**Functional interactions between gut microbiota transplantation, quercetin and high fat diet determine non-alcoholic fatty liver disease development in germ-free mice**

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**Abbreviations:** BWG: body weight gain, ControlQ: control diet supplemented with quercetin, *dC*: control diet-fed donor, *dCQ*: control diet supplemented with quercetin-fed donor, *dHFD*-: non-responder to the high fat diet donor, *dHFD*+: responder to the high fat diet donor, *dHFDQ*: higher response to quercetin with high fat diet donor, GFm: germ-free mice, HFD: high fat diet, HFDQ: high fat diet supplemented with quercetin, HOMA-IR: homeostasis model assessment of insulin resistance, LPS: lipopolysaccharide, NAFLD: Non-alcoholic fatty liver disease, NAS: NAFLD activity score, NLRP3: NOD-like receptor family pyrin domain containing 3, PCoA: principal coordinates analysis, SCFAs: short chain fatty acids, TLR-4: toll-like receptor 4.

**Keywords:** *Akkermansia* spp.; Flavonoids; Gut microbiota transplantation; Gut-liver axis; Nonalcoholic fatty liver disease (NAFLD)

**Abstract**

**Scope:** Modulation of intestinal microbiota has emerged as a new therapeutic approach for non-alcoholic fatty liver disease (NAFLD). Here we addressed whether gut microbiota modulation by quercetin and intestinal microbiota transplantation can influence NAFLD development.

**Methods and results:** Gut microbiota donor mice were selected according to their response to high fat diet (HFD) and quercetin in terms of obesity and NAFLD-related biomarkers. Germ-free recipients displayed metabolic phenotypic differences derived from interactions between microbiota transplanted, diets and quercetin. Based on the evaluation of hallmark characteristics of NAFLD, we identified that gut microbiota transplantation from the HFD-non-responder donor and the HFD-fed donor with the highest response to quercetin resulted in a protective phenotype against HFD-induced NAFLD, in a mechanism that involves gut-liver axis alteration blockage in these receivers. Gut microbiota from the HFD-responder donor predisposed to NAFLD in transplanted germ-free mice. Divergent protective and deleterious metabolic phenotypes exhibited were related to definite microbial profiles in recipients, highlighting the predominant role of *Akkermansia* genus in the protection from obesity-associated NAFLD development.

**Conclusions:** Our results provide scientific support for the prebiotic capacity of quercetin and the transfer of established metabolic profiles through gut microbiota transplantation as protective strategy against the development of obesity-related NAFLD.

**Introduction**

Non-alcoholic fatty liver disease (NAFLD) has emerged as one of the commonest liver disorders in western countries linked to the global epidemic of obesity [1]. In the last years, a significant role of intestinal microbiota in obesity and non-alcoholic fatty liver disease (NAFLD) development has been proposed. It was demonstrated that germ-free mice (GFm) are resistant to diet induced obesity.[2] Moreover, colonisation of GFm with microbiota from conventional raised donors leads to a marked increase in body fat mass and development of insulin resistance thus demonstrating that the microbiota is related to the onset of the metabolic syndrome.[3] Furthermore, gut microbiota may provide potential harmful molecules to the liver through the gut liver-axis, contributing to establish a chronic low-grade inflammation state and, promoting the NAFLD progression.[4, 5] Le Roy *et al.*[6] showed that the transplantation of faecal microbiota from donors discordant for NAFLD severity to GFm resulted in different phenotypic patterns, verifying that NAFLD susceptibility is strongly determined by gut microbiota.

Due to the crucial role of gut microbiota and the lack of a *gold standard* treatment for NAFLD, strategies to modulate it, including the use of probiotics and prebiotics, emerge as potential therapeutic approaches. Recently, it has been discussed the capacity of flavonoids to modulate gut microbiota composition, intestinal inflammation and barrier integrity.[7–9] Quercetin, flavonoid widely studied for its antioxidant and anti-inflammatory properties, has demonstrated its ability to diminish lipid accumulation in the liver, ameliorate insulin resistance and modulate lipogenic genes in animal models of metabolic syndrome and NAFLD.[10, 11] Moreover, recent studies showed that quercetin reshapes intestinal microbiota concomitant with attenuated obesity and NAFLD symptoms, suggesting a prebiotic effect of the flavonol.[12, 13] It has been also proposed that some bacterial species may be responsible of the protective effect of prebiotics. This is the case of the mucin-degrader gram-negative bacteria *Akkermansia muciniphila,* which is promoted by some prebiotic compounds and can contribute to maintain intestinal integrity preventing endotoxemia.[14, 15]

The aim of this study is to investigate the effect of different metabolic phenotypes transfer through gut microbiota transplantation, and the interplay between specific microbiota transplanted, diet and quercetin, in order to determine the protective or harmful role that they can exert against obesity-related NAFLD development.

**Experimental section**

**Animals and experimental protocol**

A model: Donor mice selection

The A model was established in a previous work of our research group [12]. Briefly, seven-weeks C57BL/6J conventional male mice were fed, after adaptation to the environment, with freely available water and diets (Research Diets, Inc. New Brunswick, NJ. USA; Supplementary Table 1): Control (10% of energy from fat; D12450J); ControlQ (control + ~~0.05% (wt./wt)~~ aglycone quercetin D14062801); HFD (60% energy from fat; D12492) and HFDQ (HFD + ~~0.05% (wt/wt)~~ aglycone quercetin; D14062802). After sixteen weeks mice were euthanized under anesthesia. The caecal and faecal contents, plasma, liver and visceral adipose tissue samples were collected. The caecal content was immediately preserved at -80°C in skim milk (10%) supplemented with cysteine as a reducing agent. All procedures were performed in accordance with the European Research Council guidelines for animal care and use and under the approval by the local Animal Ethics Committees.

Mice from the A model representing the different groups were selected to act as donors of intestinal microbiota based on obesity and NAFLD-related markers (control, *dC*; control supplemented with quercetin, *dCQ*; responder to HFD, *dHFD*+ and non-responder to HFD, *dHFD*-, and higher response to quercetin with HFD, *dHFDQ*) (Supplementary Figure 1).

B model: Transplantation of gut microbiota

Seven-week germ-free male C57BL/6J mice (Anaxem, MICALIS Institute) were colonized in a single administration by oral-gastric gavage with 250 µl of caecal content collected from donors of the A model following the protocols described by Le Roy *et al* [6]. The animals were kept in sterile and controlled environment and fed freely irradiated diets and sterile water. The mice were distributed in 20 groups according with the microbiota transplanted from donors (*dC*, *dCQ*, *dHFD+*, *dHFD-* or *dHFDQ*), the diet and quercetin supplementation (control: C, control + quercetin: CQ, HFD, or HFD + quercetin: HFDQ) (Supplementary Figure 1). After 16 weeks of treatment the animals were sacrificed under anesthesia. Plasma, caecal and faecal contents, liver and visceral adipose tissues were collected.

**Dosage information**

Quercetin was supplemented in the control and HFD in the form of aglycone quercetin (0.05% (wt/wt)) at a dosage roughly equal to 80 mg kg-1 day-1. The *in vivo* dosage was chosen according to previous studies.[11] For human equivalent dose, the dosage used in animal experiments was approximately equivalent to 6.5 mg kg-1 day-1 in humans. This dose is achievable through supplements or diets rich in fruits and vegetables.[16]

Additional Materials and Methods are included as supporting information.

**Results**

**Donor mice selection**

Caecal microbiota donors from the A model were selected out ~~of Control (~~*~~dC~~*~~), ControlQ (~~*~~dCQ~~*~~), HFD (~~*~~dHFD-~~* ~~and~~ *~~dHFD+~~*~~) and HFDQ (~~*~~dHFDQ~~*~~) groups,~~ in terms of obesity, metabolic syndrome and hepatic steatosis development, in addition to endotoxemia, gut-liver axis alteration, inflammatory response induction and lipid metabolism deregulation (Table 1). *dHFD+* showed greater body weight gain (BWG: +27%), NAFLD activity score (NAS: +41%), insulin resistance (HOMA-IR: +5%), endotoxemia (lipopolysaccharide, LPS: +9%), gut liver-axis alteration (toll-like receptor 4, TLR-4: +366%; NOD-like receptor family pyrin domain containing 3, NLRP3: +42%), inflammation (tumor necrosis factor-alpha, TNF-α: +215%) and lipid metabolism deregulation (fatty acid translocase CD36, FAT/CD36: +111%; liver X receptor alpha, LXRα: +42%) in comparison with HFD group. An opposite pattern was observed in *dHFD-* and *dHFDQ* (BWG: -26% and -58%; NAS: - 44% and -72%; HOMA-IR: -12% and -58%; LPS: -34% and -37%; TLR-4: +73% and -71%; NLRP3: -13% and -42%; TNF-α: -45% and -67%; FAT/CD36: -30% and -55%; LXRα: -20% and -37%, respectively, *vs* HFD group) (Table 1).

**Gut microbiota balance in donors**

At the phylum level, all donors were dominated by three phyla: *Firmicutes*, *Bacteroidetes* and *Proteobacteria* (Figure 1A), similarly to that showed by their corresponding groups at the A model (Supplementary Figure 2, modified from Porras *et al*[12]). Nevertheless, *dHFD-* and *dHFDQ* showed an increase in the relative percentage of *Firmicutes* and *Verrucomicrobia* phyla, respectively, in comparison to the phylotypes observed in the A model (Figure 1A and Supplementary Figure 2). At the class level, *Verrucomicrobiae* was markedly increased in *dHFDQ* compared to its corresponding A model group. Moreover, a different bacterial profile at class and genus levels between *dHFD+* and *dHFD-* was revealed. In this respect, *Clostridia* was considerably increased in *dHFD-* compared to *dHFD+.* In contrast, *Bacteroidia* and *Flavobacteriia* were reduced in *dHFD-* in comparison to *dHFD+* (Figure 1B)*.* As shown in Figure 1C, *Helicobacter* genus detection was higher in *dHFD+* in comparison to *dHFD-*, whereas *Oscillospira*, *Lactobacillus* and *Alkaliphilus* genera showed an opposite pattern. When the donor received a HFD supplemented with quercetin the profile of genera studied was similar to control donors. Furthermore, it should be pointed out that *Akkermansia*, the unique bacteria belonging to *Verrucomicrobia* phylum, was overrepresented in *dHFDQ* in comparison to the rest of donors (Figure 1C).

**Effect of diets, quercetin and intestinal microbiota transplantation from donors on obesity, insulin resistance and liver histological findings in GFm**

*dC* and *dCQ*-receiver mice fed with HFD display an increased BWG, epididymal fat accumulation (data not shown) and impaired insulin sensitivity (*dC*/HFD: +373%; +435%; +530%; *dCQ*/HFD: +395%; +450%; +675%, respectively, *vs dC*/C), which were partially reverted with quercetin supplementation (*dC*/HFDQ: -10%; -3%; -34%; *dCQ*/HFDQ: -27%; -1,3%; -28%, respectively, *vs dC*/HFD) (Figure 2A and B), as in the A model.[12] As shown in Figure 2, *dHFD-* and *dHFDQ*-receiver mice fed with HFD showed reduced BWG (-48% and -49%, respectively) and insulin resistance (HOMA-IR: -82% and -76%, respectively) in comparison withHFD-fed *dHFD+*-transplanted mice, exhibiting similar results to that shown in *dC*/C mice (Figure 2A and B).

Hepatic histopathological evaluation showed microvesicular and macrovesicular steatosis in both control recipients and *dHFD+*-receiver mice fed with HFD, resulting in an elevated NAS (*dC*/HFD: +313%, *dCQ*/HFD: +332%, *dHFD+*/HFD: +300%), compared to *dC*/C mice. Quercetin supplementation to HFD showed a protective role in the development of characteristic histological findings of NAFLD in *dC* and *dCQ* recipients (*dC*/HFDQ: -40%; *dCQ*/HFDQ: -43%, *vs* *dC*/HFD) (Figure 2). These results correlated with an increase in the hepatic triglyceride content in *dC*-receiver mice fed with HFD (*dC*/HFD: +134%, *dCQ*/HFD: +148%, *dHFD+*/HFD: +104%, *vs dC*/C), which was partially reverted by quercetin administration (*dC*/HFDQ: -20%, *dCQ*/HFDQ: -35%, *vs* *dC*/HFD). *dHFD-* and *dHFDQ*-transplanted mice showed non-pathological hepatic histological findings despite HFD feeding, reverting the increased NAS observed in *dHFD+*-recipients (*dHFD-*/HFD: -62%, *dHFDQ*/HFD: -92%, *vs* *dHFD+*/HFD) and exhibiting similar results to *dC*/C mice (Figure 2C). As expected, *dHFD-* and *dHFDQ*-transplanted mice fed with HFD showed reduced hepatic triglyceride concentration (-60% and -55%, respectively) in comparison with HFD-fed *dHFD+*-transplanted mice (data not shown).

**Effect of diets, quercetin and intestinal microbiota transplantation from donors on the bacterial community profile showed in GFm**

A total of 9,095,319 reads were obtained from all GFm caecal samples using *Illumina MiSeq* system. Each library contained an average of 110,919 reads per sample. At the phylum and class levels, the microbial composition of transplanted GFm was modified according to the diets, except for the *dHFD+*-receiver group, which showed a similar profile to their donor regardless of the diet (Figure 3A and B). As previously described,[17] the phenotype of transplanted GFm could be attributed to several key taxa. Thus, a notable increase in the detection of *Verrucomicrobia* phylum, *Verrucomicrobiae* class and *Akkermansia* genus was observed in *dC*, *dCQ*, *dHFD-* and *dHFDQ*-receiver groups fed with HFD (Figure 3). Surprisingly, in *dHFD+* recipients these phylum, class and genus were undetectable, independently of the diet (Figure 3). Quercetin supplementation in recipients fed with control diet and HFD was found to considerably modify the number of reads of *Verrucomicrobia* phylum, *Verrucomicrobiae* class and *Akkermansia* genus. In this respect, *dHFD-* and *dHFDQ*-receiver groups fed with a control diet supplemented with quercetin showed a higher detection of these phylum, class and genus in comparison to recipients fed with control diet, exhibiting a similar detection of *Akkermansia* genus to the ControlQ group from the A model. However, when *dHFD-* and *dHFDQ*-receiver groups were fed with HFD supplemented with quercetin, the profile of these recipients changed showing a reduction of *Verrucomicrobia* phyla, *Verrucomicrobiae* class and *Akkermansia* genus (Figure 3). As previously described in the A model (Supplementary Figure 2), and similarly to results concerning *Akkermansia*, a greater number of reads of *Lactobacillus* genus was observed in all receiver groups fed with HFD, except for the *dHFDQ*-receiver group (Figure 3C). In addition, the relative abundance of this genus was remarkably higher in HFD-fed *dHFD-* recipients, showing a similar profile to its donor (Figure 1). Interestingly, a higher detection of *Helicobacter* in HFD-fed *dC*-receiver group was observed, which was significantly reduced with quercetin, confirming the results previously described in the A model [12], while a protective effect of quercetin has not been detected in the rest of receiver groups. Over again, a different relative abundance of *Helicobacter* was observed between *dHFD-* and *dHFD+* in control diet-fed recipients, being increased in the HFD-responder receivers. A very remarkable finding was the absence of detection of *Helicobacter* genus in mice transplanted with microbiota from *dHFDQ,* regardless of the diet (Figure 3C). The microbiota diversity, calculated by the Shannon diversity index, seems to be reduced in recipient mice transplanted with microbiota from HFD-fed donors in comparison to *dC* and *dCQ* receivers. However, in *dHFDQ*-receiver groups microbial diversity tended to be similar to the control recipients (data not shown).

On the other hand, a Principal Coordinates Analysis (PCoA) based on Morisita Horn Index was performed showing that the caecal microbiota from *dC* and *dCQ*-receivers formed different clusters according to the first axis (55.78% and 28.04%, respectively) based on the diet and the quercetin, as previously described in the A model[12] (Figure 4). While, a clear cluster was formed with the bacterial communities from *dHFD-* and *dHFDQ*-receiver groups fed with HFD supplemented with quercetin according to the first axis (32.18% and 24.41%, respectively) (Figure 4). In contrast, the bacterial communities of *dHFD+*-recipients were dispersed without forming any group (Figure 4).

**Effect of diets, quercetin and intestinal microbiota transplantation from donors on short chain fatty acids (SCFAs) production**

Altered metabolism of SCFAs has been related to dysbiosis and compromised intestinal barrier integrity in our A model.[12] Similarly, HFD feeding in *dC* and *dCQ*-receiver groups was associated to lower acetate, propionate and butyrate production ~~(~~*~~dC~~*~~/HFD: -32%; -36%; -35%;~~ *~~dCQ~~*~~/HFD: -13%; -35%; -25%, respectively,~~ *~~vs dC/C~~*~~)~~ in comparison with *dC*/C group, meanwhile quercetin supplementation led to a partial recovery of SCFAs formation ~~(~~*~~dC~~*~~/HFDQ: +35%; +39%; +19%;~~ *~~dCQ~~*~~/HFDQ: +29%; +23%; +0.75%, respectively,~~ *~~vs dC~~*~~/HFD)~~ (Figure 5A). Interestingly, *dHFD+*-receiver groups showed a reduced SCFAs generation independently of the diet ~~(~~*~~dHFD+~~*~~/HFD, acetate: -19%; propionate: -10%; butyrate: -23%,~~ *~~vs dC~~*~~/HFD)~~ (Figure 5A). The impairment of SCFAs formation associated with HFD feeding was partially recovered when mice were transplanted with *dHFD-* and *dHFDQ* microbiota, showing an opposite pattern to *dHFD+* recipients ~~(~~*~~dHFD-~~*~~/HFD, acetate: +35%; propionate: +17%; butyrate: +37%,~~ *~~dHFDQ~~*~~/HFD, acetate: +57%; propionate: +23%; butyrate: +40%,~~ *~~vs dHFD+~~*~~/HFD)~~.

**Effect of diets, quercetin and intestinal microbiota transplantation from donors on HFD-induced endotoxemia and gut-liver axis activation**

Such as in our A model, *dC* and *dCQ*-receiver mice fed with HFD displayed endotoxemia (*dC*/HFD, LPS: +57%; ethanol: +64%; *dCQ*/HFD, LPS: +41%; ethanol: +48%, *vs dC/C*) and the concomitant overexpression of TLR-4 (*dC*/HFD: +75%, *vs dC/C*), while quercetin supplementation partially counteracts HFD-related endotoxemia and markedly reduces TLR-4 upregulation (*dC*/HFDQ, LPS: -20%; ethanol:-37%; TLR-4: -79%; *dCQ*/HFDQ, LPS: -45%; ethanol:-56%; TLR-4: -62%, *vs* *dC*/HFD) (Figure 5B-C and 6A). In contrast, in HFD-fed *dHFD+*-receiver group endotoxemia was increased in comparison to *dC*/HFD recipients (*dHFD+*/HFD, LPS: +27%; ethanol: +27%), while TLR-4 mRNA was overexpressed in *dHFD+* recipients independently of the diet (Figure 6A). Regarding to *dHFD-* and *dHFDQ*-receiver mice fed with HFD, LPS and ethanol plasma levels were significantly reduced in comparison toHFD-fed *dHFD+*-transplanted mice (*dHFD-*/HFD, LPS: -64%; ethanol: -64%; *dHFDQ*/HFD, LPS: -78%; ethanol: -78%). These results were accompanied with a significant reduction on TLR-4 upregulation in HFD-fed *dHFD-* and *dHFDQ* recipients (mRNA: -65% and -59%; protein: -72% and -55%, respectively, *vs dHFD+/*HFD) (Figure 6A).

**Effect of diets, quercetin and intestinal microbiota transplantation from donors on inflammasome activation**

In order to determine whether dysbiosis triggers inflammasome activation, hepatic expression of receptor NLRP3 was assessed. Results for *dC*-receiver mice fed with HFD were in accordance with our previous findings with a significant elevation of NLRP3 expression (+126%, *vs dC/C*), and restoration to control levels by quercetin supplementation (-57%, *vs dC*/HFD) (Figure 6B). Regarding the effect of microbiota transplantation on inflammasome activation, NLRP3 hepatic gene expression showed a significant increase in *dHFD+*-receiver groups with respect to the other receivers independently of the diet (Figure 6B). Moreover, *dHFD-* and *dHFDQ*-receiver mice showed a significant reduction on NLRP3 gene expression in comparison with *dHFD*+-transplanted mice regardless of the diet (*dHFD-*/HFD, -61%; *dHFDQ*/HFD, -55% *vs dHFD+*/HFD) (Figure 6B).

***Akkermansia* spp. relative abundance inversely correlates with obesity-associated NAFLD spectrum and related inflammasome response activation**

In order to determine the potential role of *Akkermansia* in the development of divergent metabolic phenotypes in response to HFD, correlation analyses were performed. The presence of *Akkermansia* was significantly and negatively correlated with body weight gain (p<0.001), HOMA-IR (p<0.01), NAS (p<0.001) and inflammasome activation (p<0.05) and significantly and positively correlated with butyrate production (p<0.01) in the HFD-fed receiver groups (Figure 7).

**Discussion**

In a previous study, our research group described in a HFD-based NAFLD model (A model) that intestinal dysbiosis, fundamentally at the genus level, was involved in the development of fatty liver associated to obesity, justifying the importance of intestinal microbiota modulation as a possible therapeutic approach for the prevention and treatment of this disease. In this study, we also demonstrated the protective effect of quercetin supplementation on NAFLD development in a mechanism involving its anti-inflammatory, antioxidant and prebiotic integrative effect.[12] It has been suggested the suitability of intestinal microbiota modulation by faecal microbiota transplantation as a therapeutic option in the treatment of NAFLD associated to obesity in both experimental models[6, 18, 19] and patients.[20] Thus, in the present study we investigate the effect of gut microbiota transplantation from donors selected from the A model to GFm in order to transfer functional metabolic phenotypes. We also evaluate the impact of HFD and quercetin supplementation on final gut microbiota composition and NAFLD development in the receiver groups.

Based on the results obtained in the A model, we selected caecal microbiota from donors that showed specific metabolic profiles. The high-fat diet provoked the appearance of non-responder (*dHFD-*) and responder (*dHFD+*) to HFD donors characterized by divergent metabolic phenotypes. Different response to HFD has been previously described by Le Roy *et al*.,[6] and similar opposite responders were selected as gut microbiota donors. In our study, HFD-related donors showed a distinctive microbiota composition at the phylum, class and genus levels. Interestingly, a higher detection of *Helicobacter* genuswas observed in the *dHFD+* in comparison to the *dHFD-*. The increased relative abundance of this genus in the HFD-responder donor supports its possible involvement in the pathogenesis of NAFLD, as previously suggested.[12, 21–25] Other genera, such as *Oscillospira*, *Lactobacillus* and *Alkaliphilus* revealed an opposite pattern, showing a higher detection in *dHFD-*. Recent studies have shown that *Oscillospira* genus appears to be reduced in patients with NAFLD and NASH.[26–28] Further, several clinical trials have shown an improved disease state associated to the treatment with different species of *Lactobacillus* genus as a probiotic.[29, 30] The relative abundance of these genera in *dHFD-*, in addition to its specific profile at phylum and class levels, could be related to a protective functionality against NAFLD.

In *dHFDQ*, a surprising higher detection of *Verrucomicrobia* phylum and *Verrucomicrobiae* class, as well as *Akkermansia* genus was observed in comparison to the rest of donors. Several studies have shown that *Akkermansia muciniphila* is able to protect against the increase of BWG and the development of adiposity associated to HFD,[31–33] and to improve metabolic parameters in obesity and NAFLD,[34, 35] supporting its suitability as a potential probiotic agent, as previously suggested using both viable *A. muciniphila*[36] or preparations of this bacteria[37]. Further, it has been described an increase in the relative abundance of *Akkermansia* spp. population in HFD-fed mice treated with protective natural compounds.[14, 38–41] In this regard, a positive modulatory effect of quercetin on *Akkermansia* genus has already been shown in our A model[12] justifying its possible protective role in *dHFDQ*.

In the B model, we observed that *dC* and *dCQ* recipients developed HFD-induced NAFLD characterized by increased BWG, NAFLD activity score and insulin resistance, similarly to mice fed with HFD in the A model. Also, a protective effect of quercetin on HFD-fed *dC* and *dCQ* recipients was observed, corroborating our previous findings.[12] Interestingly, microbiota transplantation from *dHFD-* and *dHFDQ* donors entails the transfer of protective metabolic phenotypes, counteracting the deleterious effect of HFD on obesity and NAFLD development. Oppositely, *dHFD+* microbiota transplantation produced insulin resistance and moderate hepatic steatosis in control diet-fed recipients, suggesting a harmful metabolic phenotype transfer associated with microbiota composition in this donor. These findings support the cause-effect relationship between observed changes in caecal microbiota from donors and the metabolic signature of the group in our model A. Thus, we were able to transfer a specific metabolic phenotype by means of microbiota transplantation from selected donors based on metabolic parameters. Donor selection is a major concern about the suitability of microbiota transplantation in clinical practice, and the results obtained suggest that evaluation of hallmark characteristics of the pathology is sufficient to reach positive outcomes; however microbial composition needs to be addressed not only to avoid potential pathogens transmission but in order to achieve a targeted beneficial microbiota profile.[42] Moreover, *dHFDQ* was equally effective protecting mice from the effect of HFD feeding as the non-responder donor, pointing the microbiota as a driving force of beneficial effects of quercetin on NAFLD development and confirming the prebiotic activity previously attributed to the flavonoid.[12, 13] The absence of an analogous protective effect of *dCQ* suggests that its prebiotic effect takes place only in the presence of HFD.

Metagenomic studies revealed that the interplay between gut microbiota phylotypes from control and protective donors with HFD in transplanted GFm resulted in an increased detection of *Akkermansia* genus, that showed an opposite pattern to that previously described in non-transplanted HFD-fed mice,[12, 31, 33, 43] which could be explained in terms of different microbiome initial conditions. Interestingly, *Akkermansia* spp. population was undetectable in *dHFD+*-receiver groups independently of the diet or quercetin supplementation, suggesting that the absence of this genus is involved in the resultant harmful phenotype. In this regard, predisposition to develop NAFLD, obesity and insulin resistance inversely correlated with *Akkermansia* relative abundance in HFD-fed receiver groups. Similar findings involving the relationship between this bacterium and body weight and glucose tolerance in rodents and humans has been previously described.[31, 34–36] Otherwise, quercetin supplementation to HFD-fed recipients showed an opposite effect on *Akkermansia* relative abundance to that exhibited in the donors’ model. A similar diminished detection of this genus was described in resveratrol-fed obese mice, in which this polyphenol develops a protective role.[44] In our study, these recipients were transplanted with particular microbiota phylotypes and maintained in isolation conditions, which could modify the response to quercetin. This finding could justify the observed absence of an additive protective effect between quercetin and caecal microbiota transplantation from *dHFD-* and *dHFDQ* donors. Moreover, quercetin supplementation to HFD seems to exert a contradictory effect on *dHFDQ* recipients, worsening the outcomes of the disease. Thus, we suggested the existence of an elaborate interaction between quercetin, HFD and transferred microbiota phylotypes which requires further investigations.

Intestinal dysbiosis associated to HFD observed in the B model resulted in an impaired intestinal SCFAs production and endotoxemia, which increased TLR ligands delivered to the liver, leading to gut-liver axis alteration and inflammasome activation in HFD-fed *dC*-receiver group, which were significantly reduced with quercetin, as we previously described in the A model.[12] Regarding the effect of different microbial profiles, *dHFD*+-receivers showed limited bacterial SCFAs production and enhanced endotoxemia and TLR-4 and NLRP3 expression independently of the diet, while in *dHFD*- and *dHFDQ*-receivers these parameters remain similar to *dC*-receivers fed with control diet even under HFD feeding. Thus, the protective and predisposing effects of *dHFD-* and *dHFDQ*, and *dHFD+* donors on NAFLD development seem to be mediated by the blockage or activation, respectively, of gut microbiota imbalance-mediated gut-liver axis alteration. *Akkermansia* presence in the gut microbiota of recipients fed with HFD could be involved in this divergent response, as suggested by the detection of an inverse relationship between the activation of inflammasome and *Akkermansia* relative abundance. This finding was accompanied by the existence of a positive correlation between *Akkermansia* spp. detection and the capacity of caecal microbiota to generate butyrate in HFD-fed receiver groups, supporting a proposed mechanism of action for *Akkermansia* that involves modulation of lipid metabolism genes through propionate and butyrate production.[45] It has been shown that the administration of HFD and *A. muciniphila* at the same time could moderate the impact of diet on metabolic endotoxemia and gut barrier impairment by regulating host-microbe interactions.[31, 34] In this regard, increased *Akkermansia* spp. relative abundance in HFD-fed receivers could exert a protective effect in a mechanism involving the improvement of intestinal bacterial imbalance and barrier integrity, as previously proposed,[14, 40, 46] suggesting that this genus can play a role as a potential strategy for preventing NAFLD development. Moreover, an increase in *Akkermansia* spp. relative abundance has been associated to polyphenols consumption in diet-induced obese models,[45, 47] reinforcing the protective effect of these substances, including quercetin, on metabolic diseases by means of intestinal microbiota modulation.

Moreover, *Helicobacter pylori* seems to contribute to the progression of NAFLD in a mechanism that could involve dysbiosis-induced endotoxemia and inflammatory response activation.[21] In HFD-fed *dC*-receivers supplementation with quercetin reduced the increased *Helicobacter* genus detection, supporting the results described in the A model.[12] Furthermore, this genus was notably absent in *dHFDQ*-receiver groups independently of the diet or quercetin supplementation, maintaining the gut microbiota phylotype exhibited by this donor. These results reinforce the protective role of quercetin on NAFLD development in a mechanism involving *Helicobacter* inhibition in these recipients. However, in view of controversial results observed in the other receiver groups, further investigations to establish the precise interaction between transplanted microbiota composition, HFD and quercetin supplementation and its influence in *Helicobacter* relative abundance are necessary.

In conclusion, results obtained indicate the existence of different microbial profiles as a consequence of the interaction between the particular microbiota transplanted from donors, diets and quercetin supplementation, leading to distinct susceptibilities to develop HFD-induced NAFLD in transplanted germ-free mice. Moreover, *Akkermansia* genus shows to play a key role in the development of the protective metabolic phenotypes in our model of NAFLD. Finally, our study provide scientific sustain of the suitability of established protective gut microbiota profiles transplantation as a potential therapeutic strategy against obesity-associated NAFLD development.

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**Contributors** DP, EN, SMF, MVGM and SSC performed most of the experiments. JLO and FJ performed statistical analysis. RJ and JGG assisted for *in vivo* models. SSC designed the experiments and supervised the study. All the authors wrote the manuscript.

**Disclosures**

The authors declare that they have no conflict of interest

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**Figure Legends**

**Figure 1** Changes in bacterial populations between donors. (A)Bar graphs representing the relative abundance of gut microbiota between donors at phylum level. (B) Bar graphs showing the microbial composition in donors at class level. (C) Boxplot representing differences in the number of reads between donors at the genus level. *dC* (control diet donor), *dCQ* (control diet supplemented with quercetin donor), *dHFD-* (non-responder to HFD donor), *dHFD+* (responder to HFD donor) and *dHFDQ* (HFD supplemented with quercetin donor).

**Figure 2** Effect of diets, supplementation with quercetin and intestinal microbiota transplantation from donors on obesity, insulin resistance and liver histological findings in germ-free mice. (A) Body weight gain at the end of the experiment. (B) HOMA-IR, homeostasis model assessment of insulin resistance. (C) Representative Hematoxylin & Eosin staining liver sections from receiver mice (×100) (right panel). NAFLD activity score (NAS) (calculated from individual scores for steatosis, lobular inflammation and ballooning) (left panel). Data are described as the means ± SEM. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 *vs dC*/C; #p<0.05, ##p<0.01, ###p<0.001 *vs dC*/HFD; ap<0.05, aap<0.01, aaap<0.001 *vs dHFD+*.

**Figure 3** Effect of diets, supplementation with quercetin and intestinal microbiota transplantation from the different donors on the bacterial community profile of germ-free recipients fed with control diet or HFD supplemented with or without quercetin. (A) Bar graph representing relative abundance of phyla in samples. (B) Bar graph showing the proportion of sequences from each sample that could be classified at the class level. (C) Boxplot showing differences in the number of reads of *Akkermansia, Helicobacter* and *Lactobacillus* genera. Statistical analysis were performed using Kruskal-Wallis followed by Mann-Whitney U test (P<0.05). Significant differences related to *Akkermansia* genus: \*p=0.029 *dC*/HFD *vs* *dC*/HFDQ, ap=0.008 *dHFD-*/CQ *vs* *dHFD-*/HFDQ and bp=0.032 *dHFD-*/HFD *vs* *dHFD-*/HFDQ. Significant differences related to *Helicobacter* genus: \*p=0.016 *dHFD+/*C *vs* *dHFD+*/CQ, ap=0.036 *dHFD+*/C *vs* *dHFD+*/HFD, bp=0.032 *dHFD+*/C *vs* *dHFD+*/HFDQ and cp=0.036 *dHFD+*/HFD *vs* *dHFD+*/HFDQ. Significant differences related to *Lactobacillus* genus: ap=0.032 *dHFD-*/C *vs* *dHFD-*/HFDQ, bp=0.008 *dHFD-/*CQ *vs* *dHFD-*/HFDQ, cp=0.036 *dHFD+*/C *vs* *dHFD+*/HFD, dp=0.036 *dHFD+*/CQ *vs dHFD+*/HFD, ep=0.036 *dHFD+*/HFD *vs* *dHFD+*/HFDQ, \*p=0.029 *dHFDQ*/C *vs* *dHFDQ*/HFDQ, #p=0.032 *dHFDQ*/CQ *vs* *dHFDQ*/HFDQ.

**Figure 4** Global effect of diets on gut microbiota composition of the different recipient groups. Principal Coordinates Analysis (PCoA) plot derived from the Morisita-Horn dissimilarity index at the genus level of *dC* (A), *dCQ* (B), *dHFD-* (C), *dHFD+* (D) and *dHFDQ* (E) recipient groups fed with control diet and HFD supplemented with or without quercetin. The percentage of the total variance explained is indicated in parenthesis in each axis.

**Figure 5** Effect of diets, supplementation with quercetin and intestinal microbiota transplantation from the different donors on SCFAs production and endotoxemia. (A) Acetate, propionate and butyrate levels were measured in caecal samples by gas chromatography-mass spectrometry (GC-MS). (B) Plasma LPS level was measured using the LAL Chromogenic Endotoxin Quantitation Kit. (C) Plasma ethanol level was measured using the colorimetric Ethanol Assay Kit. Data are described as the means ± SEM. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 *vs dC*/C; #p<0.05, ##p<0.01, ###p<0.001 *vs dC*/HFD; ap<0.05, aap<0.01, aaap<0.001 *vs dHFD+*.

**Figure 6** Effect of diets, supplementation with quercetin and intestinal microbiota transplantation from donors on gut-liver axis alteration and inflammasome initiation response. (A) Bar graphs show hepatic mRNA levels of TLR-4 determined by RT-qPCR (left panel). Representative western blot of TLR-4 protein expression in the liver of HFD-fed *dHFD-*, *dHFD+* and *dHFDQ*-receiver groups (3 samples per group are shown) (right panel). β-actin levels were used as a loading control. Bar graphs show densitometry analysis of specific bands expressed as percentage relative to *dHFD+*/HFD (100%). (B) Bar graphs show hepatic mRNA levels of NLRP3 determined by RT-qPCR. Data are described as the means ± SEM. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 *vs dC*/C; #p<0.05, ##p<0.01, ###p<0.001 *vs dC*/HFD; ap<0.05, aap<0.01, aaap<0.001 *vs dHFD+*.

**Figure 7** *Akkermansia* spp. population relative abundance correlated with body weight gain, HOMA-IR, NAFLD activity score, intestinal butyrate production and inflammasome activation. (A) Correlation analysis between *Akkermansia* and body weight gain, (B) Correlation analysis between *Akkermansia* and HOMA-IR, (C) Correlation analysis between *Akkermansia* and NAS, (D) Correlation analysis between *Akkermansia* and butyrate production, (E) Correlation analysis between *Akkermansia* and NLRP3 mRNA levels of HFD-fed mice receiver groups (*dC*/HFD, *dCQ*/HFD, *dHFD-*/HFD, *dHFD+*/HFD and *dHFDQ*/HFD). Pearson’s r correlation and corresponding P value were shown.

**Tables**

**Table 1** Caecal microbiota donors selection parameters.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | *dC* | Control | *dCQ* | CQ | *dHFD-* | HFD | *dHFD+* | *dHFDQ* | HFDQ |
| NAS | 0 | 0.33±0.21 | 0 | 0.21±0.15 | 2 | 3.55±0.57\*\*\* | 5 | 1 | 1.91±0.39\*\*## |
| Body weight gain (g) | 9.70 | 9.2±0.4 | 7.6 | 10.8±0.6 | 16.4 | 22.2±2.3\*\*\* | 28.3 | 9.42 | 17.2±2.1\*\*# |
| Liver weight (g) | 1.3 | 1.5±0.1 | 1.35 | 1.38±0.05 | 1.38 | 1.8±0.3 | 1.8 | 1.3 | 1.5±0.1# |
| Food intake (g/day) | 3.45 | 3.3±0.1 | 3.12 | 3.29±0.06 | 2.98 | 2.9±0.1 | 3.2 | 2.93 | 2.9±0.1\* |
| Fasting glycemia (mg/dl) | 99 | 111.7±7.3 | 107 | 103.2±7.8 | 150 | 170.6±18\*\*\* | 180 | 101 | 154.8±12\*\*# |
| Fasting insulinemia (ng/ml) | 0.45 | 0.4±0.4 | 0.48 | 0.35±0.03 | 1.14 | 1.6±0.5\*\*\* | 2.05 | 0.89 | 1.1±0.2## |
| HOMA-IR | 2.39 | 2.7±1.6 | 2.36 | 2.36±1.3 | 15.13 | 17.2±2.8\*\*\* | 18.15 | 7.17 | 11±1.5\*\*## |
| Liver TG (g/mg prot) | 69.5 | 76.9±9.5 | 75 | 74.3±4.2 | 153 | 219.9±27.4\*\*\* | 332 | 72 | 157.17±20.47\*\*## |
| LPS (U/ml) | 1.85 | 2.2±0.1 | 1.76 | 1.9±0.1 | 2.5 | 3.8±0.21\*\* | 4.15 | 2.4 | 2.5±0.31## |
| Ethanol (nM) | 0.055 | 0.067±0.0026 | 0.055 | 0.062±0.0034 | 0.069 | 0.09±0.0029\*\* | 0.098 | 0.066 | 0.069±0.0027# |
| TLR-4 mRNA | 0.61 | 1±0.1 | 0.66 | 0.99±0.1 | 11.7 | 15.16±3.3\*\*\* | 70.6 | 4.31 | 4.9±0.6\*\*### |
| NLRP3 mRNA | 0.85 | 1±0.081 | 0.84 | 0.94±0.04 | 1.1 | 1.27±0.08\*\* | 1.8 | 0.73 | 0.86±0.09## |
| TNF mRNA | 0.35 | 1±0.098 | 0.68 | 0.9±0.1 | 1.82 | 3.3±0.56\*\* | 10.4 | 1.1 | 1.16±0.17## |
| FAT/CD36 mRNA | 0.86 | 1±0.098 | 0.56 | 0.94±0.095 | 1.65 | 2.36±0.34\*\*\* | 4.99 | 1.07 | 1.62±0.19\*## |
| LXR mRNA | 0.83 | 1±0.1 | 1.1 | 0.97±0.1 | 0.96 | 1.2±0.54\*\* | 1.7 | 0.76 | 0.97±0.06## |
| FAS mRNA | 0.53 | 1±0.085 | 1.2 | 1.1±0.3 | 0.63 | 1.35±0.56\*\*\* | 1.43 | 0.8 | 1.045±0.022### |

Data are means ± SEM (n=6 mice per group). \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 *vs* Control; #p<0.05, ##p<0.01, ###p<0.001 *vs* HFD. LXR, liver X receptor alpha; FAT/CD36, fatty acid translocase CD36; FAS, fatty acid synthase; TLR-4, toll-like receptor 4; TNF- tumor necrosis factor; NLRP3, NOD-like receptor family pyrin domain containing 3.