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Original article

# Protective effect of quercetin on high-fat diet-induced non-alcoholic fatty liver disease in mice is mediated by modulating intestinal microbiota imbalance and related gut-liver axis activation

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## ABSTRACT

Gut microbiota is involved in obesity, metabolic syndrome and the progression of nonalcoholic fatty liver disease (NAFLD). It has been recently suggested that the flavonoid quercetin may have the ability to modulate the intestinal microbiota composition, suggesting a prebiotic capacity which highlights a great therapeutic potential in NAFLD. The present study aims to investigate benefits of experimental treatment with quercetin on gut microbial balance and related gut-liver axis activation in a nutritional animal model of NAFLD associated to obesity. C57BL/6J mice were challenged with high fat diet (HFD) supplemented or not with quercetin for 16 weeks. HFD induced obesity, metabolic syndrome and the development of hepatic steatosis as main hepatic histological finding. Increased accumulation of intrahepatic lipids was associated with altered gene expression related to lipid metabolism, as a result of deregulation of their major modulators. Quercetin supplementation decreased insulin resistance and NAFLD activity score, by reducing the intrahepatic lipid accumulation through its ability to modulate lipid metabolism gene expression, cytochrome P450 2E1 (CYP2E1)-dependent lipoperoxidation and related lipotoxicity. Microbiota composition was determined *via* 16S ribosomal RNA Illumina next-generation sequencing. Metagenomic studies revealed HFD-dependent differences at phylum, class and genus levels leading to dysbiosis, characterized by an increase in *Firmicutes/Bacteroidetes* ratio and in Gram-negative bacteria, and a dramatically increased detection of *Helicobacter* genus. Dysbiosis was accompanied by endotoxemia, intestinal barrier dysfunction and gut-liver axis alteration and subsequent inflammatory gene overexpression. Dysbiosis-mediated toll-like receptor 4 (TLR-4)-NF-κB signaling pathway activation was associated with inflammasome initiation response and reticulum stress pathway induction. Quercetin reverted gut microbiota imbalance and related endotoxemia-mediated TLR-4 pathway induction, with subsequent inhibition of inflammasome response and reticulum stress pathway activation, leading to the blockage of lipid

*Abbreviations:* ALT, alanine aminotransferase; C/EBPα, CCAAT/enhancer binding protein alpha; CHOP, CCAAT-enhancer-binding protein homologous protein; CYP2E1, cytochrome P450 2E1; DAMPs, danger-associated molecular patterns; FABP1, fatty acid binding protein 1; FAS, fatty acid synthase; FAT/CD36, fatty acid translocase CD36; FFA, free fatty acid; FOXA1, forkhead box protein A1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRP78, 78 kDa glucose-regulated protein; HFD, high fat diet; HOMA-IR, homeostasis model assessment of insulin resistance; IAP, intestinal phosphatase alkaline; IL-6, interleukin 6; LPO, lipid peroxidation; LPS, lipopolysaccharide; LXRα, liver X receptor alpha; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; NF-κB, nuclear factor kappa B; NLRP3, NOD-like receptor family pyrin domain containing 3; PAMPs, pathogen-associated molecular patterns; PRRs, pattern recognition receptors; SCFAs, short-chain fatty acids; SREBP-1c, sterol regulatory element binding protein 1c; TG, triglycerides; TLR, Toll-like receptor; TNF-α, tumor necrosis factor; UPR, unfolded protein response

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metabolism gene expression deregulation. Our results support the suitability of quercetin as a therapeutic approach for obesity-associated NAFLD *via* its anti-inflammatory, antioxidant and prebiotic integrative response.

## 1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is the commonest form of liver disease in the Western countries [1]. NAFLD is associated with obesity and represents the hepatic manifestation of metabolic syndrome [2]. It ranges from simple hepatic lipid accumulation (steatosis) to steatohepatitis (NASH) when combined with inflammation, which can lead to cirrhosis, hepatocellular carcinoma and death related to liver morbidity [3]. Despite advances in this field, the molecular mechanisms of progression from steatosis to NASH remain obscure. The current and most accepted theory proposed for understanding the pathogenesis of NAFLD is the multiple parallel hits hypothesis. The "multiple hit" hypothesis conceives a complex interplay between multiple events acting in parallel with genetic predisposition, providing a more accurate explanation of the pathogenic mechanisms involved in NAFLD. Thus, NAFLD development and progression depend on changes in lipid metabolism, derived from the induction of fatty acid biosynthesis and transport that contributes to the intrahepatic lipid accumulation associated with insulin resistance, accompanied by oxidative stress-mediated lipotoxicity and proinflammatory cytokines gene expression, among others mechanisms [4–6].

A relationship has been reported between intestinal microbiota dysbiosis, barrier function and immune response and liver diseases [4,7]. Thus, gut microbiota is involved in obesity, metabolic syndrome and in the progression of NAFLD [8–10]. In NAFLD, alteration of gut microbiota and increased intestinal permeability enhance exposure of the liver to gut-derived bacterial products inducing chronic endotoxemia and associated gut-liver axis alteration [8,10]. Thereby, gut microbiota composition determination adds information to classical predictions of NAFLD severity and suggests novel targets for pre/probiotics therapies [11]. In this respect, it has been described that gut microbiota modulation with prebiotics improved obesity, metabolic syndrome and fatty liver [12].

Previous studies have established the modulatory capacity of polyphenols on the gut microbial community [13–15]. Natural compounds with antioxidant and anti-inflammatory capacity present in the diet such as flavonoids, including quercetin, appear to be capable of reducing hepatic lipid accumulation, which gives them a great therapeutic potential in NAFLD [5]. Recently, it has been indicated that quercetin may have the ability to modify the gut microbial balance [16], suggesting a prebiotic capacity. Thus, the use of flavonoids as quercetin in NAFLD might be considered as a potential strategy to modulate intestinal bacterial composition.

The present study aimed to investigate the potential benefits of the experimental treatment with quercetin on HFD-fed mice restoring host-microbial balance and regulating endotoxemia-related immune-mediated inflammatory signaling mechanisms and lipid metabolism alteration in the pathogenesis of nonalcoholic liver disease. Our results would enable the design of quercetin administration-based novel therapeutic approaches to manipulate gut microbiota to treat obesity-associated NAFLD.

## 2. Materials and methods

### 2.1. Animals and treatments

Seven-weeks-old male C57BL/6J mice were fed with a standard diet to their adaptation to the environment and later distributed in 4 groups (10 mice per group) according with the following diets (Research Diets, Inc. New Brunswick, NJ. USA): (1) Control (10% of energy from fat; D12450J); (2) Control Q (10% of energy from fat +0.05% (wt/wt) aglycone quercetin D14062801) (3) HFD (60% energy from fat; D12492) and (4) HFDQ (60% energy from fat +0.05% (wt/wt) aglycone quercetin; D14062802). Mice were fed freely available diets and water, and housed under controlled conditions of temperature, humidity and lighting. Body weight and food intake were monitored weekly. After 16 weeks, mice were euthanized, plasma, liver, small intestine and adipose tissue samples were collected and weighed. The right posterior lobe of the liver was fixed in 10% formalin and the remaining liver was snap frozen.

All procedures were approved by the local Animal Ethics Committees in accordance with the European Research Council guidelines for animal care and use.

### 2.2. Histopathology and fluorescence microscopy

Formalin-fixed and paraffin-embedded liver samples were sectioned and stained with hematoxylin and eosin (H & E). Lesions were evaluated by a histological scoring system for non-alcoholic fatty liver disease proposed by Kleiner et al. [17]. The NAFLD activity score (NAS) was used as a tool toprovide a numerical score evaluating semi-quantitatively 3 histological features: steatosis (0 − 3), lobular inflammation (0 − 3) and hepatocellular ballooning (0 − 2). Samples with scores more than 5 were correlated with a diagnosis of NASH, and score less than 3 were diagnosed as "not NASH." Histological analysis was measured by two objective expert examiners blinded to experimental design protocol. Frozen liver tissue samples were sectioned and stained with 1 μg/ml Bodipy 493/503 and Bodipy 581/591 C11 (Invitrogen, Carlsbad, CA, USA) combined with DAPI for nuclei staining, to analyze lipid accumulation or lipoperoxidation (LPO), respectively. The sections were imaged using a Nikon Eclipse Ti inverted microscope (Nikon, Amstelveen, The Netherlands).

### 2.3. Biochemical analysis

Plasma levels of triglycerides (TG), and alanine aminotransferase activity (ALT) were analyzed by the Instrumental Techniques Laboratory of the University of León using standard techniques. Plasma levels of insulin and interleukin (IL)-6 were determined by specific ELISA kits according to the manufacturer's instructions (Millipore, Darmstadt, Germany). Plasma glucose levels were measured with the Accu-Chek (Roche Diagnostics, Almere, The Netherlands) after an 8-h fast. The homeostasis model assessment of insulin resistance (HOMA-IR) was used to calculate the insulin resistance using the formula:

$$\text{HOMA}-\text{IR} = \text{Fasting glucose (mg/dl)} \times \text{Fasting insulin } (\mu U/\text{ml})/405$$

### 2.4. Plasma endotoxemia

Plasma lipopolysaccharide (LPS) and ethanol quantification were performed following the instructions of the commercial kits (LAL Chromogenic Endotoxin Quantification Kit, Thermo Scientific, and Ethanol Colorimetric Assay Kit, Biovision, respectively).

### 2.5. Measurement of liver triglycerides and free fatty acids

Triglycerides and free fatty acids (FFA) levels in the hepatic tissue were analyzed after liver homogenization following the guide provided by the company Biovision Research Products (Mountain View, CA, USA).