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A gut microbiota-independent mechanism shapes the bile acid pool in mice with MASH

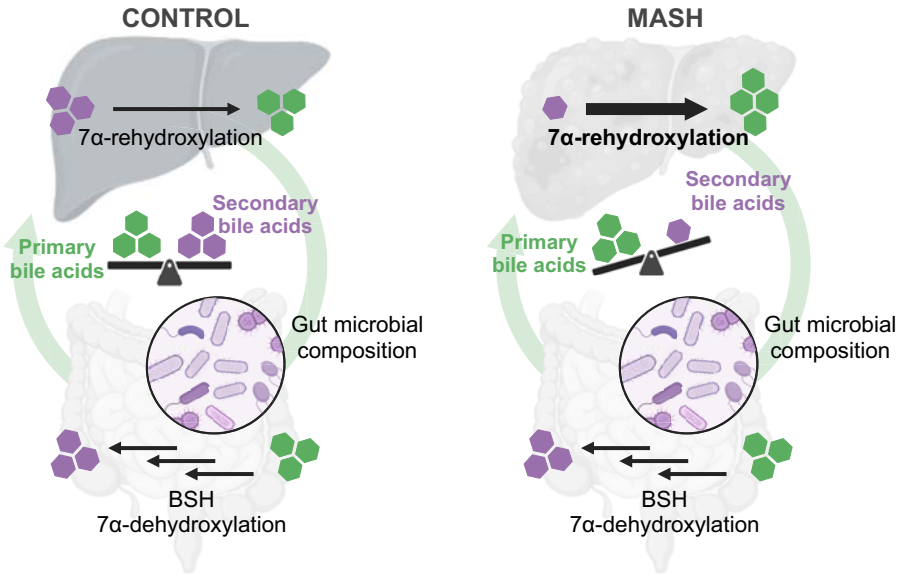
Authors

Justine Gillard, Martin Roumain, Corinne Picalausa, …, Giulio G. Muccioli, Laure B. Bindels, Isabelle A. Leclercq

Correspondence

isabelle.leclercq@uclouvain.be (I.A. Leclercq).

Graphical abstract



Highlights:

- Mice with metabolic dysfunction-associated steatohepatitis (MASH) exhibit reduced levels of secondary 7α-dehydroxylated bile acids.
- Gut microbial composition is unchanged and does not explain bile acid alterations in mice with MASH.
- Similar microbial bile salt hydrolase and 7α-dehydroxylating activities do not explain bile acid alterations in mice with MASH.
- In MASH livers, the induction of bile acid 7α-rehydroxylase activity leads to a reduction in secondary 7α-dehydroxylated bile acid levels.

Impact and implications:

Although changes in bile acid levels are implicated in the development of metabolic dysfunction-associated steatohepatitis (MASH), the precise mechanisms underpinning these alterations remain elusive. In this study, we investigated the mechanisms responsible for the changes in bile acid levels in mouse models of MASH. Our results support that neither the composition nor the metabolic activity of the gut microbiota can account for the alterations in the bile acid pool. Instead, we identified hepatic 7α-rehydroxylation of secondary bile acids as a gut microbiota-independent factor contributing to the reduced levels of secondary bile acids in mice with MASH. Further investigation is warranted to understand bile acid metabolism and its physiological implications in clinical MASH. Nonetheless, our findings hold promise for exploring novel therapeutic interventions for MASH.

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Research article

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A gut microbiota-independent mechanism shapes the bile acid pool in mice with MASH

Justine Gillard[1,2], Martin Roumain[3], Corinne Picalausa[1], Morgane M. Thibaut[2], Laure-Alix Clerbaux[1], Anne Tailleux[4], Bart Staels[4], Giulio G. Muccioli[3], Laure B. Bindels[2,5,†], Isabelle A. Leclercq[1,*,†]

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Background & Aims: An imbalance between primary and secondary bile acids contributes to the development of metabolic dysfunction-associated steatohepatitis (MASH). The precise mechanisms underlying changes in the bile acid pool in MASH remain to be identified. As gut bacteria convert primary bile acids to secondary bile acids, we investigated the contribution of the gut microbiota and its metabolizing activities to bile acid alterations in MASH.

Methods: To disentangle the influence of MASH from environmental and dietary factors, high-fat diet fed *foz/foz* mice were compared with their high-fat diet fed wildtype littermates. We developed functional assays (stable isotope labeling and *in vitro* experiments) to extend the analyses beyond a mere study of gut microbiota composition (16S rRNA gene sequencing). Key findings were confirmed in C57BL/6J mice were fed a Western and high-fructose diet, as an independent mouse model of MASH.

Results: Although mice with MASH exhibited lower levels of secondary 7α-dehydroxylated bile acids (3.5-fold lower, $p = 0.0008$), the gut microbial composition was similar in mice with and without MASH. Similar gut microbial bile salt hydrolase and 7α-dehydroxylating activities could not explain the low levels of secondary 7α-dehydroxylated bile acids. Furthermore, the 7α-dehydroxylating activity was unaffected by *Clostridium scindens* administration in mice with a non-standardized gut microbiota. By exploring alternative mechanisms, we identified an increased bile acid 7α-rehydroxylation mediated by liver CYP2A12 and CYP2A22 enzymes (4.0-fold higher, $p < 0.0001$), that reduces secondary 7α-dehydroxylated bile acid levels in MASH.

Conclusions: This study reveals a gut microbiota-independent mechanism that alters the level of secondary bile acids and contributes to the development of MASH in mice.

Introduction

Bile acids flow along an enterohepatic cycle.[1] Primary bile acids (cholic [CA] and chenodeoxycholic [CDCA] acids in humans) are synthesized from cholesterol in hepatocytes. The presence of a hydroxyl group at the C7 position of the sterol backbone is the signature feature of all primary bile acids. After conjugation to taurine or glycine, bile acids are secreted into the bile and released into the intestinal lumen, where they interact with gut microorganisms.[2] After cleavage of glycine or taurine conjugates by microbial bile salt hydrolases (BSHs), the bile acid backbone is further modified by bacteria to yield secondary bile acids.[2] The quantitatively most important modification is the conversion of primary bile acids (CA and CDCA) to 7α-dehydroxylated secondary bile acids (deoxycholic [DCA] and lithocholic [LCA] acids, respectively) by the multistep 7α-dehydroxylation.[2] Most of the bile acids present in the gut are reabsorbed into the portal circulation and brought back to the liver, where they are mixed with newly synthesized bile acids.

Gut microorganisms, mainly bacteria, thus ensure the diversification of the bile acid pool by converting primary bile acids to secondary bile acids. Accordingly, the bile acid pool of germ-free mice is only composed of conjugated primary bile acids[3–5] and treatment with antibiotics considerably depletes secondary bile acids.[4,5] Changes in the composition of the gut microbiota are often correlated to alterations in the composition of the bile acid pool.[6–10] The causality is however rarely established. Most of the studies focus on taxonomical composition rather than on functional capacity (gene expression) or function (enzymatic activities) of the gut microbiota. Deducing activity toward bile acids from taxonomic information might be inappropriate. Indeed, whereas a small subset of low abundance bacterial species (*e.g. Clostridium scindens*) is known to convert primary bile acids to secondary bile acids,[11] activity varies between one species to another[12] and other yet unknown bacteria or a collaborative metabolism by several bacteria might also produce secondary 7α-dehydroxylated bile acids. Therefore, the investigation of both the composition and

* Corresponding author. Address: Avenue Mounier 53/B1.52.01, 1200 Woluwe-Saint-Lambert, Belgium. Tel: +32 2 764 52 73.
E-mail address: isabelle.leclercq@uclouvain.be (I.A. Leclercq).
† Shared co-last authorship.
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function of the gut microbiota is essential to decipher the potential contribution of the gut bacteria to bile acid alterations.

Metabolic dysfunction-associated steatohepatitis (MASH) is the progressive form of metabolic dysfunction-associated steatotic liver disease (MASLD), the most common chronic liver disease worldwide,[13] in which the balance between primary and secondary bile acids is altered.[1,14] Although there is no established bile acid signature in individuals with MASH,[1,14] several studies reported reduced concentrations of secondary bile acids.[7,15–19] Considering that secondary 7α-dehydroxylated bile acids are potent activators of the bile acid receptors Takeda G protein-coupled receptor 5 (TGR5) and Farnesoid X receptor (FXR) that control several immuno-metabolic processes,[20] low concentrations of secondary bile acids might foster MASLD progression. As MASH is also associated with gut dysbiosis,[6,21–23] we postulated that impaired transformation of bile acids by gut microorganisms reduces the proportion of secondary 7α-dehydroxylated bile acids in the bile acid pool and, by dampening the activation of FXR and TGR5, contributes to MASH pathogenesis.

In this study, we explored both the composition and function of the gut microbiota to decipher its contribution to bile acid changes in the context of MASH. This led us to identify an alternative mechanism, independent of the gut microbiota, that significantly affects the balance between primary 7α-hydroxylated and secondary 7α-dehydroxylated bile acids in MASH livers.

Materials and methods

Animal models

Six-week-old male *foz/foz* (*Alsm1*$^{-/-}$, n = 8) and wildtype (WT, *Alms1*$^{+/+}$, n = 6) mice on a NOD.B10 background were fed a high-fat diet (HFD; D12492, ResearchDiets (NewBrunswick, USA)) *ad libitum* for 12 weeks. HFD-fed *foz/foz* mice (FOZ) were used as a model of obesity, insulin resistance, and MASH, whereas HFD-fed WT littermates served as controls, as previously described.[24–26] *Foz/foz* and WT mice were co-housed, except when specifically indicated.

Eight-week-old male C57BL/6J mice were fed a normal diet (ND; SAFE diets A03) or a Western diet containing 0.5% cholesterol (D05011404, ResearchDiets (New Brunswick, USA)) and 30% fructose in drinking water for 20 weeks (n = 10/group). After 20 weeks, C57BL/6J fed a Western and high-fructose diet (WFD) exhibited MASH, whereas C57BL/6J fed a ND had no liver disease, as previously described.[25]

An independent cohort of 6-week-old male *foz/foz* mice was divided in two groups based on their body weight and glycemia (n = 8/group). Mice were then fed an HFD and received by oral gavage a suspension of *Clostridium scindens* ATCC35704 (CS, 10^9 colony forming units (CFU)) or vehicle (culture medium) three times a week for 12 weeks. Fresh fecal pellets were collected at the beginning and at the end of the experiments.

Mice were kept under conventional conditions in individually ventilated cages and submitted to 12 h light/12 h dark cycles. Animal care was provided in accordance to the guidelines for humane care for laboratory animals as per the European regulations and data reported in conformity with ARRIVE guidelines. The study protocol was approved by the university ethics committee for the use of experimental animals under the references 2016/UCL/MED/016 and 2020/UCL/MD/018.

Bacterial culturing

CS (DSM5676, DSMZ (Braunschweig, Germany)) and *Neglectibacter timonensis* SN17 (CSUR P2265, CSUR (Marseille, France)) were cultured in brain–heart infusion broth (BHI, 37.0 g/L) supplemented with yeast extract (5.0 g/L) and L-cysteine HCl (0.5 g/L) at pH 7.0, in an anaerobic chamber (Coy Laboratory Product (Grass Lake, USA)) with a gas mix of 5% hydrogen and 95% nitrogen. When used to measure the 7α-dehydroxylation of bile acids, bacteria were grown in sterile BHI medium with 100 μM CA for 48 h to induce the *bile acid inducible* (*bai*) operon and the 7α-dehydroxylating activity. The stationary culture was centrifuged and then resuspended in 1 ml of fresh BHI. When used to prepare the suspension containing 10^9 CFU/200 μl for administration to mice, a stationary culture of CS was centrifuged and resuspended in fresh BHI. The suspension was then directly administered to mice, with no contact with oxygen.

Bile acid metabolizing activities

Microbial bile salt hydrolase

Taurocholic acid (TCA) hydrolase activity was measured as previously described.[27] Frozen cecal samples were weighed, homogenized in sterile PBS, and centrifuged. Proteins in the supernatant were precipitated by the addition of saturated ammonium sulfate, centrifuged, and the protein-containing pellet resuspended in sodium acetate buffer (50 mM, pH 4.7). Next, proteins (50 μg/reaction, in duplicate) were incubated with the taurocholic acid-2,2,4,4-deuterium (TCA-d4, 400 μM final concentration, Sigma Aldrich (St Louis, USA)) for 15 min at 37 °C. The reaction was terminated by adding cold acetone, constituting the first step in the quantification of bile acids.

Microbial bile acid 7α-dehydroxylating and 7α-rehydroxylating activities

The 7α-dehydroxylation and 7α-rehydroxylation of bile acids by bacteria collected from cecal contents and specific cultured bacteria (CS and *N. timonensis* SN17) were assessed. For bacteria from cecal contents, ceca were collected and transferred directly to an anaerobic chamber (Coy Laboratory Product (Grass Lake, USA)). From there, all steps were carried out under strict anaerobic conditions. Cecal contents were then collected, weighed, resuspended in sterile oxygen-free PBS, filtered (100 μm) and centrifuged. For cultured bacteria, culture broth was centrifuged directly. The bacterial pellet was suspended in 1.0 ml of fresh BHI. The fresh bacterial suspension (100 μl, in triplicate) was then incubated with cholic acid-2,2,4,4-deuterium (CA-d4, 100 μM final concentration, Toronto Research Chemicals (North York, Canada), Fig. S5A) or deoxycholic acid-2,2,4,4-deuterium (DCA-d4, 100 μM final concentration, Eurisotop (Saint Aubin, France)) for 15 or 30 min, respectively, at 37 °C to assess bile acid 7α-dehydroxylation or 7α-rehydroxylation, respectively. Blanks were BHI without bacteria in which the corresponding deuterated bile acid was added. The reaction was terminated by freezing in liquid nitrogen.

Hepatic bile acid 7α-rehydroxylating activity

Taurodeoxycholic acid (TDCA) 7α-rehydroxylating activity was measured as previously described.[28] Microsomes were isolated from frozen liver samples homogenized in a Tris–HCl

buffer (1 M, pH 7.4) containing EDTA 0.1 mM and sucrose 250 mM. After a first centrifugation to discard cellular debris, the supernatant was centrifuged at 7,000 × *g* for 20 min to pellet and discard the mitochondrial fraction. Then, the supernatant was ultra-centrifuged at 105,000 × *g* for 1.5 h. The microsomal fraction-containing pellet was homogenized in a potassium phosphate buffer (0.1 M, pH 7.4) containing EDTA 1 mM, KCl 50 mM, and dithiothreitol 5 mM. Mouse C57BL/6J liver microsomes were purchased from Tebu-bio (098M5000 (Le Perray, France)). Microsomal proteins (0.4 mg/ml) were then incubated for 20 min at 37 °C with TDCA 0.2 mM in presence of an NADPH regenerating system (NADPH 1.2 mM, NADP 1.3 mM, glucose-6-phosphate 3.6 mM, $MgCl_2$ 3.3 mM, and glucose-6-phosphate dehydrogenase 400 U/L). The reaction was quenched by adding cold acetone, constituting the first step in the quantification of bile acids. The functionality of the in-house isolated microsomes was assessed by measuring CYP3A4 enzymatic activity using the Luciferin-IPA P450-Glo Assay (V9001, Promega (Madison, USA), Fig. S10).

Quantification of bile acids

Quantification of deuterated bile acids was performed using a validated LC-MS method.[29] Proteins present in the samples were precipitated by the addition of acetone. After centrifugation, the supernatant was collected and evaporated under a nitrogen stream. The residues were then resuspended in methanol and analyzed by LC-MS using an LTQ-Orbitrap XL coupled to an Accela HPLC system (ThermoFisher Scientific (Waltham, USA)). The mass spectrometer was equipped with an electrospray ionization source used in negative mode. Analyte separation was performed on an Ascentis Express C-18 column (2.7 μm, 4.6 × 100 mm, Sigma-Aldrich (St Louis, USA)) maintained at 40 °C, using a gradient of water and acetonitrile in the presence of formic acid. Data are expressed as a ratio between the signal (area under the curve) of the analyte to the signal of its internal standard.

Additional information on animal models, gut microbiota analyses, *C. scindens* viability assays, bile acid profiling, TGR5 activation reporter assay, tissue mRNA analyses, histological analyses, and statistics can be found in the Supplemental methods and CTAT table.

Results

The overall composition of the gut microbiota is similar between mice with and without MASH

We used HFD-fed *foz/foz* mice (FOZ) that exhibited the hepatic characteristics of MASH and compared them with their WT co-housed littermates fed the same HFD that do not display liver disease (Fig. 1A–C). After 12 weeks of HFD feeding, the portal bile acid pool of FOZ with MASH was characterized by a lower concentration of secondary bile acids (22 ± 9% *vs.* 34 ± 7% of the portal bile acids in WT controls, $p = 0.0195$, Fig. 1D, Table S2) and a lower ratio between secondary and primary bile acids (Fig. 1E). Because secondary 7α-dehydroxylated bile acids are the most potent TGR5 activators, this resulted in a lower TGR5 activation capacity of portal blood (Fig. 1F), as we previously published.[25] The experimental conditions, which involved co-housing, the use of littermate controls and the administration of an identical diet to both groups created a setting that isolates the effects of MASH on the gut microbiota, while minimizing the influence of the environmental and dietary factors.[30] Importantly, co-housing had no significant impact on liver disease and bile acid composition in FOZ, as evaluated by the measurement of TGR5 activation capacity by portal plasma (Fig. S1A and B).

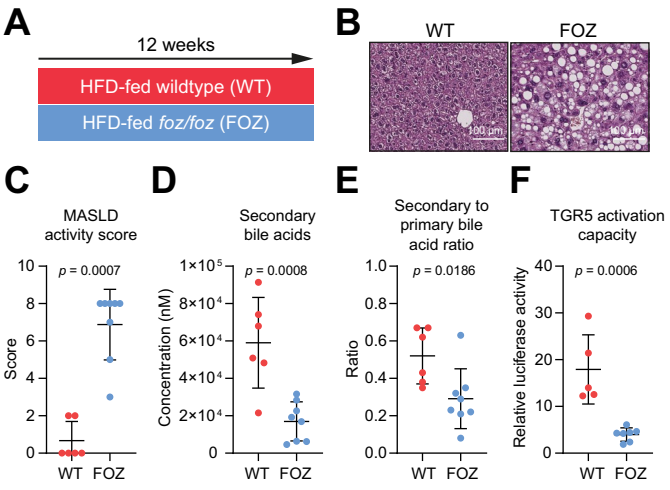


Fig. 1. FOZ mice with MASH are characterized by a depletion of secondary bile acids compared with WT controls. (A) After 12 weeks of HFD feeding, FOZ exhibit MASH while their co-housed WT littermates have no liver disease (n = 6–8/group). (B) Representative H&E staining of liver sections. (C) MASLD activity score. Mann–Whitney test. (D) Concentration of secondary bile acids in portal plasma. (E) Ratio of secondary to primary bile acids in portal plasma. (F) TGR5 activation by the portal plasma in the cell reporter assay. Data are normalized against the relative luciferase activity of the medium without plasma. Mean ± standard deviation. Unpaired two-tailed *t* tests for (D), (E), and (F). FOZ, HFD-fed *foz/foz* mice; HFD, high-fat diet; MASH, metabolic dysfunction-associated steatohepatitis; MASLD, metabolic dysfunction-associated steatotic liver disease; TGR5, Takeda G-protein coupled receptor 5; WT, wildtype.

We then analyzed the microbial composition of the cecal contents (see Supplemental data for methodological details). Importantly, no significant differences were observed in terms of weight and total bacterial load of the cecal contents when comparing FOZ and WT mice (Fig. S2A and B). The diversity within each cecal sample (*i.e.* α-diversity) was not different between FOZ and WT (Fig. S3A–F). MASH did not have an impact on the taxonomic diversity among the samples (Fig. S4A and B). The gut microbial composition at the phylum, family and genus levels were similar, regardless of whether mice had MASH or not (Fig. 2A and B, Fig. S4A and B, Table S3). Only the Bacteroidaceae family and the *Bacteroides* genus were more abundant in FOZ than in WT, although these differences did not reach statistical significance after correcting for the false discovery rate (Fig. 2B, Table S3).

At the amplicon sequence variant (ASV) level (Fig. 2C), the mouse genotype did not explain the variance present in the dataset (PERMANOVA, $R^2 = 8.9\%$, $p = 0.21$). Nevertheless, the relative abundance of three ASVs was increased (*p*-value level, aldex effect <-1, Fig. 2D and E). ASV 26 (assigned to Ruminococcaceae), ASV 36 (assigned to *Bacteroides*) and ASV 70 (assigned to Ruminococcaceae) were more abundant in FOZ than in WT cecal samples (Fig. 2D). In contrast, the lowly abundant ASV 353 was reduced, with a detection rate of 38% among the FOZ samples and 100% of the WT samples (*p*-value level, aldex effect >1, Fig. 2E). The relative abundance of ASV

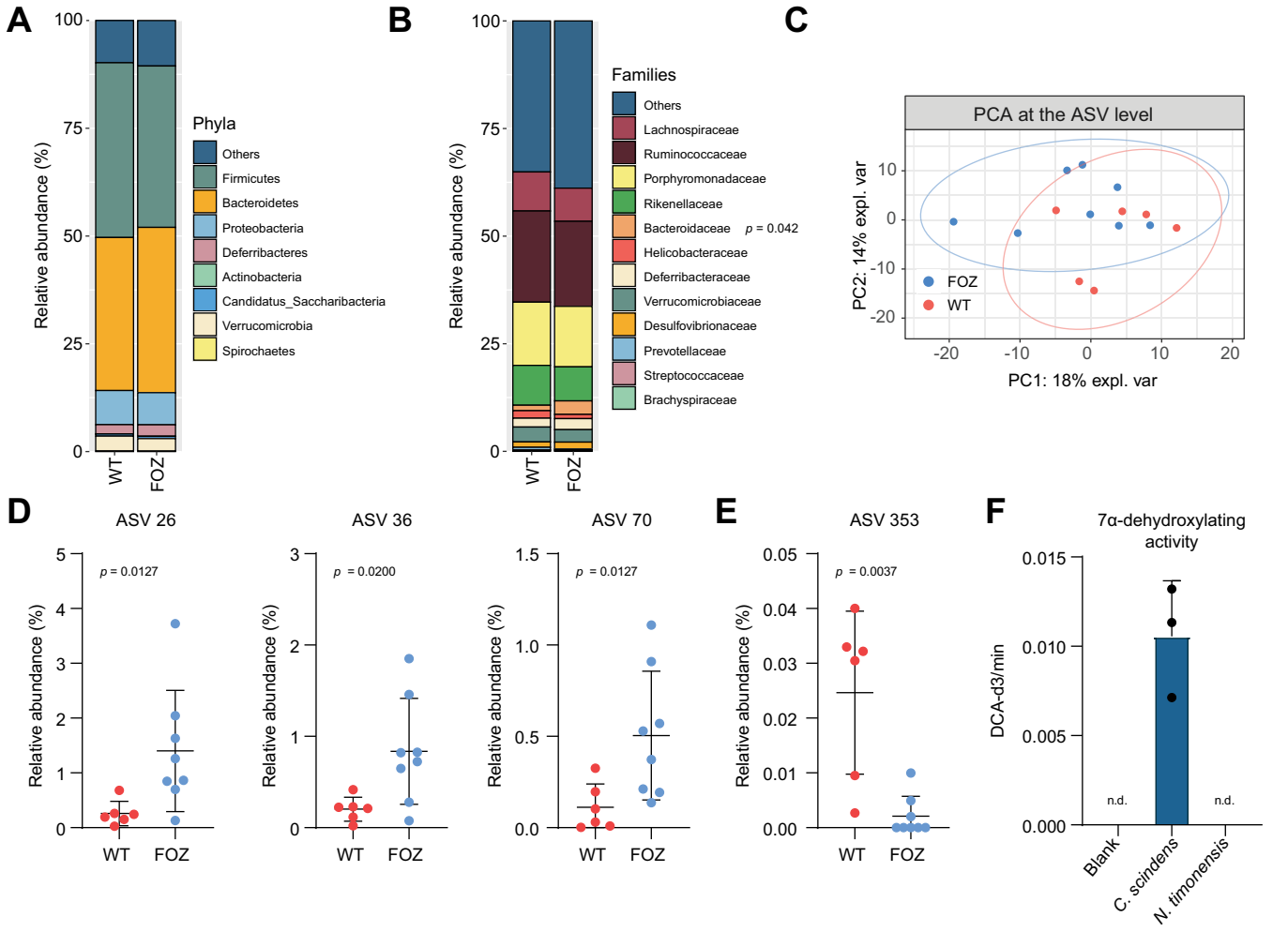


Fig. 2. The gut microbiota composition is similar between mice with and without MASH. (A, B) Mean relative abundances of phyla and families in the cecal contents of WT and FOZ mice (n = 6–8/group). For phyla, 'other' corresponds to unassigned taxa. For families, taxa with a mean relative abundance below 0.1% (*Coriobacteriaceae*, *Lactobacillaceae*, *Peptostreptococcaceae*, and *Clostridiales Incertae Sedis XIII*) were additionally included. Mann–Whitney tests followed by false discovery rate correction. (C) PCA for the visualization of correlations among samples at the ASV level. (D, E) Relative abundances of ASVs differentially expressed between FOZ (blue dots) and WT samples (red dots). Unpaired two-tailed *t* tests. (F) Cholic acid 7α-dehydroxylating activity of specific bacterial strains, expressed as DCA-d3 produced per minute after incubation of CA-d4 with the culture broth (blank), *Clostridium scindens* ATCC35704 and *Neglectibacter timonensis* SN17. One-way ANOVA followed by *post hoc* Bonferroni correction, n = 3/condition. Mean ± standard deviation. ASV, amplicon sequence variant; CA-d4, cholic acid-2,2,4,4-deuterium; DCA-d3, deoxycholic acid-2,2,4-deuterium; FOZ, HFD-fed *foz/foz* mice; MASH, metabolic dysfunction-associated steatohepatitis; ND, normal diet; PCA, principal component analysis; WT, wildtype.

353 positively correlated with the concentration of secondary bile acids and with the secondary to primary bile acid ratio in portal blood of mice (Fig. S2C). We thus hypothesized that the bacterial species assigned to this ASV possesses the ability for bile acid 7α-dehydroxylation, which could explain that its lower prevalence leads to a reduced production of secondary bile acids. The ASV 353 was assigned to the *Neglectibacter* genus, with the only described strain *N. timonensis* SN17, a bacterium phylogenetically close to *Clostridium leptum* that carries out the 7α-dehydroxylation of bile acids.[31,32] We investigated whether *N. timonensis* SN17 is able to convert CA to DCA *in vitro*. The incubation of *N. timonensis* SN17 with CA in strict anaerobic conditions failed to produce DCA, in contrast to CS, used as a positive control (Fig. 2F and Fig. S5), indicating that the gut bacterium *N. timonensis* SN17, to which the ASV 353 was assigned, does not carry bile acid 7α-dehydroxylation.

Collectively, these data support that the overall gut microbial composition is not altered in FOZ with MASH. Therefore, the observed reduction in secondary bile acids in FOZ mice is not attributed to alterations in the gut microbial composition.

Bacterial bile acid metabolizing activities are similar whether mice have MASH or not

A similar gut microbial composition does however not rule out functional changes. We thus investigated by functional assays the bile acid metabolizing activities of cecal bacteria in mice with MASH, characterized by a depletion in secondary bile acids compared with controls (Fig. 1). A decrease in BSH and/or 7α-dehydroxylating activities in FOZ would explain the lower concentration of secondary bile acids. The cleavage of glycine or taurine conjugates of bile acids by BSHs is a gateway step for

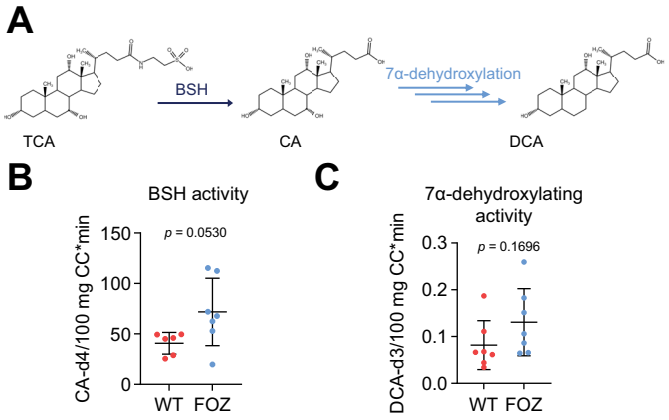


Fig. 3. Microbial bile acid metabolizing activities are similar whether mice have MASH or not. (A) Deconjugation of taurocholic acid (TCA) by the microbial bile salt hydrolase (BSH) is followed by the multistep 7a-dehydroxylation of CA to form DCA. (B) TCA hydrolase activity of FOZ and WT cecal contents, expressed as CA-d4 produced after incubation of TCA-d4 with cecal proteins. (C) CA 7α-dehydroxylating activity of FOZ and WT cecal contents, expressed as DCA-d3 produced after incubation of CA-d4 with cecal bacteria. n = 6–7/group. Mean ± standard deviation. Unpaired two-tailed *t* tests. BSHs, bile salt hydrolases; CA, cholic acid; CA-d4, cholic acid-2,2,4,4-deuterium; DCA, deoxycholic acid; DCA-d3, deoxycholic acid-2,2,4-deuterium; FOZ, HFD-fed *foz/foz* mice; MASH, metabolic dysfunction-associated steatohepatitis; TCA, taurocholic acid; WT, wildtype.

subsequent transformations, amongst which the 7α-dehydroxylation of unconjugated primary bile acids is the most significant in shaping the bile acid pool composition (Fig. 3A).[33] The BSH activity, quantified as the CA-d4 formed following the incubation of TCA-d4 with proteins extracted from cecal samples, was not different between WT and FOZ cecal samples (Fig. 3B). This indicates that CA is effectively deconjugated and thus made available for further modifications, such as 7α-dehydroxylation. To evaluate bile acid 7α-dehydroxylation, we isolated bacterial cells from fresh cecal samples, incubated them with CA-d4, and quantified the formation of DCA-d3. As for the BSH activity, the 7α-dehydroxylation by cecal bacteria was not lower in FOZ than in WT mice (Fig. 3C). Here again, the weight of the cecal content did not differ between groups ($p = 0.1214$).

Another hypothesis, albeit less likely given the anaerobic milieu, is that gut bacteria convert 7α-dehydroxylated bile acids back to 7α-hydroxylated bile acids (Fig. S6A) and that this process is heightened in mice with MASH. This hypothesis stemmed from the observation of a time-dependent production of CA-d3 by cecal bacteria upon incubation with CA-d4 (Fig. S6B). We speculated that CA-d4 was 7α-dehydroxylated to DCA-d3, with a deuterium lost during the process, and that DCA-d3 was then converted back to CA-d3 by cecal bacteria. In addition, the formation of CA-d3 was higher by cecal bacteria of FOZ than WT (Fig. S6C), providing an explanation for the low concentration of secondary bile acids in FOZ. To test our hypothesis, bacteria from fresh cecal samples were incubated with DCA-d4. The hypothesis was dismissed as the incubation of cecal bacteria, whether from WT or FOZ, with DCA-d4 did not yield CA-d4 (Fig. S6D).

In conclusion, similar bile acid metabolizing activities by gut microbes in FOZ and WT align with the similar composition of their gut microbiota. Therefore, the low concentration of secondary bile acids in the context of MASH is not attributed to diminished bile acid transformation by gut bacteria in FOZ.

Administration of *Clostridium scindens* to FOZ mice does not potentiate the 7α-dehydroxylating activity of their gut microbiota

We previously reported that increasing the concentration of DCA (the main 7α-dehydroxylated bile acid) restored FXR and TGR5 signaling and offered protection against MASH.[25] We postulated that increasing gut microbial 7α-dehydroxylation of bile acids in FOZ mice would also achieve this goal. FOZ mice therefore received a fresh suspension of CS via oral gavage for a duration of 12 weeks (Fig. 4A), as CS exhibits a high 7α-dehydroxylating activity.[12,34] CS administration was previously reported to increase the secondary bile acid levels in antibiotics-treated and gnotobiotic mice.[35–38]

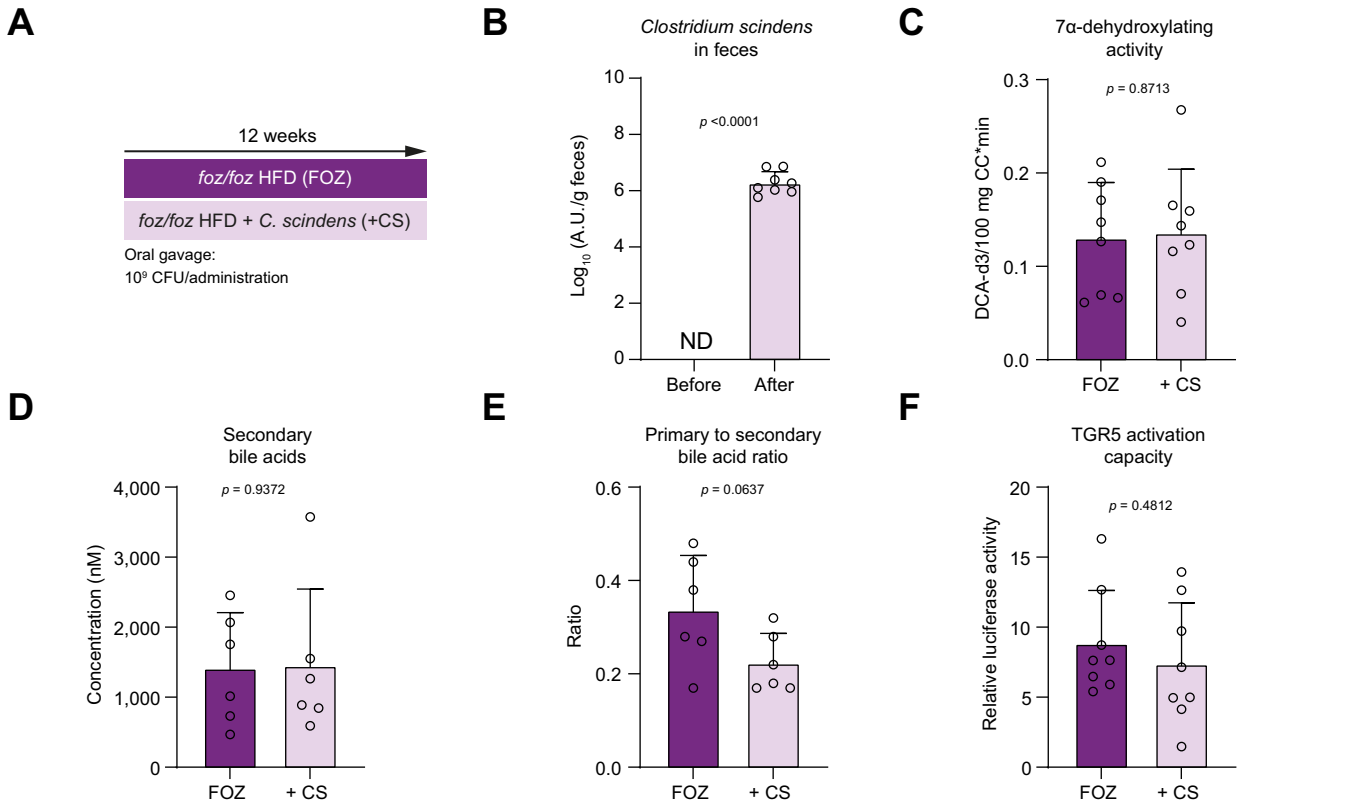
First of all, as CS is an obligate anaerobe, its viability was validated by monitoring its growth at each stage during the preparation of the gavage suspension (Fig. S7A). The viability after gavage was also evaluated by collecting the cecal contents 6 h after a single administration of CS to mice. Bacteria were then inoculated in selective culture medium to select for CS. The increasing load of CS over time in the culture of cecal bacteria from mice treated with the bacterial strain, but not in the culture of cecal bacteria from mice that received vehicle, confirmed that CS remains alive in the intestinal tract (Fig. S7B).

The administration of CS to FOZ mice for 12 weeks massively increased the load of the bacterial strain detected in feces (Fig. 4B). However, it did not enhance the 7α-dehydroxylating activity of the cecal content (Fig. 4C). The measurement of the 7α-dehydroxylating activity was 16 h after the final gavage, a timing that might have overlooked any temporary increase that occurred immediately after the gavage. A repeated transitory elevation of the 7α-dehydroxylating activity might however change the bile acid pool, as bile acids are recycled and even minor alterations can accumulate over time. Bile acids were thus analyzed in the portal plasma of FOZ mice receiving vehicle or CS. The concentration of secondary bile acids and the ratio between secondary and primary bile acids were not increased by the administration of CS (Fig. 4D and E). The TGR5 activation capacity of the portal plasma remained similar whether FOZ mice received CS or not (Fig. 4F).

In conclusion, the 7α-dehydroxylating activity of the gut microbiota was not significantly influenced by the administration of CS to FOZ mice, indicating that adding CS in a complex and rich bacterial environment negligibly enhances the basal 7α-dehydroxylating activity.

Reconversion of secondary to primary bile acids increases in MASH livers

Secondary bile acids formed by microbial 7α-dehydroxylation are readily absorbed into the portal circulation and returned to the liver. In murine hepatocytes, secondary 7α-dehydroxylated bile acids can be reconverted to primary bile acids (Fig. 5A), as they express cytochrome P450 (CYP) 2A12 which rehydroxylates bile acids (whether conjugated or not) at the C7 position.[28] This reaction might also be catalyzed by CYP2A22, which is highly homologous to CYP2A12.[28] We therefore hypothesized that 7α-rehydroxylation of secondary bile acids by



Fig, 4. Administration of CS to FOZ does not enhance the 7α-dehydroxylating activity of their gut microbiota. (A) From the start of the HFD, FOZ received a fresh suspension of 10^9 CFU of CS by oral gavage three times a week for 12 weeks (n = 8/group). (B) Fecal load of CS before and after the treatment. Paired two-tailed *t* test. (C) CA 7α-dehydroxylating activity of cecal contents of FOZ treated or not with CS. The activity is expressed as DCA-d3 produced after incubation of CA-d4 with cecal bacteria. Unpaired two-tailed *t* test. (D) Concentration of secondary bile acids in portal plasma (n = 6/group). Mann–Whitney test. (E) Ratio of secondary to primary bile acids in portal plasma (n = 6/group). Unpaired two-tailed *t* test. (F) TGR5 activation by the portal plasma in the cell reporter assay. Data are normalized against the relative luciferase activity of the medium without plasma. Unpaired two-tailed *t* test. Mean ± standard deviation. CA, cholic acid; CA-d4, cholic acid-2,2,4,4-deuterium; CFU, colony forming units; CS, *Clostridium scindens* ATCC35704; DCA-d3, deoxycholic acid-2,2,4-deuterium; FOZ, HFD-fed *foz/foz* mice; HFD, high-fat diet; TGR5, Takeda G-protein coupled receptor 5.

hepatic enzymes explains the low concentration of secondary bile acids in MASH.

The twofold higher gene expression of *Cyp2a12* and *Cyp2a22* in FOZ livers (Fig. 5B) prompted us to assess the 7α-rehydroxylation capacity of FOZ and WT livers. Microsomal fractions were isolated from frozen livers. We incubated them with TDCA and quantified the formation of TCA (Fig. 5A). The enzymatic activity did not vary between groups (Fig. S8A and B). However, because liver weight is substantially higher in FOZ than in WT (Fig. S8C), the 7α-rehydroxylation capacity of FOZ livers was much higher than that of WT livers (Fig. 5C). The 7α-rehydroxylation capacity correlated positively with hepatic gene expressions of *Cyp2a12* and of *Cyp2a22* (Fig. 5D), supporting that these two enzymes catalyze the reconversion of secondary 7α-dehydroxylated bile acids to primary bile acids. The ratio of secondary to primary bile acids in portal blood also significantly decreased in parallel to the 7α-rehydroxylation capacity (Fig. 5E).

To confirm that the 7α-rehydroxylation of secondary bile acids by liver enzymes significantly shapes the bile acid composition in MASH, we used an independent mouse model of MASH. C57BL/6J mice were fed a WFD for 20 weeks to induce MASH and compared with their healthy counterparts fed an ND (Fig. 6A–C). Comparable to the changes in FOZ, C57BL/6J fed a WFD showed bile acid alterations (Fig. 6C), as we previously published.[25] The enhanced conversion of secondary 7α-dehydroxylated bile acids to primary bile acids in the MASH livers was confirmed by the higher gene expression of *Cyp2a12* and of *Cyp2a22*, and the higher 7α-rehydroxylation capacity of livers from WFD-fed mice, compared with ND-fed mice (Fig. 6D–F, Fig. S9).

In conclusion, the hepatic 7α-rehydroxylation of bile acids is a gut microbiota-independent mechanism that alters the balance between primary and secondary 7α-dehydroxylated bile acids in mice with MASH.

Discussion

In this study, we analyzed the composition and function of the gut microbiota to decipher the underlying mechanisms behind the bile acid alterations observed in MASH. We provide evidence that the significant depletion of secondary 7α-dehydroxylated bile acids in murine MASH is not attributable to an impaired gut microbiota. In the FOZ mouse model of MASH, both microbial composition and bile acid metabolizing activities were similar to those of WT without liver disease. Instead, our data reveal that the reconversion of secondary 7α-dehydroxylated bile acids to primary 7α-hydroxylated bile acids, catalyzed by liver enzymes, is enhanced in MASH, and substantially alters the enterohepatic bile acid pool. Importantly, our study

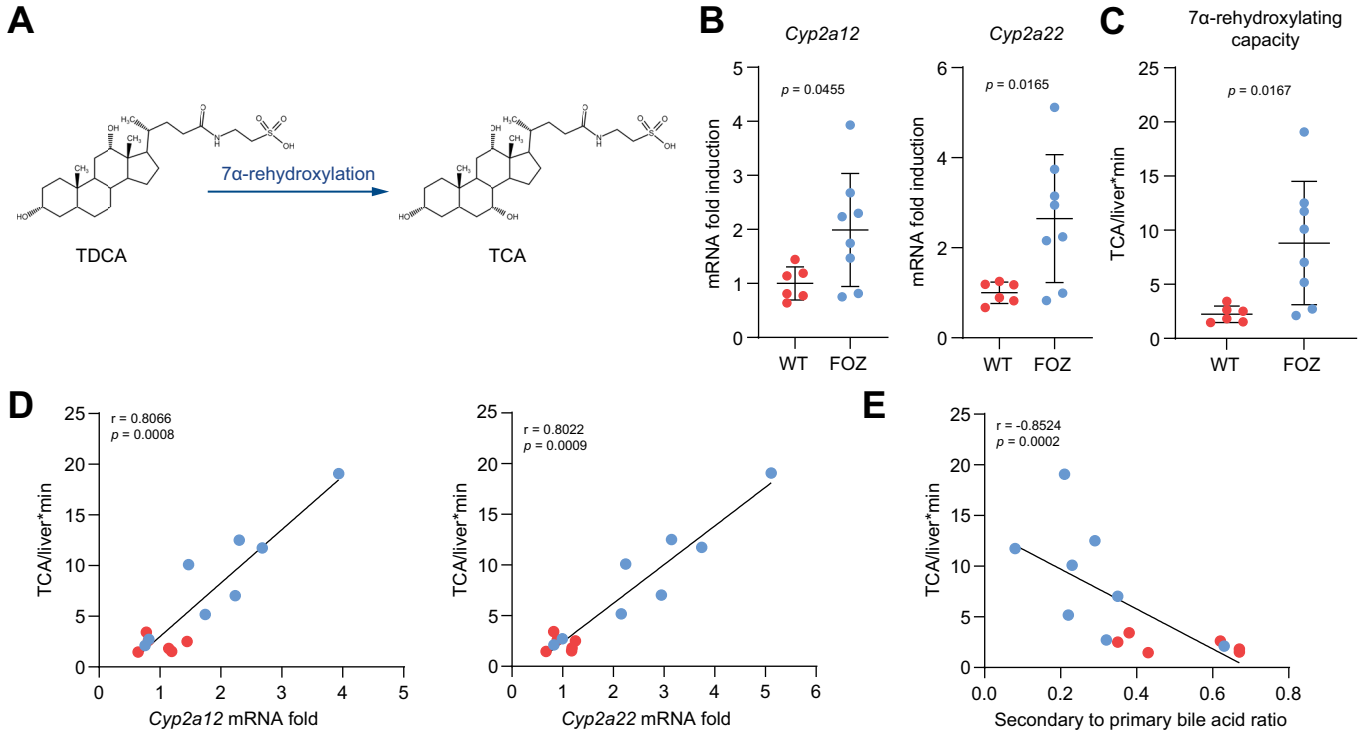


Fig. 5. Reconversion of secondary bile acids to primary bile acids is enhanced in FOZ livers. (A) TDCA is rehydroxylated to TCA at the C7 position by liver enzymes. (B) Relative gene expression of *Cyp2a12* and *Cyp2a22* in FOZ and WT livers. Unpaired two-tailed t tests. (C) TDCA 7α-rehydroxylation capacity of FOZ and WT livers, expressed as TCA produced after incubation of TDCA with liver microsomal proteins, normalized to liver weight. Unpaired two-tailed *t* test. Spearman correlations between TDCA 7α-rehydroxylation capacity and (D) the relative gene expression of *Cyp2a12* and of *Cyp2a22* in FOZ and WT livers, or (E) the ratio of secondary to primary bile acids in portal plasma. WT are shown in red and FOZ in blue. Mean ± standard deviation; n = 6–8/group. CYP, cytochrome P450; FOZ, HFD-fed *foz/foz* mice; TCA, taurocholic acid; TDCA, taurodeoxycholic acid; WT, wildtype.

emphasizes the impact of factors beyond the gut bacterial metabolism that regulate the balance between primary and secondary bile acids.

The use of littermates co-housed and fed with the same HFD as controls established a unique experimental setting in which the contribution of the gut microbiota to MASH could be isolated from that of the environment and dietary composition. FOZ (*i.e.* HFD-fed *foz/foz* mice) are one of the few mouse models of MASH that exhibit both the dysmetabolic and the histopathological criteria for human MASH and whose littermate controls are also fed an HFD.[39] The use of littermates and co-housing mitigate bias related to developmental programming and environmental exposure.[30] In the present study, housing conditions accounted for 11.9% of the variance in microbial composition (PERMANOVA, p = 0.016, ASV level), whereas genetic background did not significantly contribute to this variance (PERMANOVA, R2 = 8.9%, p = 0.21, ASV level). However, the severity of liver disease and bile acid alterations were not different whether mice were housed together or not. This reinforces that the gut microbiota does not contribute to liver disease and bile acid alterations in this context.

Although FOZ and WT mice had a similar overall gut microbial composition, FOZ still exhibited an altered portal bile acid pool characterized by low concentrations of secondary bile acids compared with WT.[25] Therefore, we investigated the transformation of bile acids by gut microbes, with an emphasis on 7α-dehydroxylation. In contrast to the numerous microorganisms expressing a BSH gene (over 117 genera among 12 phyla in the human microbiota),[40,41] the few identified bacterial strains that can perform bile acid 7α-dehydroxylation are Gram-positive anaerobes found in low abundance.[33,42] These strains are estimated to represent only one millionth of the total gut microbes in humans.[33,42] Many of them are part of the *Clostridium* genus – for instance, *Clostridium scindens* ATCC35704, *Clostridium hylemonae* TN271, *Clostridium hiranonis* TO931, and *Clostridium leptum* VPI 10900[2,12] – but numerous other strains within the genus do not convert primary bile acids to secondary bile acids. It is likely that other unidentified bacterial strains also carry out this enzymatic function. To illustrate, *Faecalicatena contorta* S122 was recently reported to efficiently convert CA to DCA.[43] The production of secondary 7α-dehydroxylated bile acids might also occur through a collaborative metabolism involving several bacteria. As a result, the abundance of 7α-dehydroxylating bacteria cannot be accurately determined through taxonomic profiling. Detection of genes that encode enzymes involved in the 7α-dehydroxylation is not optimal, as this pathway includes seven enzymes and one transporter, which are encoded by eight genes within the *bai* operon.[33] We attempted to quantify the *baiCD* gene, an Fe–S flavoenzyme that catalyzes the two crucial reductive steps of the pathway,[33] using qPCR and primer sequences reported in the literature.[44–46] However, we failed to achieve a specific amplification. To address these limitations, we developed functional assays to assess the metabolism of bile acids by gut microbes. In our study, we assessed BSH and 7α-dehydroxylating activities of cecal contents and not of fecal samples, because the cecum is the gut compartment where the majority of DCA is produced *in vivo*,[38] and where anaerobic conditions are maintained to ensure the survival and growth of the anaerobes.[47] We

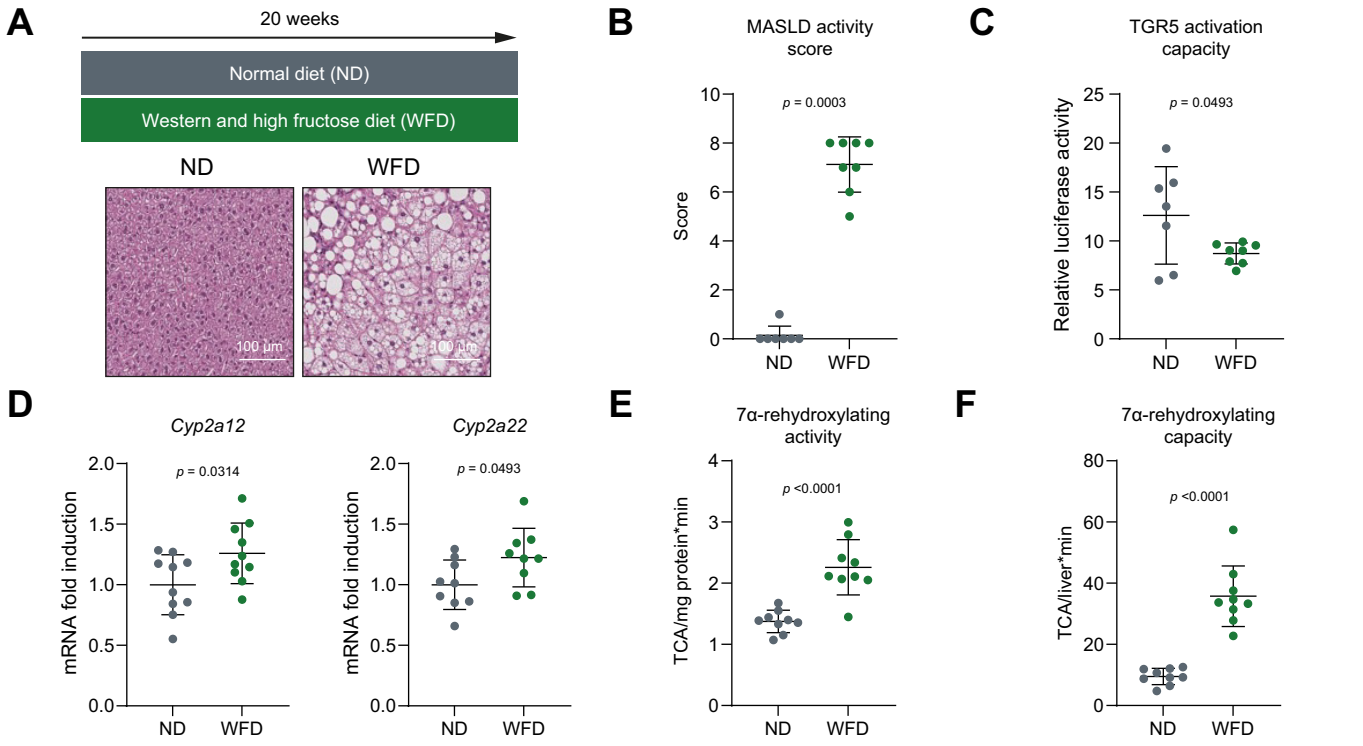


Fig. 6. Validation of the findings in an independent mouse model of MASH. (A) After 20 weeks of WFD feeding, C57BL/6J mice exhibit MASH (green dots). ND-fed C57BL/6J have no liver disease (grey dots) (n = 8–10/group). Representative H&E staining of liver sections. (B) MASLD activity score. Mann-Whitney test. (C) TGR5 activation by the portal plasma. Data are normalized against the relative luciferase activity of the medium without plasma. Unpaired two-tailed *t* tests. (D) Relative gene expression of *Cyp2a12* and *Cyp2a22* in livers of ND- and WFD-fed mice. Unpaired two-tailed *t* tests. (E) TDCA 7α-rehydroxylating activity, expressed as TCA produced after incubation of TDCA with liver microsomal proteins. Unpaired two-tailed *t* test. (F) TDCA 7α-rehydroxylation capacity of livers, expressed as TCA produced after incubation of TDCA with liver microsomal proteins, normalized to liver weight. Unpaired two-tailed *t* test. Mean ± standard deviation. CYP, cytochrome P450; MASH, metabolic dysfunction-associated steatohepatitis; MASLD, metabolic dysfunction-associated steatotic liver disease; ND, normal diet; TGR5, Takeda G-protein coupled receptor 5; TDCA, taurodeoxycholic acid; WFD, Western and high fructose diet.

used CA, conjugated or not to taurine, as a substrate, as CA is the main primary bile acid in FOZ and WT mice (35 ± 8% and 25 ± 5% of the portal bile acid pool, respectively) and as taurine is the main bile acid conjugate in mice.[25] Reduced deconjugation of primary bile acids in FOZ mice would result in less unconjugated primary bile acids available for their subsequent 7α-dehydroxylation, thus explaining the lower concentration of secondary bile acids. We found that BSH activity was not different between WT and FOZ cecal samples. Reduced conversion of primary bile acids to 7α-dehydroxylated secondary bile acids in FOZ would also explain the lower concentration of secondary bile acids in FOZ mice, but bile acid 7α-dehydroxylating activity was not lower in FOZ than in WT mice. Thus, our study proves that BSH and 7α-dehydroxylating activities cannot explain the low concentrations of secondary bile acids in FOZ with MASH, indicating that other mechanisms underly the low levels of secondary bile acids.

Although *C. scindens* administration modified the bile acid pool composition in mice with a simplified gut microbiota (*i.e.* germ-free, antibiotic-treated or gnotobiotic mice[35–38]), we did not observe this in mice with a non-standardized gut microbiota, at a higher or equal dose of *C. scindens* (*i.e.* 10^9 CFU, three times a week[35–38]). The absence of change might have several explanations. First, the main variation lies in the mouse model and the complexity of its gut microbiota. Although the treatment successfully elevated the load of *C. scindens*, the increment of 7α-dehydroxylating activity was likely biologically negligible compared with the basal 7α-dehydroxylating activity of the gut microbiota. Second, if primary bile acids are not deconjugated because of low BSH activity, they cannot be converted to secondary bile acids, even if the 7α-dehydroxylating activity is high. However, we showed that BSH activity was not a limiting factor for low 7α-dehydroxylation in *foz/foz* mice. Third, gut microorganisms might hydroxylate bile acids at the C7 position to reconvert secondary 7α-dehydroxylated bile acids to 7α-hydroxylated bile acids. However, we tested and invalidated this hypothesis. Alternatives strategies to enhance secondary bile acids within the bile acid pool should therefore be considered.

Having ruled out a putative 7α-rehydroxylation of secondary bile acids by gut microbes, we focused on the liver as a site of reconversion of secondary bile acids to primary bile acids. Indeed, the lower concentration of secondary bile acids in mice with MASH could be explained by higher bile acid 7α-rehydroxylation. Increased bile acid 7α-rehydroxylation, as suggested by the upregulation of *Cyp2a12* and *Cyp2a22* in MASH livers, was confirmed by the 7α-rehydroxylation assay in two independent mouse models of MASH. Our data pinpoint the induced reconversion of secondary 7α-dehydroxylated bile acids to primary 7α-hydroxylated bile acids as a novel explanation, independent of gut bacteria metabolism, for the altered

bile acid pool in MASH. To date, there is no documented information in the literature regarding the regulation of CYP2A12 and CYP2A22. We previously reported an induced CYP7A1-dependent bile acid synthesis in mice with MASH, which compensates the lower total bile acid concentration arriving to the liver but increases the proportion of primary bile acids in mice with MASH.[25] This induction is controlled by FXR, the main regulator of bile acid synthesis, but other regulatory mechanisms might also influence the hepatic production of bile acids. The effects are likely to be amplified by the several daily enterohepatic cycles, which results in a reduction of secondary 7α-dehydroxylated bile acids in mice with MASH.

The findings of our preclinical study need to be contextualized with the clinical data available in the literature. Although there are considerable discrepancies between studies on the gut microbiota in MASH subjects, with heterogeneous results at the phylum, family and genus level,[23] alterations in the gut microbiota have been reported and described in individuals with MASH.[6,21–23] In our study, we did not find major differences in the gut microbial composition of *foz/foz* mice with MASH. This might be the result of the experimental conditions used in our study to minimize the effects of confounding factors (*e.g.* diet, housing) on the gut microbiota. In mice fed a WFD, gut microbiota was not assessed. However, gut microbiota was likely changed because of the diet difference and might therefore contribute to the altered bile acid levels in this model. In contrast, human cohorts are composed of individuals characterized by numerous confounding factors influencing the gut microbiota composition (*e.g.* anthropometric and metabolic parameters, diet, treatment), which are, however, often not controlled. Globally, changes in gut microbial function in individuals with MASH remain poorly defined, despite considerable efforts to explore them. The use of functional assays could answer simple but yet unelucidated questions in human MASH about the contribution of microbial bile acid metabolizing activities to bile acid alterations. Importantly, whether the bile acid 7α-rehydroxylation activity occurs in humans in unclear. Although the sequences encoding *Cyp2a12* and *Cyp2a22* are not present in human genome, and functional orthologs have not been identified,[48] Xie *et al.*[49] have reported that LCA is 7α-rehydroxylated to CDCA upon incubation with human liver microsomes, suggesting that human livers also complete bile acid 7α-rehydroxylation. In the event that the 7α-rehydroxylation of secondary bile acids is definitely established in human livers, pharmacological modulation of this activity might open up innovative therapeutic possibilities for diseases such as MASH, in which secondary 7α-dehydroxylated bile acids display low levels and/or confer beneficial effects.

In conclusion, this study identifies hepatic 7α-rehydroxylation of secondary bile acids as a mechanism that significantly affects the bile acid composition in mice with MASH and contributes to disease progression. This challenges the conventional notion that the gut microbiota is the sole factor controlling the balance between primary and secondary bile acids. Further work is warranted to further characterize bile acid metabolism and the physiological processes involved in their modulation in clinical MASH.

Affiliations

[1]*Laboratory of Hepato-Gastroenterology, Institute of Experimental and Clinical Research, Université Catholique de Louvain, Brussels, Belgium;* [2]*Metabolism and Nutrition Research Group, Louvain Drug Research Institute, Université Catholique de Louvain, Brussels, Belgium;* [3]*Bioanalysis and Pharmacology of Bioactive Lipids, Louvain Drug Research Institute, Université Catholique de Louvain, Brussels, Belgium;* [4]*University of Lille, Inserm, CHU Lille, Institut Pasteur de Lille, Lille, France;* [5]*Welbio department, WEL Research Institute, Wavre, Belgium*

Abbreviations

ASV, amplicon sequence variant; bai, bile acid inducible; BHI, brain–heart infusion broth; BSH, bile salt hydrolase; CA, cholic acid; CA-d4, cholic acid-2,2,4-deuterium; CA-d4, cholic acid-2,2,4,4-deuterium; CDCA, chenodeoxycholic acid; CFU, colony forming units; CS, Clostridium scindens ATCC35704; DCA, deoxycholic acid; DCA-d4, deoxycholic acid-2,2,4,4-deuterium; DCA-d3, deoxycholic acid-2,2,4-deuterium; FOZ, HFD-fed foz/foz mice; FXR, Farnesoid X receptor; HFD, high-fat diet; LCA, lithocholic acid; MASH, metabolic dysfunction-associated steatohepatitis; MASLD, metabolic dysfunction-associated steatotic liver disease; ND, normal diet; PCA, principal component analysis; TCA, taurocholic acid; TCA-d4, taurocholic acid-2,2,4,4-deuterium; TDCA, taurodeoxycholic acid; TGR5, Takeda G-protein coupled receptor 5; WFD, Western and high-fructose diet; WT, wildtype.

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Conflicts of interest

BS is a scientific advisor of Genfit. The authors declare no other competing interests.

Please refer to the accompanying ICMJE disclosure forms for further details.

Author contributions

Conception and design: JG, IAL, LBB. Acquisition of data: JG, MR, CP, MMT, AT. Analysis and interpretation of data: JG, IAL, LBB. Drafting the article: JG, IAL, LBB. Revising the article: all authors.

Data availability statement

The raw 16S rRNA gene sequencing dataset is available in the SRA repository under project ID PRJNA1037347. Other data are available from the corresponding author, IAL, upon request.

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Supplementary data

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Author names in bold designate shared co-first authorship

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Keywords: Bile acids; Enterohepatic cycle; 7α-Dehydroxylation; 7α-Rehydroxylation; Gut microbiota; *Clostridium scindens*; Liver metabolism; Cytochrome P450; Metabolic dysfunction-associated steatohepatitis; CYP2A12; CYP2A22.
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