A network involving gut microbiota, circulating bile acids and hepatic metabolism genes that protects against NAFLD

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**Abbreviations**:

BA, bile acid; Bsep, bile salt export pump; Car, constitutive androstane receptor; Cd36, cluster of differentiation 36 / fatty acid translocase; Cebpa, CCAAT/enhancer binding protein (Cebp) alpha; Cyp7a1, cytochrome P450 family 7 subfamily A member 1; Cyp8b1, cytochrome P450, family 8, subfamily b, polypeptide 1; DCA, deoxycholic acid; Fatp5, fatty acid transport protein 5; Fxr, farnesoid X receptor; GFm, germ-free mice; Gnmt, glycine N-methyltransferase; HDCA, hyodeoxycholic acid; HFD, high-fat diet; HFDQ, HFD + 0.05% (wt/wt) aglycone quercetin; Hnf4a, hepatocyte nuclear factor 4 alpha; HOMA-IR, homeostasis model assessment of insulin resistance; LPS, lipopolysaccharide; Lxra, liver X receptor alpha; Mat1a, S-adenosylmethionine synthase isoform type-1; MCA, muricholic acid; Mdr2, multidrug resistance protein 2; NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD Activity Score; NASH, nonalcoholic steatohepatitis; Ntcp, sodium-dependent uptake transporter; Pxr, pregnane X receptor; SCFAs, short chain fatty acids; Shp, small heterodimer partner; TCA, taurocholic acid; TDCA , taurodeoxycholic acid; TGR5, takeda G-protein-coupled receptor 5; TG, triglyceride; THDCA, taurohyodeoxycholic acid; TωMCA, tauro-omegamuricholic acid; UPLC-MRM-MS, ultra‐performance liquid chromatography-multiple reaction monitoring-mass spectrometry; αMCA, alphamuricholic acid; βMCA , betamuricholic acid; BSH, bile salt hydrolase.

# ABSTRACT

Gut microbiota contributes to nonalcoholic fatty liver disease (NAFLD) development and progression by multiple mechanisms, not yet completely understood. In this study we investigate the differences between germ-free mice (GFm) transplanted with protective or non-protective intestinal microbiota against NAFLD. Caecal samples from 4 donor mice (control diet, high-fat diet (HFD) responder, HFD non-responder and quercetin-supplemented HFD) with different grade of obesity, metabolic syndrome and NAFLD, were transplanted to GFm mice, which were placed on control diet or HFD for 16-weeks. Gut microbiota genera (n=11 from 4 phyla), serum bile acids (BAs) (n=24) and liver mRNAs (n=24) were quantified. Multivariate analysis showed that GFm colonized with caecal samples from HFD-non-responder and quercetin-supplemented HFD donors clustered together (protected phenotype), whereas GFm colonized with microbiota from control and HFD-responder mice established another cluster (non-protected phenotype). The protected phenotype was associated with loss of *Bacteroides* (*Bacteroidetes* phylum), increased *Desulfovibrio* (*Proteobacteria* phylum) and alterations in *Firmicutes* phylum (increased *Oscillospira* and decreased *Oribacterium*) in the gut. This was paralleled by lower abundance of primary BAs (-muricholic acid (MCA) and -MCA) and increased abundance of secondary BAs (deoxycholic acid (DCA), tauro--MCA and derivatives) in serum, and with a specific gene expression profile characterized by repression of hepatic lipogenic and BA synthesis genes (Lxra, Cebpa, Car, Pxr, Gnmt, Mat1a, Fat/Cd36 and Cyp7a1), and induction of BA transporter genes (Ntcp, Fatp5, Mdr2 and Bsep). Correlation analysis demonstrated strong associations between the altered gut microbiota genera or the altered liver mRNAs, and the modified serum BAs, pointing to these BAs as the signaling mediators between gut and liver in a coordinated response against NAFLD. In conclusion, protective gut microbiota associates with increased synthesis of specific secondary BAs, which likely inhibit lipogenic pathways and enhance biliary flux in the liver, revealing a novel cross-talk between gut and liver, via serum BAs, that promote a protective hepatic phenotype against NAFLD.

# INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is one of the most important causes of hepatic alteration worldwide and will likely emerge as the leading reason of end-stage liver disease in the near future, thus placing a growing strain on health-care systems. NAFLD has a global prevalence of 24% and involves a high risk of liver-related morbidity and mortality along with metabolic comorbidities (Younossi et al. 2018). NAFLD covers a wide spectrum of histologic lesions, ranging from isolated hepatic steatosis to nonalcoholic steatohepatitis (NASH), the latter characterized by the presence of lobular inflammation and hepatocyte ballooning, with or without fibrosis (Chalasani et al. 2018). NASH, with a prevalence among biopsied NAFLD patients of 59% (Younossi et al. 2016), and advanced fibrosis have been associated with a risk of evolution to cirrhosis and hepatocellular carcinoma, and increased liver-related and cardiovascular mortality (Goldberg et al. 2017; Chalasani et al. 2018).

In the last few years the pathogenesis concept of NAFLD has evolved to the multiple parallel hit hypothesis, considering factors that act simultaneously as insulin resistance, oxidative stress, deregulation of lipid metabolism, release of inflammatory cytokine or endoplasmic reticulum stress (Tilg and Moschen 2010; Buzzetti et al. 2016). In addition, germ-free mice-based studies have demonstrated that intestinal microbiota influences weight gain, body composition and insulin sensitivity (Backhed et al. 2004; Bäckhed et al. 2007). Similarly, it has been shown that NAFLD is a transmissible condition by means of intestinal microbiota transplantation (Le Roy et al. 2013). Underlying mechanisms are not completely understood yet, but they include dysbiosis-induced deregulation of the intestinal barrier integrity and alterations in the metabolism of dietary and endogenous compounds, among others (Porras et al. 2018).

The gut microbiota not only determines a more or less efficient harvesting of nutrients and energy from the ingested food but also produces numerous metabolites that signal to regulate host metabolism, including ethanol, short chain fatty acids (SCFAs), secondary BAs and choline derivatives (Wahlström et al. 2016; Porras et al. 2018). BAs are produced in the liver from cholesterol and metabolized in the intestine by the gut microbiota. This interplay modulates the signaling properties of BAs via the Farnesoid X receptor (FXR) and Takeda G-protein-coupled receptor 5 (TGR5) receptors, which regulate numerous metabolic pathways in the host. Conversely, BAs can modulate gut microbiota composition both directly and indirectly through activation of innate immune genes in the small intestine (Wahlström et al. 2016).

Although the interplay between gut microbiota and BAs metabolism has been known for over 60 years, several recent studies with paradoxical findings (e.g. beneficial effects by both intestinal-specific FXR activation and inhibition) highlight the importance of further investigating this interaction in the regulation of host physiology and disease (Taylor and Green 2018).

Due to the outstanding role of gut microbiota in NAFLD, new strategies to modulate it emerge as potential therapeutic approaches. Among them, administration of substances with prebiotic properties as nutritional supplements, probiotics or faecal microbiota transplantation have been tested with promising results (Porras et al. 2018). Thus, quercetin, a flavonoid widely studied for its antioxidant and anti-inflammatory properties, has shown a potential prebiotic effect modulating gut microbiota composition in animal models of metabolic syndrome and NAFLD (Pisonero-Vaquero et al. 2015; Porras et al. 2017). Moreover, in a recent study, caecal microbiota transplantation from both non-responder to HFD and quercetin-treated donors triggered a protected metabolic phenotype that strongly determine susceptibility to NAFLD development, by mechanisms not yet completely understood (Porras et al. 2019). The aim of this study is to investigate the impact of protective and non-protective microbiota transfer from different donor mice into GFm on gut microbiota, circulating BAs and liver BAs- and lipid-metabolism genes to characterize the key players in the gut-liver axis that promote protection against NAFLD.

# METHODS

**Animals and Experimental Protocol**

C57BL/6J conventional male mice were fed Control (10% of energy from fat; D12450J); HFD (60% energy from fat; D12492) or HFDQ (HFD + 0.05% (wt/wt) aglycone quercetin; D14062802) diets for 16-weeks (Research Diets, Inc. New Brunswick, NJ. USA). Mice were euthanized under anesthesia and the caecal contents were immediately preserved at -80°C in skim milk (10%) supplemented with cysteine (0.5 g/l) as a reducing agent. Four donor mice (*dC*, *dHFD+*, *dHFD-* and *dHFDQ*) were selected based on their differences in terms of obesity, metabolic syndrome and NAFLD severity (Supplementary Figure 1). *dHFD+* showed more body weight gain, insulin resistance, steatosis and NAFLD Activity Score (NAS) than *dHFD-* and dHFDQ (Supplementary Table 1).

Germ-free male C57BL/6J mice (Anaxem, MICALIS Institute) were colonized by a single oral-gastric gavage with 250 µl of caecal content collected from donors. The mice were distributed in 8 groups according with the microbiota transplanted (*dC*, *dHFD+*, *dHFD-* or *dHFDQ*) and the diet (control: C or high-fat: HFD) (Supplementary Figure 1). After 16 weeks of treatment the animals were sacrificed under anesthesia. Plasma, caecal content and liver tissue were collected as previously indicated (Porras et al. 2019).

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.

**Histopathology**

To analyse the degree of steatosis and steatohepatitis, paraffin-embedded liver samples were sectioned in a microtome followed by hematoxylin and eosin stain (H & E). Liver lesions were evaluated according with the NAFLD Activity Score (NAS) proposed by Kleiner et al. NAS provide a numerical score corresponding to 3 histological features: steatosis (0−3), lobular inflammation (0−3) and hepatocellular ballooning (0−2). Scores more than 5 were diagnosed of NASH, and score less than 3 were associated with “no NASH.”

**Biochemical analysis**

Plasma glucose concentration was analysed with the Accu-Chek (Roche Diagnostics, Almere, The Netherlands) after an 8-h fast. Insulin resistance was calculated by the homeostasis model assessment of insulin resistance (HOMA-IR) using the formula:

HOMA−IR = Fasting glucose (mg/dl) × Fasting insulin (μU/ml)/405

Endotoxemia was determined quantifying plasma levels of lipopolysaccharide (LPS) and ethanol following the instructions of the commercial kits (LAL Chromogenic Endotoxin Quantification Kit, Thermo Scientific, and Ethanol Colorimetric Assay Kit, Biovision, respectively). Liver triglycerides (TGs) were analysed by the Instrumental Techniques Laboratory of the University of León using standard techniques.

**Gut microbiota and bioinformatic analysis**

Genomic DNA purification, PCR amplification and 16 sRNA sequencing were performed according to protocols described elsewhere (Porras et al. 2019). Bacterial taxonomical composition was determined using BaseSpace Application 16S Metagenomics v1.0 (Illumina Inc.). Processed reads were then clustered in Operational Taxonomic Units (OTUs) using UCLUST with a similarity threshold of 0.97 and were subsequently aligned using PyNast against 16S reference database GreenGenes (version 13.8) using default parameters. All the microbiota analyses were estimated using Vegan package (https://cran.r-project.org/web/packages/vegan) in R software (R Development Core Team, 2011).

**Serum bile acid profiling**

Serum BAs were profiled by a UPLC-MRM-MS method as described previously (García-Cañaveras et al. 2012). Briefly, 50 μL of serum and plasma samples were spiked with deuterated internal standards. Then proteins were precipitated and samples were dried and reconstituted in 50 μL methanol:water (50:50, V/V). Samples were analysed using an Acquity UPLC system (Waters, UK) equipped with an Acquity UPLC BEH C18 column (1.7μm, 2.1 x 100 mm; Waters). The MS analysis was performed using a Waters Xevo TQ-S mass spectrometer (Waters) with an ESI source working in the negative-ion mode. This method, validated according to the Food and Drug Administration guidelines, allows the quantification of 12 non-conjugated, 8 glycine-conjugated, and 11 taurine-conjugated BA, using 5 additional deuterated BA as internal standards in a single analytical run. These analyses were performed in the Analytical Unit, Core Facility, IIS Hospital La Fe in Valencia, Spain.

**RNA purification, reverse transcription and real time PCR**

Frozen mouse liver samples (25–50 mg) were placed in 2-ml tubes containing CK14 ceramic beads (Precellys) and 800 ml of RLT buffer (Qiagen). Then, liver tissues were homogenized twice for 10 seconds at 6000 rpm at 4°C in a Precellys 24 dual system equipped with a Criolys cooler. Tubes were centrifuged at 3000g for 5minutes at 4°C. Next, total RNA was extracted from the supernatants with the RNeasy Plus Mini Kit (Qiagen), according to manufacturer´s instructions. The final concentrations of RNA were determined by Nanodrop (ND-1000) spectrophotometer.

Twenty-four mouse genes were selected for a panel representative of hepatic BA and lipid metabolism, transport and regulation. For RT-qPCR gene expression analyses, total RNA (1µg) was reverse transcribed using the Moloney murine leukemia virus reverse transcriptase (Invitrogen) and oligo-dT14 (Pérez et al. 2003). Diluted cDNA (2.5 ng cDNA equivalents per reaction) was amplified with a rapid thermal cycler (LightCycler Instrument LC480, Roche Applied Science) in 9.5 µl of LightCycler DNA Master SYBR Green I (Roche Applied Science) and 0.5 µM of each primer (Sequences of each primer are provided in Supplementary Table 2). Data were normalized using the geometric mean of the mRNA concentration of the reference genes Actb, Rplp0 and Tbp as internal controls. Relative mRNA levels were calculated and expressed as fold induction over controls (value=1.0), taking into account the efficiency of amplification for each gene (Pfaffl 2001).

**Statistical analysis**

All statistical analyses were performed using SPSS 22.0 (Chicago, IL, USA) or GraphPad PRISM (La Jolla, CA, USA) software. When multiple comparisons were made, the statistical significance of the mean differences was evaluated by one-way ANOVA followed by Tukey HD test, by two-way ANOVA followed by Bonferroni test, or by nonparametric Kruskal-Wallis test followed by Mann-Whitney U test, as indicated. Comparisons between two groups were made by the Student's t-test. P < 0.05 was assumed statistically significant.

# RESULTS

Effect of diets and intestinal microbiota transplantation from donors on obesity, insulin resistance and NAFLD development in GFm

*dC*-receiver mice fed with HFD (*dC*/HFD) displayed increased body weight gain and impaired insulin sensitivity (+373%; +530%, respectively) vs *dC*-receiver mice fed with control diet (*dC/*C). Conversely, *dHFD-*/HFD and *dHFDQ*/HFD mice showed reduced body weight gain (-48% and -49%, respectively) and insulin resistance (HOMA-IR: -82% and -76%, respectively) in comparison with *dHFD+*/HFD mice.

Hepatic histopathological evaluation showed microvesicular and macrovesicular steatosis in both *dC*/HFD and *dHFD+*/HFD mice, concomitant with an increase in hepatic triglycerides (+134% and +104%, respectively) and in NAS score (+313% and +300%, respectively), compared to *dC*/C mice (Supplementary Figure 2).

*dHFD*-/HFD and *dHFDQ*/HFD mice showed non-pathological hepatic histology despite HFD feeding, being protected from the increase in NAS observed in *dC*/HFD and *dHFD+*/HFD mice, and exhibiting similar results to *dC*/C mice. As expected, *dHFD-*/HFD and *dHFDQ*/HFD mice showed reduced hepatic triglyceride content (-60% and -55%, respectively) in comparison with *dHFD+*/HFD mice (Supplementary Figure 2).

Therefore, *dHFD*- and *dHFDQ*-transplanted mice are protected against HFD-induced NAFLD. In contrast, mice transplanted with *dC* and *dHFD+* were not protected and developed NAFLD.

# Multivariate analysis of GFm colonized with protective and non-protective microbiota

Hierarchical cluster analysis of eight-groups of mice based on more than 60 variables including liver genes (n=24 mRNAs), circulating BAs (n=24), ethanol, LPS, and gut microbiota genera (n=11 from 4 phyla) showed that colonized GFm clustered in three main groups: (**1**) *dC*/C and *dHFD+*/C, (**2**) *dHFD-*/C and *dHFDQ*/C and (**3**) all colonized GFm on HFD (Figure 1).

In the 3rd group, two subclusters were visible: (**3.1**) *dHFDQ*/HFD and *dHFD-*/HFD and (**3.2**) *dC*/HFD and *dHFD+*/HFD (Figure 1).

Thus, GFm colonized with caecal samples from non-responder HFD (*dHFD-*) or quercetin supplemented HFD (*dHFDQ*) donors clustered together (protected phenotype), whereas GFm colonized with microbiota from control (*dC*) and HFD responder mice (*dHFD+*) established other clusters (non-protected phenotype).

A supervised Partial Least-Squares Discriminant Analysis (PLS-DA) generated a well-performing model (Accuracy:0.976, R2:0.9325, Q2:0.8511, Latent Variables:2) to discriminate between NAFLD-protected and non-protected mice (Figure 2A). The 25 best discriminating variables included liver genes, BAs, ethanol, and bacteria genera/phyla (Figure 2B).

# Gut microbiota profiles in NAFLD-protected and non-protected mice

Considering gut microbiota composition from caecal samples from GFm recipients colonized with microbiota from the different donors, we observed a new gut microbiota profile associated with mice protected against NAFLD. Regarding variations at the phylum level, we found that the protected phenotype was associated with a significant decrease in *Bacteroidetes*, whereas *Firmicutes* showed a non-significant increase (Figure 3A). Thus, the ratio *Firmicutes/Bacteroidetes* was significantly increased in protected mice (Figure 3B). The protective phenotype was associated with the loss of two genera in the gut, *Bacteroides* and *Oribacterium*, and with significant increases in *Desulfovibrio* and in *Oscillospira* (Figure 3C).

# Liver gene expression profiles in NAFLD-protected and non-protected mice

The mRNA level of the two principal hepatic BAs transporters, Ntcp (basolateral) and Bsep (canalicular), were significantly induced in GFm colonized with protective microbiota (HFD- and HFDQ) regardless of the diet (C or HFD) (Figure 4A).

Moreover, the hepatic expression of Fatp5/Slc27a5 (a liver-specific BA-CoA ligase) and the canalicular biliary transporters Mdr2/Abcb4 and Mrp2/Abcc2 were significantly increased in mice protected against NAFLD (Figure 4B).

Results indicate that colonization of GFm with protective microbiota (HFD- and HFDQ) triggers an enhanced hepatic bile flow.

Regarding BAs synthesis, protected mice showed reduced expression of Cyp7a1, whereas Cyp8b1 expression did not change. The repression of Cyp7a1 was independent of Shp (Figure 4C).

Several key transcription factors related to lipid metabolism were also downregulated in the livers of GFm colonized by protective microbiota. Specifically, we observed lower hepatic mRNA levels of the lipogenic nuclear receptors, Lxra and Pxr, and the adipogenic transcription factor Cebpa (Figure 5A and 5B). Moreover, the nuclear receptors Car and Hnf4a, both related to lipid and BA metabolism, were also downregulated in protected mice (Figure 5A and 5B).

Finally, some key genes related with fatty liver were also repressed in the group of mice protected from NAFLD. Cd36, Mat1a and Gnmt mRNAs were significantly downregulated in the livers of protected mice (Figure 5C).

# Serum BAs, ethanol and LPS levels in NAFLD-protected and non-protected mice

Our results indicate that colonization of GFm with specific caecal samples protects against NAFLD by stablishing a particular gut microbiota that, in turn, influences gene expression in the liver, where BA flow is enhanced and lipogenic factors are repressed. Among the several metabolic signals that may participate in this communication in the gut-liver axis we have investigated BAs, ethanol and LPS.

We performed an extensive serum BAs profile by UPLC-MRM-MS and found significant differences between protected and non-protected mice in the primary and secondary BAs concentrations (lower abundance of primary and increased abundance of secondary, Figure 6A). However, the level of total, conjugated and unconjugated BAs was similar in both groups (data not shown).

Regarding specific BA species, protected mice showed lower serum levels of MCA and MCA (primary) and higher levels of their secondary derivatives MCA and HDCA (Figure 6B). Similarly, protected mice showed a decrease in TCA (primary) that was mirrored by an increase in its secondary derivative DCA and TDCA (Figure 6C).

Finally, and in agreement with previous results (Porras et al. 2019), ethanol and LPS levels were also reduced in the sera of protected mice (Supplementary Figure 3).

Results suggest that the protective gut microbiota (dHFD- and dHFDQ) influences the profile of serum BAs by promoting the synthesis of specific secondary BAs. These metabolites, along with lower ethanol and LPS levels could adapt transcriptomic programs in the liver to protect against lipid accumulation and NAFLD.

Associations among gut microbiota, liver gene expression and serum metabolites.

We wanted to know first whether the different microbiota genera/phyla associate with the expression of specific genes in the liver. Pearson correlation analyses showed that the relative abundancy of the genus *Bacteroides* in the gut strongly correlates with most of the genes with significant differences in liver (Table 1). For example, more *Bacteroides* (i.e. in non-protected mice) associated with more lipogenic gene expression (e.g. Rpearson=0.82 with Cebpa) and with lower expression of BA transporter genes (e.g. Rpearson=-0.64 with Bsep) (Table 1). *Oribacterium*, the other genus being more abundant in non-protected mice, also positively associated with some lipogenic genes such as Cd36 (Rpearson=0.61) (Table 1). Conversely, *Desulfovibrio* and *Oscillospira*, which were more abundant in protected mice, correlated positively with Ntcp, Bsep and Fatp5 and negatively with Car and Mat1a (Table 1). Therefore, results support that different microbiota profiles distinctly influence the expression of liver genes related with lipid metabolism and bile flow.

Next, we hypothesized that circulating BAs, ethanol and LPS could be the signaling mediators in this gut-liver axis communication. If so, these metabolites should correlate with both bacterial genera and liver genes. Indeed, Pearson correlation analyses between serum BAs and gut microbiota genera/phyla demonstrate that the increase in *Desulfovibrio* and decrease in *Bacteroides* and *Oribacterium* observed in NAFLD-protected mice strongly correlates with less primary (/MCA (*Bacteroides*) and TCA(*Oribacterium*)) and more secondary (TDCA and THDCA (*Bacteroides*) and TMCA (*Oribacterium* and *Desulfovibrio*)) BAs (Table 2).

Strong correlations were also observed between liver gene expression and serum BAs. The decreased activity of lipogenic genes in protected mice associated with lower levels of primary MCA and MCA. Whereas upregulation of Bsep, Fatp5 and Mrp2/Abcc2 associated with higher levels of secondary DCA and TDCA (Table 3). It is also worth mentioning the strong correlation of Cd36 with TCA (0.830) and DCA (-0.604), and that of Mat1a with THDCA (-0.52). Regarding serum ethanol and LPS, positive correlations with Car and Cyp7a1 and negative correlations with Ntcp and Bsep were also observed (Table 3).

Results allow concluding that the increase in *Desulfovibrio* and the loss of *Bacteroides* and *Oribacterium* in protected mice strongly correlated with reduced primary (TCA, MCA and MCA) and increased secondary (TDCA, TMCA and THDCA) BAs in serum. In addition, these primary BAs correlated positively with hepatic lipogenic genes and negatively with hepatic biliary transporters; whereas secondary BAs show an opposite association. Besides BAs, ethanol and LPS from gut microbiota may also influence some lipogenic and BA transport genes.

# DISCUSSION

BAs have emerged as signaling molecules with important roles in metabolism, inflammation and fibrosis. Beyond lipid emulsification, BAs are capable of stimulating signaling pathways related to lipid and insulin homeostasis through FXR and TGRF5 (Chávez-Talavera et al. 2017; Arab et al. 2017), thus connecting BAs with prevalent metabolic diseases such as NAFLD. Moreover, a bidirectional relationship between BAs and gut microbiota has been described. In this regard, chemical and bactericidal properties of BAs can alter microbiota composition, whereas some bacterial genera possess enzymes that can modify the BA profile (Ridlon et al. 2014).

The disturbance of the gut microbiota (dysbiosis) and the intestinal epithelial barrier has also been associated with many diseases, including NAFLD. GFm are considered a suitable model to investigate in depth the pathogenic mechanisms of these diseases. In the present study, GFm were colonized with caecal microbiota from control (*dC*), HFD-fed (*dHFD+* and *dHFD-* as responder and non-responder to diet, respectively) and HFD-fed supplemented with quercetin (*dHFDQ*) donors. In a previous study, we investigated whether gut microbiota transplantation can influence NAFLD; and provided scientific support for the prebiotic capacity of quercetin and the transfer of favorable metabolic profiles through microbiota transplantation as a protective strategy against NAFLD (Porras et al. 2019). However, mechanistic insights remained to be elucidated. Due to the strong link between intestinal microbiota and BA metabolism, we focused on circulating BAs as well as liver BA and lipid metabolism genes, and their relationship with NAFLD development. Results suggest that protective gut microbiotas (*dHFD-* and *dHFDQ*) influence the profile of serum BAs by promoting the synthesis of several secondary BAs. These metabolites, along with lower ethanol and LPS levels adapt transcriptomic programs in the liver to protect against lipid accumulation and NAFLD.

Multivariate analysis confirms the aforementioned clustering of GF recipients on a protected and a non-protected phenotype. Regarding circulating BAs, the main difference between the two phenotypes was a reduction of primary and enhanced secondary in the protected group. This is in agreement with a recent study which described increased primary and reduced secondary BAs in plasma of NAFLD and NASH patients (Puri et al. 2018). Previous studies have also reported an increase in serum (Kalhan et al. 2011) and fecal (Mouzaki et al. 2016) primary BAs in NAFLD patients. Furthermore, supplementation of a HFD with soybean protein isolate increased the secondary/primary BA ratio in mice, conferring metabolic benefits (Watanabe et al. 2018). This increase in primary BAs in NAFLD could be attributed to an enhanced synthesis through CYP7A1, the rate-limiting enzyme in this process, that has been reported to be overexpressed in NAFLD patients and HFD-fed rats (Jiao et al. 2017; Puri et al. 2018). In the present study, CYP7A1 downregulation has been shown in protected mice, justifying the reduction in primary αMCA and βMCA. Moreover, we also observed downregulation of BA transporters NTCP and BSEP in the livers of non-protected mice. Reduced BSEP expression could as well be responsible for the increase in serum primary BAs (Puri et al. 2018) and has also been associated with hepatic lipid accumulation in mice (Arab et al. 2017).

Although alterations in BAs synthesis and transport sustain the increase in primary BAs in the non-protected phenotype, a different mechanism must be involved in the increase in secondary/primary ratio observed in the protected phenotype. Transformation of primary BAs into its secondary derivatives is carried over by the intestinal microbiota. It is noteworthy that GFm lack secondary BAs, as they are produced due to metabolism of primary BAs by commensal bacteria (Sayin et al., 2013). Thus, differences in secondary BA pattern in our GF model depends on the microbiota transplanted. Metagenomic studies revealed differences in intestinal microbiota composition between protected and non-protected mice. At the phylum level the protected phenotype showed increased *Firmicutes* and decreased *Bacteroidetes* relative abundance, resulting in a higher *Firmicutes/Bacteroidetes* ratio. Enriched *Firmicutes* population has been related to increased ability to produce secondary BAs by the gut microbiota (Ridlon et al. 2014). Moreover, microbial signature of the protected phenotype was associated with reduced *Bacteroides*, and *Oribacterium* and increased *Desulfovibrio*, and *Oscillospira*; and some of these genera have potential implications in NAFLD development. The role of *Bacteroides* in NAFLD is controversial, as conflicting results have been reported in diet-induced obese rodents (Chang et al., 2015; Song et al., 2017; Yin et al., 2018; Kong et al., 2019). However, increased *Bacteroides* abundance was described in all stages of NAFLD in a HFD-streptozotocin mouse model (Xie et al. 2016). Moreover, it has been described in NAFLD patients the existence of an association between *Bacteroides* genus and the worsening of the disease and progression to NASH (Boursier et al. 2016). No relation between *Oribacterium* and NAFLD has been described to date. Nonetheless, this genus could be related to liver disease as increased relative abundance of *Oribacterium* has been found in salivary microbiota of primary sclerosing cholangitis patients (Iwasawa et al. 2018). On the contrary, *Oscillospira* has been related to a healthy metabolic profile, being diminished in obese adults and pediatric NAFLD patients (Del Chierico et al. 2017; Kashtanova et al. 2018).

More relevant to this study is the functionality of the above-mentioned genera, specially their role in BAs processing. *Bacteroides* is one of the few genera that its known to have bile salt hydrolase (BSH) activity (Chen et al. 2018), the enzymatic activity which drives the process of deconjugation of BAs. However, in this study in spite of a significant difference in the relative abundance of this genus between the two phenotypes, no differences were found in the conjugated/unconjugated BAs ratio. Nonetheless, *Bacteroides* relative abundance was positively correlated with primary unconjugated bile BAs (αMCA and βMCA) and negatively correlated with conjugated secondary BAs (TDCA and THDCA), which is in agreement with its BSH activity. Furthermore, *Bacteroides* and its related BAs profile was associated with increased expression of lipogenic genes, including Lxrα, Cebpα and Gnmt. Lxrα activation results in enhanced lipid synthesis in the liver through subsequent activation of lipogenic genes (e.g. FAS and ACC), but also induces BAs synthesis trough Cyp7A1 (Wang and Tontonoz 2018), thus pointing a link between *Bacteroides* abundance, primary-enriched BA pool and enhanced lipid synthesis in the non-protected phenotype. Regarding Cebpα, it is known that p300-C/EBPα/β complexes activate the promoters of five genes that drive triglyceride synthesis, and that this pathway is activated in the livers of patients with NAFLD (Jin et al. 2013). *Bacteroides* also positively correlated with Gnmt and Mat1a, two key genes involved in the control of S-adenosylmethionine levels, which are directly related to the abundancy of phosphatidylcholine (van der Veen et al. 2017). Both low and high levels of phosphatidylcholine lead to NAFLD (Jacobs et al. 2013).

On the other hand, *Desulfovibrio* was positively associated with secondary BAs. No primary BAs-processing activity has been described yet for *Desulfovibrio* species, but it has been shown that in sulfide-rich conditions, a derivative of sulfate-reducing bacteria like *Desulfovibrio*, could stimulate secondary BAs formation (Ridlon et al. 2016). *Oscillospira* was also correlated with the secondary DCA. *Oscillospira* belongs to the *Ruminococcaceae* family, which along with *Coriobacteriaceae, Clostridiaceae, Lachnospiraceae* are the bacterial families known to produce secondary BAs (Just et al. 2018). A recent study carried out in hypercholesterolemic mice supplemented with a probiotic *Lactobacillus* strain reported an association of *Oscillospira* with reduced cholesterol levels and increased BAs excretion, postulating an enzymatic activity for BAs processing in this genus (Lye et al. 2017). Moreover, *Oscillospira* correlated with secondary BAs in gallstone patients, who showed higher levels of 7α-dehydroxylating bacteria (Wells et al. 2000), suggesting that this activity could be present in *Oscillospira* (Keren et al. 2015).

Both *Desulfovibrio* and *Oscillospira* were also positively associated with Ntcp and Bsep expression in the liver, which are upregulated in the protective phenotype, while *Oribacterium* showed an opposite pattern. Downregulation these liver BAs transporters could be associated with NAFLD progression (Okushin et al. 2016). Bsep is under the control of Fxr, and Fxr activity, in turn, is largely dependent on specific BAs. In the protected phenotype, the increase of some secondary BA such as DCA and TDCA (Fxr agonists (Wang et al. 1999; Makishima et al. 1999)) concomitantly with a decrease of primary BA such as αMCA and βMCA (Fxr antagonists (Sayin et al. 2013; Hu et al. 2014)) would lead to activated Fxr and increased Bsep expression. Altogether, our results suggest that the protected phenotype is related to a more efficient Fxr signaling and enhanced enterohepatic circulation of BAs, which, in turn, potentially prevents hepatotoxicity by BA.

Hepatic fatty acid uptake is also determinant to NAFLD development as 60% of hepatic triglycerides comes from non-esterified fatty acids in plasma (Kawano and Cohen 2013). Gut microbiota and BA pool could be as well relevant in the regulation of this process. *Oribacterium* (enriched in non-protected mice) was found to be strongly correlated with TCA and Cd36, a fatty acid translocase involved in the incorporation of fatty acids. Enhanced expression of Cd36 has been observed in NAFLD (Miquilena-Colina et al. 2011). On the contrary, Fatp5 was inversely correlated with TCA and positively correlated with DCA and *Oscillospira* (enriched in protected mice). Fatp5 is a multifunctional protein involved in the uptake of long-chain fatty acids and in the activation of BA with CoA prior to amidation. Fatp5 was upregulated in the protected phenotype but this likely would not result in more fatty acid uptake as BA such as DCA are potent inhibitor of Fatp5 (Nie et al. 2012), thus attributing antisteatotic properties to this BA.

Our results demonstrate strong associations between specific gut bacteria and liver genes, between gut bacteria and serum BAs and between liver genes and serum BAs (Tables 1-3). However, a remaining question is whether these pair associations are consistent when gut, serum and liver are considered altogether. A more in depth analysis demonstrate consistent linkages among these three parties (Figure 7). Thus, the strong correlation between *Bacteroides* and lipogenic genes (Lxra, Cebpa and Gnmt) could be explained by the positive associations of this genus with MCA and MCA, and its negative associations with TDCA and THDCA (Figure 7A). Conversely, the significant correlations of *Oribacterium*, *Desulfovibrio* and *Oscillospira* with Ntcp and Bsep could be explained by the significant association of these genera with secondary BA, and with TCA and DCA (Figure 7B). Finally, the decreases in gut *Oribacterium* and *Bacteroidetes* observed in NAFLD-protected mice could explain the downregulation of liver Cd36 and the upregulation of liver Mrp2/Abcc2 through decreased TCA (Figure 7A) and increased TDCA (Figure 7B), respectively.

In conclusion, protective gut microbiota against NAFLD associates with increased activity in the synthesis of secondary BAs, which likely inhibit lipogenic pathways and enhance biliary flux in the liver, suggesting a cross-talk between gut and liver, via serum BAs, to establish a protective hepatic phenotype. Further studies are needed to unequivocally prove causal relationships and intracellular pathways involved.

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