. 2B).

HFD-triggered hepatocellular injury can lead to microvescicular steatosis and hypertrophy, which are key clinical features in the development of NAFLD [40, 41]. After H&E staining, parameters of hepatocytes damage were evaluated using the NAS score (Fig. 2C). The NAS score was significantly higher in mice fed the HDF compared to all the other dietary groups (Fig. 2C), indicating that BJe supplementation could prevent HFD-induced liver injury and steatosis. Because of the important observed increases of all markers of liver injury, we next evaluated liver fibrosis by measuring TGF 1β and COL 1A1 protein levels by Western blot. The content of both proteins was high (48%) in HF liver compared to the C group. Mice from the HF-BJe group had lower TGF 1β and COL 1A1 protein levels (29% and 34%, respectively) compared to HF mice (Fig. 2D). All together, these data indicate that BJe can protect against liver steatosis, inflammation and fibrosis induced by HFD consumption.

3.4 BJe supplementation mitigates HFD-induced intestinal permeabilization and endotoxemia

Consumption of HFD can lead to alterations of enterocyte TJs and to intestinal permeabilization. Increased intestinal permeability can lead to the paracellular transport of luminal LPS to the circulation. Increased circulating LPS, called metabolic endotoxemia, can lead to systemic and tissues inflammation [3, 42]. Intestinal permeability was evaluated after 8 weeks on the corresponding diets by measuring the paracellular transport of FITC-dextran (4 kDa). FITC-dextran transport was 2-fold higher in the HF mice compared to the C group, which was not observed in HF-Bje mice (Fig. 3A). Consistently, plasma LPS levels were 37% higher in the HF group compared to the C group (Fig. 3B). In HFD-fed mice supplemented with BJe, plasma LPS concentration was significantly lower (43%) compared to the HF group (Fig. 3B). These data suggest that daily BJe supplementation mitigates endotoxemia by mitigating HFD-induced intestinal permeabilization.

3.5 BJe supplementation improves HFD-induced decreased tight junction protein levels in the ileum

Intestinal permeability is caused by alterations of TJ structure and function. We next evaluated by Western blot the ileal levels of major TJ protein components, i.e. claudin-1, occludin and ZO-1 After 12 weeks on the HFD, a significant reduction of ZO-1, occludin and claudin-1 protein levels (49%, 41% and 48%, respectively; Fig. 3C) were observed in the HF compared to the C group. Mice fed the HFD and supplemented with BJe showed an increase in TJ proteins levels compared to the HF group (84%, 111% and 134%, respectively; Fig. 3C). Results suggest that the observed increase in intestinal permeabilization associated by HFD consumption and mitigated by BJe supplementation is in part due to variations in TJ protein expression.

3.6 BJe supplementation modulates HFD-induced hepatic upregulation of TLR-4 and its downstream signaling pathway

LPS binds to the TLR4 receptor and activates pro-inflammatory signaling cascades including myeloid differentiation primary response gene 88 (MyD88), nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK). Mice fed the HFD for 12 weeks showed in the liver a 45% increased protein levels of TLR-4 compared to the control group, which was prevented by supplementation with BJe (Fig. 4A). Downstream, mice fed the HFD had higher liver MyD88 protein levels compared to all other groups (Fig. 4B). NF-κB activation was evaluated by measuring phosphorylation of IKK by Western blot. After 12 weeks on the diets, phosphorylation levels of IKKα/β (Ser176/180) and JNK 1/2 (Thr183/Tyr185) were higher (67% and 20 %, respectively) in the HF group compared to the control group. Both changes were mitigated by supplementation with BJe (Fig. 4B). All these

outcomes suggest that supplementation with BJe could mitigate HFD-induced liver inflammation via downregulation of the TLR-4 signaling pathway.

3.7 Effects of BJe supplementation on HFD-induced liver inflammation

Activation of NF-κB regulates the transcriptions of genes involved in the production of pro-inflammatory proteins. Thus, we next evaluated by Western blot the liver protein levels of the chemokine MCP-1, cytokines TNF-α and interleukin IL-6, inducible nitric oxide synthase (iNOS), and of the macrophage infiltration marker F4/80 (Fig. 5). 12-week consumption of the HFD caused an increase in the levels of all these proteins involved in the pro-inflammatory process, which was prevented by BJe supplementation (Fig. 5).

4. Discussion

Diets rich in fats and carbohydrates, typical of Western lifestyle, are among the main causes of obesity, insulin resistance and NAFLD onset, which is considered as one of the emerging causes of liver disease worldwide [39, 43]**.** This paper shows that supplementation of mice with BJe can mitigate HFD-induced liver steatosis and inflammation in mice, in part by protecting the intestinal barrier from permeabilization, which subsequently prevents LPS paracellular transport, endotoxemia, and liver TLR4-mediated activation of signalling cascades (NF-κB, JNK1/2) that promote liver inflammation and steatosis.

Consistent with previous findings [35, 44], we observed that 12-week consumption of a HFD caused: i) liver inflammation, as indicated by increased levels of the chemokine MCP-1, cytokines TNF-α, and IL-6 and NOS2, with evidence of tissue macrophage infiltration as indicated by an increase of F4/80 protein levels; ii) liver damage (increased ALT plasma levels); and iii) steatosis and fibrosis. All these events were prevented by supplementation with BJe. A large body of evidence associates NAFLD to different conditions including obesity, intestinal permeabilization, inflammatory bowel diseases [45] and cardiometabolic disorders [46,

[47]. The interaction between the gut and the liver (gut-liver axis) is key to the pathogenesis of NAFLD [48]. A negative gut-liver interaction can occur upon loss of intestinal barrier function. Thus, a permeable intestinal epithelium allows the passage, via paracellular transport, of bacteria and toxins from food and bacteria (LPS) to the circulation, leading to systemic inflammation which causes fat accumulation and inflammation in the liver [49-51 52, 53]. In fact, previous evidence showed that the preservation of the intestinal barrier and mitigation of endotoxemia are critical for the prevention of steatosis in the context of HFD and obesity [54]. Additionally, overfeeding and consumption of HFD is associated with increase endotoxin blood levels and correlates with energy intake in healthy subjects and individuals with type 2 diabetes [55, 56]. At the liver, LPS binds to TLR4 receptors present both in resident phagocytes (Kupffer cells) as well as in recruited macrophages. Accordingly, in the current model we observed that HFD consumption was associated with the upregulation of pro-inflammatory and pro-fibrotic cascades downstream TLR4, (i.e., MyD88, NF-κB, and MAPK) [57]. The crosstalk of these signaling pathways can further increase systemic and local inflammation [58, 59]. TLR4 activation causes an increased production of proteins involved in the inflammatory process such as chemokines, cytokines, and enzymes that produce reactive nitrogen and oxygen species. In agreement with the above mechanisms, we currently observed that BJe supplementation prevented HFD-induced intestinal permeabilization and endotoxemia, which led to the mitigation of the HFD-induced hepatic TLR-4 increased upregulation and of the consequent activation of the downstream signalling pathways MyD88, NF-κB, and JNK1/2. Such mechanism of action is further supported by a recent study showing that BJe alone and together with albedo and pulp fiber, prevented endotoxemia in rats fed a HFD [60]. In addition, it has been observed that neohesperidin, a compound rich in BJe, prevented intestinal permeability and endotoxemia in mice fed a HFD [61].

The protective capacity of BJe on intestinal permeability could be in part due to the preservation of the TJ structure and function. TJs protein constitute a dynamic complex composed by different proteins, including occludin, ZO-1, and claudins, which act as "gate/fence" mechanism, allowing the passage of water and ions in between epithelial cells (paracellular transport) but preventing the passage of larger molecules [62]. After 12 weeks on the HFD, we observed alterations in the ileum TJ structure, as evidenced by a decrease of ZO-1, occludin and claudin-1 protein levels. BJe supplementation prevented the loss of TJ caused by the chronic consumption of HFD. These findings are in agreement with previous studies showing that HFD consumption reduces the expression of intestinal TJs proteins, leading to intestinal permeability [63, 64]. Accordingly, Azuma et al [65] and Lu et al [61] showed that naringenin and neohesperidin, respectively, mitigated intestinal barrier damage in mice. The beneficial effects of BJe supplementation on TJ integrity could be in part due to its anti-inflammatory and antioxidant capacity [65-67].

While the action of BJe preventing HFD/obesity-induced intestinal barrier emerges as a central mechanism in its capacity to mitigate liver alterations, other mechanisms can be also involved. Thus, HFD-associated liver insulin resistance is a contributing factor to the development of NAFLD [68]. Furthermore, TLR4 can be activated by excess fatty acids [69] and hepatocyte lipotoxicity *per se* can promote the differentiation of infiltrating monocytes into pro-inflammatory macrophages [70]. Supporting, an anti-NAFLD action of BJe supplementation through metabolic effects, we observed that BJe ameliorated metabolic parameters, such as plasma glucose, insulin, total cholesterol and TG. Accordingly, in a mouse model of NAFLD [30] and in rat models of type 2 diabetes [32, 71] and hyperlipidaemia [72, 73], BJe supplementation prevented insulin resistance, hyperlipidaemia, and liver damage. Evidence from clinical trials show that consumption of bergamot polyphenolic fraction reduced serum cholesterol, TG and glycemia in metabolic syndrome patients [74, 75, 76]. Mollace et al. [53] recently demonstrated that

rats fed a HFD supplemented with bergamot polyphenolic fraction showed changes in lipoprotein size and that their re-arrangement could be due to the restoration of the gut microbiota profile. Furthermore, mice fed a HFD and supplemented with neohesperidin and naringin, the most abundant flavonoids present in BJe, improved obesity-associated dyslipidemia, hyperglycemia, and hepatic lipid accumulation in part via modulation of the microbiota profiles [61, 77]. Interestingly, some of these beneficial metabolic effects can be in part explained by other actions at the gastrointestinal tract, i.e. the regulation of total bile acid secretion and BJe's capacity to modulate the gut microbiota [60, 72]. In fact, Miceli and co-workers [66] were the first to report that the administration of BJ for 30 consecutive days prevented the diet-induced hyperlipidemia in rats, hypothesizing that its hypocholesterolemic effect might be related to the increase in fecal neutral sterols and total bile acids excretion.

Overall, our results confirm the BJe capability of counteracting liver damage in HFD-fed mice, a recognized experimental model for studying possible remedies for ailments related to Western-style diets. Our study offers a new key to understanding the mechanisms underlying the protective effects of the BJe, supporting their pivotal role in the restoration of the intestinal barrier functionality. This occurs by protecting the integrity of TJs that in turn prevents endotoxemia and the consequent liver damage triggered by the TLR-4 signaling cascade. This is the first report suggesting that, at least in part, the hepato-protective effects of the flavonoid pool of BJ arise from its capability of restoring intestinal health, laying the foundations for further in-depth investigations in human studies.

Author Contributions: G.E.L. performed the experiments, analyzed the data, and wrote the manuscript; M.N. conceived the study, drafted the paper, and provided the BJe composition; E.C. supervised the experimental procedures and data analysis, designed and conceived the study, wrote the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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Legend to Figures

Figure. 1. Effects of BJe supplementation on body weight gain. A- Experimental study design; **B- Body weight gain** of mice fed with control diet (C) (empty circle) (BJe; empty triangles), the control diet supplemented with 50 mg BJe/kg BW (BJe) (empty triangles), a HFD (HF) (black circle), and HFD supplemented with 50 mg BJe/kg BW (HF-BJe) (full triangles). Results are shown as means ± SEM and are the average of 8-10 animals/group. *Values are significantly different compared to the other groups ($p<0.05$, Repeated Measures ANOVA).

Figure. 2. Effects of BJe supplementation on liver steatosis, hepatic damage and fibrosis in HFD-fed mice. Mice were fed the different diets as described in the methods. **A-** Liver images; **B-** Liver triglycerides (TG) content; **C-** NAFLD activity score (NAS) evaluated as described in methods; **D-** proteins involved in fibrosis, i.e. TGF 1β and COL 1A1, were evaluated by Western Blot and values were normalized to GADPH levels (loading control). Results are shown as mean ± SEM of 8-10 animals/group analyzed by one-way ANOVA test. *Values are significantly different compared to the other groups ($p<0.05$, one-way ANOVA test).

Figure 3. Effects of BJe supplementation on intestinal permeability and endotoxemia in HFD-fed mice. Mice were fed the different diets as described in the methods. **A-** Intestinal permeability evaluated by the FITC-dextran assay after 8 weeks on the diets as described in methods; **B-** Plasma endotoxins was measured in the plasma at the end of the study as described in methods. C- Tight junction proteins (ZO-1, occludin, and claudin-1) levels were measured by Western blot. Values were normalized to HSC-70 (loading control). Results are shown as mean ± SEM of 8-10 animals/group. *Values are significantly different compared to the other groups ($p<0.05$, one-way ANOVA test).

Figure 4. Effects of BJe supplementation on liver TLR-4 and downstream signaling pathways activation in HFD-fed mice. Mice were fed the different diets as described in the methods. After 12 weeks on the corresponding diets the following parameters were measured in liver: A- TLR-4 protein levels; B- MyD88, NF-κB (phosphorylation of IKK Ser176/180) and MAPK (phosphorylation of JNK Thr183/Tyr185) activation were measured by Western blot. Bands were quantified and values referred to GADPH (loading control for TLR-4 and MyD88) and total protein levels (IKK, JNK). Results are shown as mean ± SEM of 8-10 animals/group. *Values are significantly different compared to the other groups ($p<0.05$, one-way ANOVA test).

Figure 5. Effect of BJe on hepatic inflammation in HFD-fed mice. Mice were treated as described in material and methods. After 12 weeks on the corresponding diets the following inflammatory parameters were measured in liver by Western blot: MCP-1, TNF-α, IL-6, NOS2, and F4/80. Bands were quantified and values referred to GADPH (loading control). Results are

shown as mean ± SEM of 8-10 animals/group. *Values are significantly different compared to the other groups ($p<0.05$, one-way ANOVA test).

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Data Availability Statements

The data supporting this article “A flavonoid-rich extract of bergamot juice improves high-fat diet-induced intestinal permeability and associated hepatic damage in mice” have been included as part of the Supplementary Information.

Figure 1

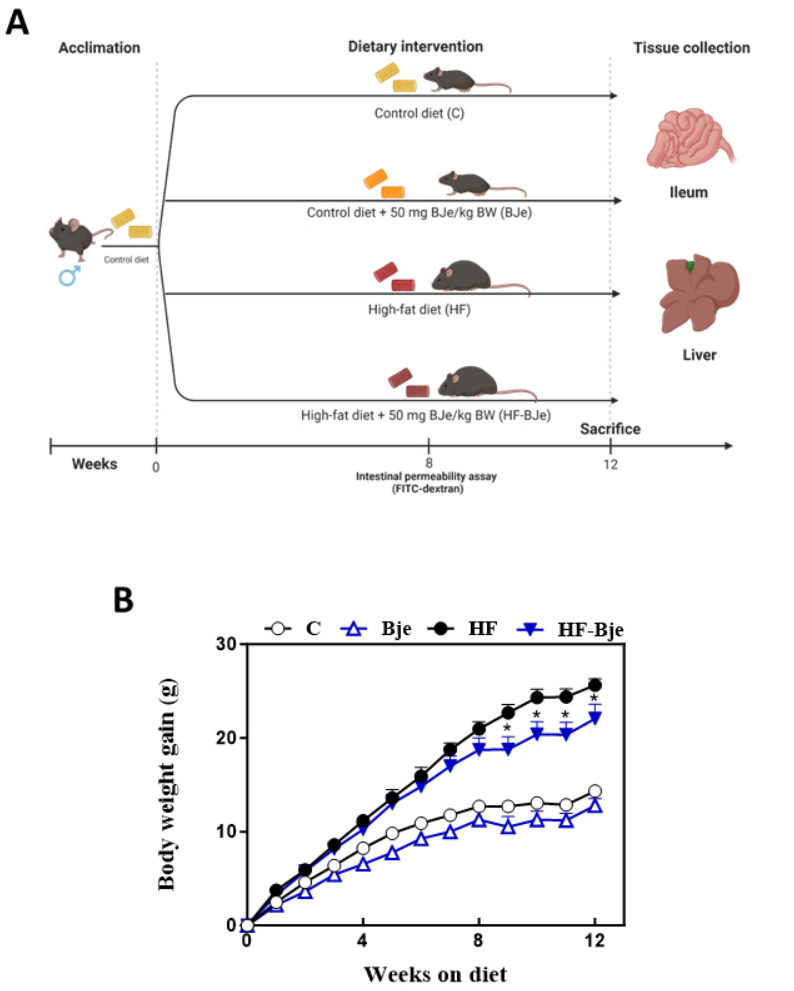

Figure 1

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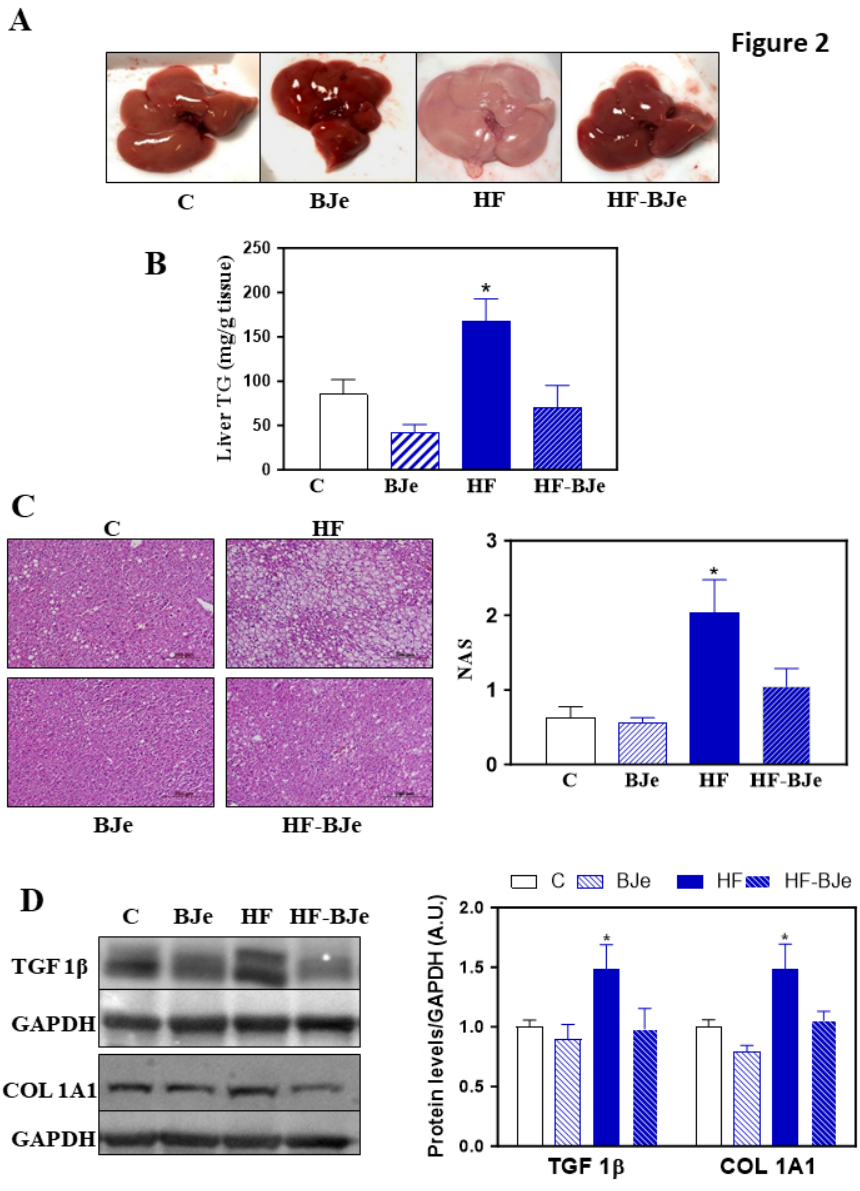
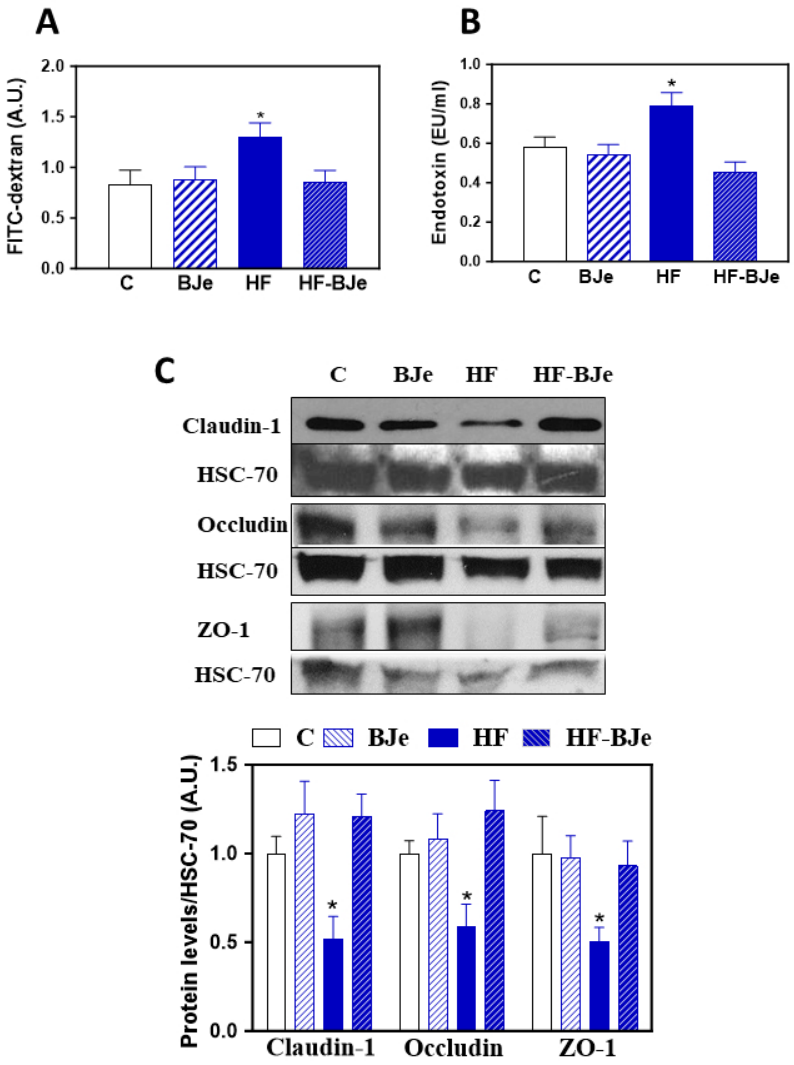


Figure 2

Figure 2

190x254mm (96 x 96 DPI)

Figure 3



190x254mm (96 x 96 DPI)

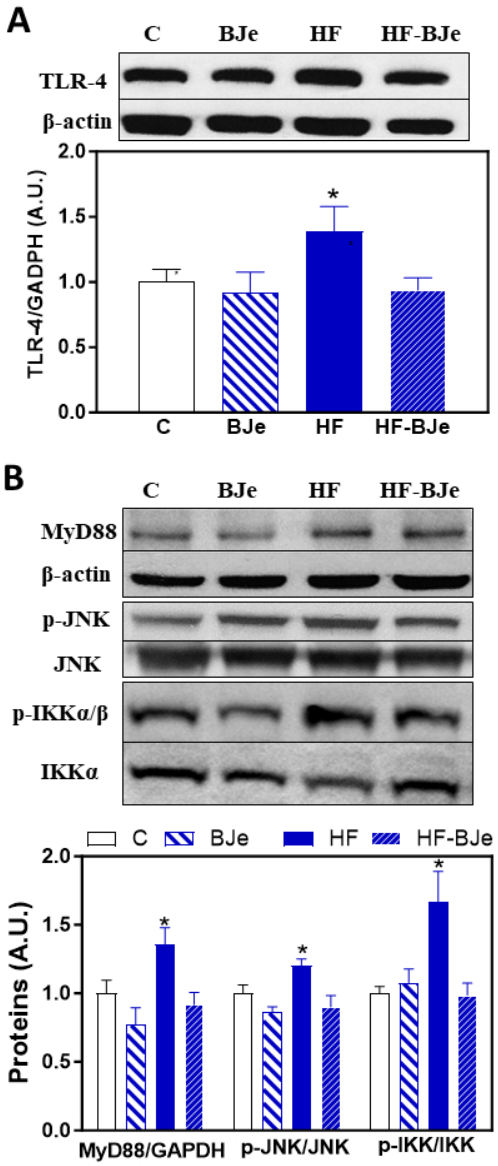


Figure 4

Figure 4

190x254mm (96 x 96 DPI)

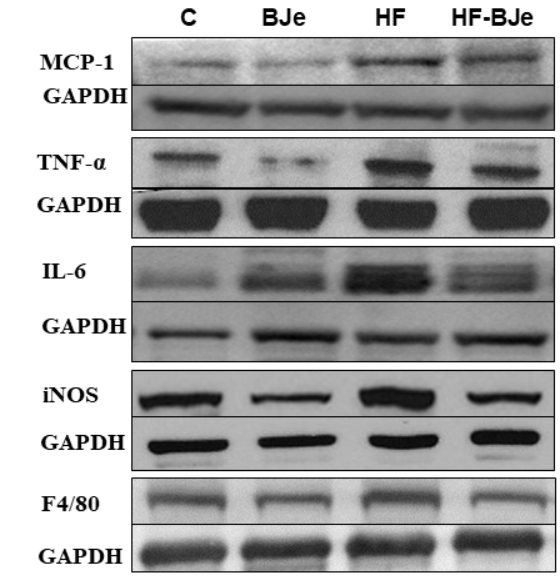


Figure 5

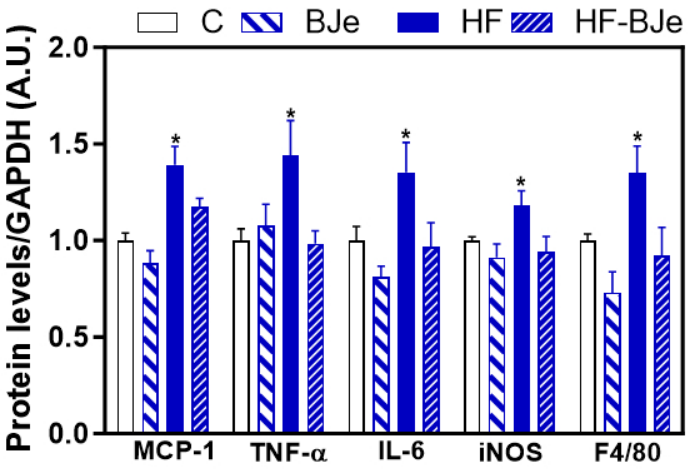


Figure 5

190x254mm (96 x 96 DPI)

Compounds	mg/g dry weight
Naringin	114
Neoeriocitrin	109
Neohesperidin	95
Brutieridin	46
Melitidin	24

Table 1. Flavonoids composition of BJe.

Parameters	C	BJe	HF	HF-BJe
Food intake (g/d)	3.0 ± 0.9^{a}	2.7 ± 0.3^{a}	2.5 ± 0.9^{b}	2.0 ± 0.7^{b}
Caloric intake (kcal/g/d)	11.1 ± 3.3^{a}	10.0 ± 1.0^{a}	12.8 ± 4.6^{a}	10.2 ± 3.6^{a}
Body weight (g)	36.3 ± 0.9^{a}	35.0 ± 0.8^{a}	46.9 ± 1.0^{b}	43.7 ± 1.4^{b}
Glucose (mg/dl)	$191 \pm 7^{a,c}$	194 ± 7^{a}	266 ± 11^{b}	229 ± 10^{c}
Insulin (IU/ml)	1.6 ± 0.2^{a}	1.8 ± 0.3^{a}	4.5 ± 0.4^{b}	3.1 ± 0.8^{c}
Total cholesterol (mg/dl)	174 ± 9^{a}	170 ± 16^{a}	252 ± 9^{b}	217 ± 10^{a}
Triglycerides (mg/dl)	60 ± 4^{a}	$70 \pm 5^{a,c}$	103 ± 6^{b}	87 ± 5^{c}
ALT (mU/ml)	13.2 ± 0.4^{a}	12.8 ± 0.4^{a}	17.6 ± 2.0^{b}	13.5 ± 0.9^{a}
Liver weight (g)	1.72 ± 0.09^{a}	1.5 ± 0.04^{a}	2.16 ± 0.19^{b}	1.73 ± 0.18^{a}
Visceral fat (g)	0.65 ± 0.07^{a}	0.57 ± 0.03^{a}	1.76 ± 0.13^{b}	1.23 ± 0.16^{c}
Subcutaneous fat (g)	2.27 ± 0.19^{a}	2.05 ± 0.16^{a}	5.41 ± 0.20^{b}	4.38 ± 2.26^{c}

Table 2. Body weight, food and caloric intake, liver weight, and metabolic parameters. Metabolic parameters of mice fed for 12 weeks with control or high fat diet, with or without BJe supplementation (50 mg/kg body weight). Fasted glucose and insulin as well as cholesterol, triglycerides and ALT were measured in plasma. Results are shown as means ± SEM of 8-10 animals/group. Different superscripts are significantly different compared to the other groups ($p<0.05$, one-way ANOVA test).