

Serum cholic acid and cecal *Faecalibaculum* increase in a male-specific manner in a murine hepatocellular carcinoma model

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Abstract Hepatocellular carcinoma (HCC) has a higher incidence in males and is a leading cause of cancer-related deaths, which lacks effective therapies and surveillance markers. Using a murine model of bile acid excess (farnesoid X receptor and small heterodimer partner double knockout, DKO), which phenocopies many aspects of HCC, including sex differences, we investigated the links between sex, bile acid metabolism, and microbial composition. Unexpectedly, the increase in the ratios of carcinogenic deoxycholic acid (DCA) was similar between DKO males with HCC and cancer-resistant DKO females. However, both taurine-conjugated and free cholic acid (CA) sharply increased in the serum of DKO males, with free-CA comprising 65% of the bile acid pool, whereas DKO female serum was mostly comprised of conjugated bile acids. Unlike such sex differences in DKO serum composition, conjugated bile acids were predominant in the hepatic BA pool irrespective of the sex or genotype, as expected. Fecal and cecal microbiota—many of which harbor bile acid transformation/deconjugation capacity—were altered in DKO mice in a sex-specific manner. Untargeted fecal metabolite analysis showed differences in bile acids, phospholipids, and oxidized fatty acids between the genotypes, with DKO females excreting more sulphated and oxidized CA than tumor-bearing DKO males. Further analysis revealed a direct correlation between unconjugated CA levels with the abundance of the microbial genus *Faecalibaculum* in the DKO HCC model. These findings suggest distinct sex-specific changes in cecal and fecal microbiota, and BA composition may be leveraged in combination as a potential tool for HCC surveillance.

Liver cancer has the third highest mortality rate among cancers worldwide, with higher prevalence and mortality, particularly in men (1). Hepatitis B and C (HBV and HCV, respectively), aflatoxin exposure, alcoholic liver disease (ALD), and metabolic diseases, such as non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH), are major risk factors for hepatocellular carcinoma (HCC) (2, 3). Patients with HCC and associated risk factors mentioned above display aberrant bile acid signaling as well as high circulating and hepatic bile acids (4–6). Moreover, the expression of bile acid homeostatic regulators, farnesoid X receptor (FXR) and small heterodimer partner (SHP), is downregulated in patients with HCC (7–10). Hence, in this study, we used the murine model of bile acid dysregulation (global *Fxr Shp* double knockout (DKO) mice) that develops spontaneous HCC in a male-predominant manner (8, 11).

Over the last decades, bile acid metabolism and signaling have been actively studied (12–16). Typically, primary bile acids synthesized in the liver are modified by the intestinal microbiota to secondary bile acids (17), which have been linked to HCC development (18–21). Here, we set out to investigate if there are sex-specific bile acid or gut-microbiota changes that may correspond with hepatic carcinogenesis by measuring gene expression, analyzing bile acid composition, performing untargeted fecal metabolomics, and examining cecal and fecal microbiota.

METHODS

Animal use

Mice were housed in conventional or IVC flow cages (indicated for each specific study) containing corn cob

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bedding and companion square enrichment. The facility is temperature-controlled at 23°C and on a 12-h light/dark cycle. Food and water were provided ad libitum. All studies using experimental animals were approved by the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee at the University of Illinois Urbana-Champaign, and these experiments followed the guidelines for animal care and use set by the National Institute of Health.

Male and female C57BL/6 wild-type (WT) and whole-body *Fxr*/*Shp* double knockout (DKO) mice were used in this study and housed in conventional cages. Mouse tails were collected and digested in 100:1 DirectPCR (tail) lysis reagent (ThermoFisher Scientific; cat. no. NC9724951) and proteinase K (20 mg/ml; 10 mM Tris-HCl, pH 8.0, 1 mM CaCl₂, and 30% glycerol) to confirm genotype. Samples were heated to 95°C for 10 min to inactivate Proteinase K. Two separate PCR reactions were used for verifying global *Fxr* KO and *Shp* KO. Three primers were used for genotyping the deletion of *Fxr*: forward 1: 5'-TCTCTTTAAGTGAT-GACGGGAATCT-3', forward 2: 5'-GCTCTAAGGAGAGTCACTTGTGCA-3', reverse: 5'-GCATGCTCTGTTTCATAAACGCCAT-3'. WT mice had a product size of 249 bp, while *Fxr* KO had a product size of 291 bp. Thermal cycling parameters for verifying *Fxr* were 95°C for 5 min, 25 cycles of 95°C for 45s, 60°C for 45s, and 72°C for 30s, with a final extension at 72°C for 5 min. Products were run on a 1.4% agarose gel for adequate separation. Four primers were used for genotyping the deletion of *Shp*: Gal-5': 5'-CTAGCTAGAGGATCCCCGGGTACC-3', Gal-3': 5'-AATTTCGGCTCTGGCCTTCCTGTAG-3', Exon 1A: 5'-CTCTGCAGGTCGTCCGACTATTCTG-3', and Exon 1B: 5'-CCTCGAAGGTCACAGCATCCTG-3'. WT mice had a product size of 300 bp for primer set Exon 1A and 1B, and *Shp* KO had a product size of 500 bp for primer set Gal 5' and Gal 3'. Thermal cycling parameters for verifying *Fxr* were 95°C for 5 min, 25 cycles of 95°C for 45s, 56°C for 45s, and 72°C for 30s, with a final extension at 72°C for 5 min. Products were run on a 1% gel.

Mice were maintained on a chow diet (Envigo, cat no. TD-07571) until 12–13 months of age ($n = 6$ –10). At 22:00 the night before sacrifice, fecal samples were collected from mice, immediately flash frozen in liquid nitrogen, and stored at -80°C until use. Mice had continued access to food and were sacrificed starting at 9:00. Serum, liver, cecum (containing content), and ileum (last 5 cm of small intestine with content removed with a 1x PBS wash) were collected for analysis. For serum, blood was collected retro-orbitally into serum tubes (Becton Dickinson and Company, cat no. 365978), and samples were incubated at room temperature for at least 30 min and then centrifuged at 10,000 g for 3 min. All tissue was flash-frozen and stored at -80°C until use.

Histology

Liver tissue from male and female WT and whole body DKO was fixed in 10% neutral buffered formalin for approximately 24 h and then stored in 70% ethanol until processing to be embedded into paraffin. Five μm sections were made for staining. Standard protocols were followed for hematoxylin & eosin (H&E) staining.

Targeted metabolomics for bile acid composition

Serum and liver samples were sent to the Washington University School of Medicine Metabolomics Facility for targeted metabolomics of their bile acid compositions. The

liver samples were homogenized in water (4 ml/g tissue). A protein precipitation procedure was used to extract bile acids from 0.05 ml of liver homogenate and 0.05 ml of serum in the presence of deuterated internal standards (d4-TMCA, d4-CA, d4-GCDCA, d4-GCA, d4-CDCA, d4-DCA, d4-LCA, d4-UDCA, d4-GUDCA, d4-GDCA, d4-GLCA, d4-TUDCA, d4-TLDC, d4-TCA, d4-TCDC, d6-TDCA). The 9–11-point standard curves for bile acids in the surrogate matrix were prepared in duplicate. The calibrators were injected at the beginning and end of the run. The samples with bile acids above the upper limit of quantification (ULOQ), the high end of the calibration curve, were also diluted 10-fold or 25-fold with water before extraction. The bile acids were separated by column-switching high-performance liquid chromatography (HPLC) on a Security Guard Gemini C18 (4 $\times$ 3 mm) and ACE Excel Super C18 column (3 μm , 50 $\times$ 4.6 mm). Bile acids and their internal standards were detected by an AB Sciex 4000QTRAP mass spectrometer equipped with an ESI probe in the negative multiple-reaction monitoring (MRM) mode. Data processing was conducted with Analyst 1.6.3 (AB Sciex). The calibrators that deviated by more than 15% of nominal concentrations for all calibrators except for 20% for the lower limit of quantification (LLOQ), which is the low end of the calibration curve, were excluded from construction of the calibration curve. A limitation of this analysis is that we only used d4-TMCA as an internal standard for all MCA measurements, and hence it is possible the TMCA measurements may not be as accurate. We were also unable to separate the α -, β -, and ω -isomers of MCA or TMCA. The bile acids in the liver were normalized to tissue wet weight. Analysis was carried out to determine the percent composition of each bile acid and the total concentration in each tissue.

Untargeted fecal bile acid composition

Metabolites from 10 mg mL⁻¹ murine fecal slurries were extracted by adding ice-cold methanol to a final concentration of 70% (v/v), followed by overnight incubation at 4°C. Samples were centrifuged at 10,000 $\times g$ for 5 min to pellet cell debris, and the extract supernatant was diluted 1:1 in 50% methanol (v/v) prior to liquid chromatography-tandem mass spectrometry analysis (LC-MS/MS).

Liquid chromatography was performed using a Vanquish Autosampler (Thermo Scientific) and an Acquity UPLC BEH C-18 column, 2.1 mm $\times$ 100 mm (Waters). Mass spectrometry was performed using a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific) in positive ion mode. All analyses used a 10 μl injection volume, 0.4 ml min⁻¹ flow rate, and 60°C column temperature. Samples were eluted using a linear solvent gradient of water (A) and acetonitrile (B), each containing 0.1% formic acid, across a 12-min chromatographic run as follows: 0–1 min, 2% B; 1–8 min, 2%–100% B; 8–12 min, 100% B; 10–12 min, 2% B. Data were collected using electrospray ionization in positive mode. MS1 data were collected using a 35,000 resolution, AGC target of 1e6, maximum iteration time of 100 ms, and a scan range set from 100 to 1500 m/z during (min 1–10). Data-dependent MS2 spectra were collected for the top 5 most abundant peaks identified in MS1 survey scans.

Raw files were converted to mzXML format via GNPS Vendor Conversion and submitted to the Global Natural Products Social Molecular Networking Database (GNPS, University of California at San Diego) for molecular networking and spectral identification (22, 23).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Following the manufacturer's protocol, RNA was extracted from frozen liver tissue using TRIzolTM Reagent (Invitrogen, Waltham, MA, catalog number: 15,596-018). Liver tissue was homogenized with 1.0 mm diameter Zirconium oxide beads (Next Advance, Troy, NY) in 1 ml of TRIzolTM Reagent using a Bullet Blender Storm (Next Advance, Troy, NY, cat no. BBY24 M) at speed 9 for 1 min. cDNA was made following the manufacturer's protocols for DNase I (New England Biolabs®, Inc., Ipswich, MA, cat no. M0303S) and Maxima H Reverse Transcriptase (Fisher, Thermo ScientificTM, Middletown, VA, cat no. FEREP0743). Sixty ng of cDNA was used for each qPCR reaction. SYBR green was used for the qPCR reactions and analyzed by quantifying $\Delta\Delta C_T$, the Livak method (24). Mouse primers used for the qPCR reactions can be found in Supplemental Table 1.

16S rRNA full-length sequencing for fecal samples

DNA extraction. Fecal and cecal samples were snap-frozen in liquid nitrogen and then stored at -80°C until DNA extraction for full-length 16S rRNA sequencing. For DNA extraction, the Qiagen QIAmp PowerFecal Pro DNA Kit (Hilden, Germany, cat no. 51804) was used following the manufacturer's protocol with modifications. Before starting, the tubes containing beads were weighed, samples were added, and the tubes were weighed again. Samples remained on dry ice until homogenization in CD1 buffer. Homogenization was carried out using the Bullet Blender Storm (Next Advance, Troy, NY, cat no. BBY24 M) for one minute at speed 9 for three rounds to ensure adequate homogenization. Samples rested on ice for 4 min between each round. The remainder of the DNA extraction followed the manufacturer's protocol, and samples were eluted off the MB spin column with water. DNA was quantified using a SpectraDrop micro-volume microplate for the SpectraMax M2 Multi-Detection Reader (Molecular Devices, LLC). Approximately 50 ng of DNA was run on a 1% agarose gel to ensure no degradation of samples. DNA was diluted to 10–50 ng/ml in 15 μl . Diluted samples were submitted to the Roy J. Carver Biotechnology Center for library preparation and sequencing.

PacBio full-length 16S rRNA sequencing methods.

Pooling and amplification. To create 16S rRNA amplicons, 2.5 ng of genomic DNA was used following the published PacBio 16S rRNA protocol. Briefly, 25 μl reactions were set up using barcoded primers (Forward: AGRGTTYGATYMTGGCTCAG*, Reverse: RGYTACCTTGTTACGACTT*, * notes 5' barcode extension). Each reaction consisted of 12.5 μl of Kappa Hotstart Ready Mix (KAPA Biosystems, Inc), 1.5 μl of water, 3 μl for each 16S rRNA forward and reverse primer (2.5 μM), and 5 μl of DNA (0.5 ng/ μl). Amplification was completed using these PCR cycling parameters: 95°C for 3 min for denaturing, 25 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 60 s, followed by a 4 $^{\circ}\text{C}$ hold. The Qubit Broad Range kit (ThermoFisher Scientific) was used to measure the products and then run on an Agilent Fragment Analyzer (Agilent Technologies) for QC. Samples were then pooled according to their concentration and cleaned twice with 0.6 volumes of magnetic beads. Before library preparation, the cleaned pooled samples were checked on the Agilent Fragment Analyzer.

Library preparation and sequencing. Library construction and sequencing in the PacBio Sequel IIe were performed at the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. The 16S amplicons were generated with the barcoded Full-Length 16S primers from Pacific Biosciences following their protocol. The forward 16S rRNA sequence is 5'-AGRGTTYGATYMTGGCTCAG-3', and the reverse sequence is 5'-RGYTACCTTGTTACGACTT-3'. The individually barcoded amplicons were pooled and converted to a library with the SMRTBell Express Template Prep kit 3.0. The library was quantitated with Qubit and then run on a Fragment Analyzer (Agilent) to confirm the presence of DNA fragments of the expected size. The library was sequenced on a SMRTcell 8M on a PacBio Sequel IIe with 12 hs movie time. The circular consensus analysis and demultiplexing were done using SMRTLink V10.1 with the following parameters: min-passes 3 -min-rq 0.999.

Predicted functionality of microbial samples

PICRUSt2 was used to impute the functional potential for each sample (picrust2_pipeline.py) (25). Default parameters were used for PICRUSt2 analysis. Annotated gene abundance tables were filtered to retain only genes involved in secondary bile acid metabolism. Linear models were used to quantify relationships between genes involved in bile acid transformation, animal sex, and animal genotype. The estimated marginal means were calculated for each model (parameters – sex and genotype) using the emmeans package in R. P-values were adjusted using the Tukey method.

Statistical analysis

Analysis of 16S rRNA for fecal samples. Data files from the Roy J. Carver Biotechnology Center were extracted, and the MD5 sum was run to ensure a complete transfer of the files. The total reads obtained for each fecal sample were between 16,000 and 61,000. Fastq files were used for analysis. RStudio, version 2022.12.0.353 (<http://www.posit.co/>), an integrated development environment, was used to run analysis through R, version 4.2.2 (<https://www.R-project.org/>). Primers were removed from the sequence files. DADA2, version 1.26.0 (26), was used for dereplicating, denoising, and chimera removal, and to make an ASV table and a taxonomy table. Taxonomy was assigned using the Silva Database, version 138.1 (27). Statistical analysis for 16S rRNA fecal samples was completed on rarefied counts using the package vegan, version 2.6–4 (<https://cran.r-project.org/web/packages/vegan/index.html>) for the adonis test to analyze beta diversity and the package MASS, version 7.3.58.1 (28) for the additive two-way ANOVA (genotype + sex) analyzing specific ASV for differences between each group. For visualization of phyla and genera, phyloseq, version 1.42.0 (29) was used. All plots were made using ggplot2, version 3.4.1 (30).

Two-way ANOVA analyses. For all other analyses, GraphPad Prism (GraphPad Software, San Diego, CA) was used for the two-way ANOVA with a Tukey's post hoc test for bile acid concentration analysis and bile acid-related genes qPCR analysis. Data were reported as mean $\pm$ standard error (SEM). P-values are reported for the two-way ANOVA analyses. For the post-hoc analyses, P-values less than 0.05 are reported and noted as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

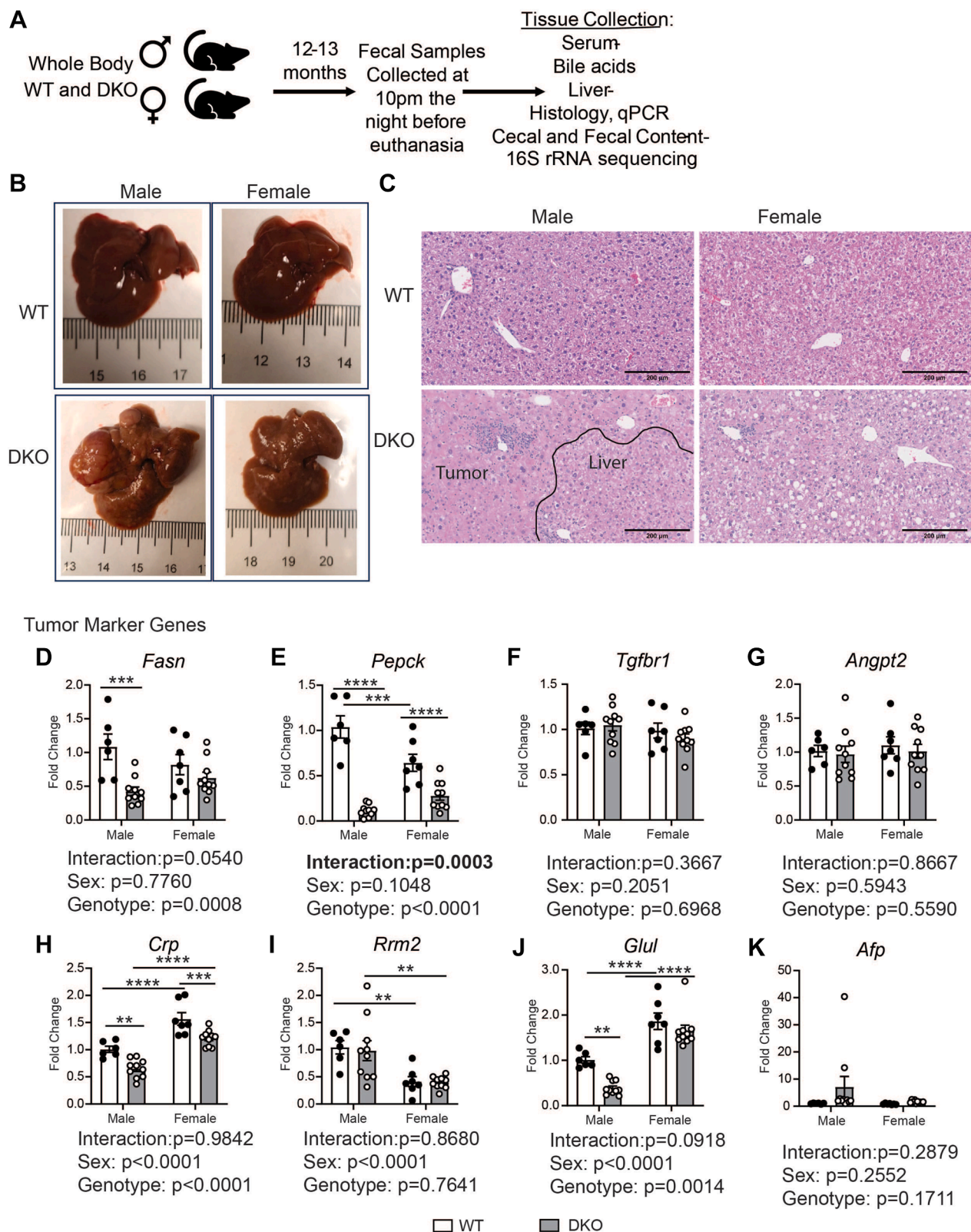


Fig. 1. DKO mice mimic several features of clinical hepatocellular carcinoma; male and female wild-type (WT) and *Fxr/Shp* double knockout (DKO) mice were aged to 12–13 months. **A:** Schematic of our studies and analysis. **B–C:** Representative gross liver images and liver histology with hematoxylin and eosin (H&E) staining. Tumor margins are highlighted with a solid black line. Gene expression of known tumor markers from the livers of these experimental cohorts (**D**) *Fasn*, (**E**) *Pepck*, (**F**) *Tgfb1*, (**G**) *Angpt2*, (**H**) *Crp*, (**I**) *Rrm2*, (**J**) *Glul*, and (**K**) *Afp*. A two-way ANOVA was used with an uncorrected Fisher's LSD *post hoc* test where significance was noted at $P < 0.05$ (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). Each bar is the mean $\pm$ SEM, and $n = 6$ –11 mice per group.

Between 3-11 mice were used for each group, and the specific n numbers are reported for each analysis.

RESULTS

Correlations. Correlations between bile acid composition and microbial genera were assessed using Spearman's correlation in RStudio (version 2022.12.0.353) (<http://www.posit.co/>).

Loss of *Fxr/Shp* increased serum primary bile acid levels in cancer-bearing male mice

We aged WT and DKO mice of both sexes, as one-year-old male DKO mice spontaneously developed

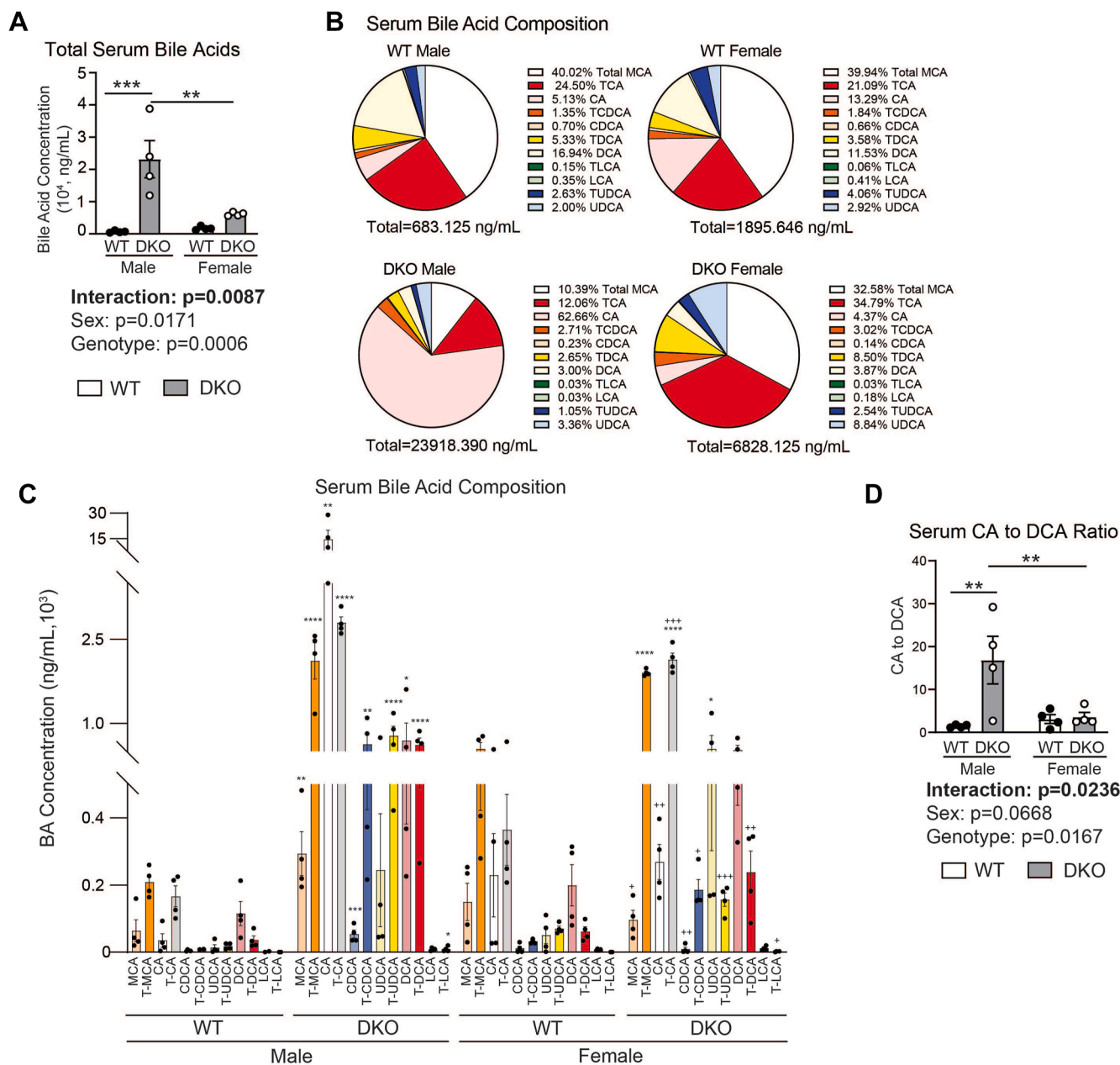


Fig. 2. Male DKO mice have an increased proportion of circulating unconjugated cholic acid. A: Total serum bile acid concentration in 12-13-month-old male and female wild-type (WT) and *Fxr/Shp* double knockout (DKO). B: Proportion of conjugated and unconjugated primary and secondary bile acids in all groups. C: Composition of individual bile acids (muricholic acid: MCA, taumuricholic acid: T-MCA, cholic acid: CA, taurocholic acid: T-CA, CDCA: chenodeoxycholic acid, T-CDCA: taurochenodeoxycholic acid, ursodeoxycholic acid: UDCA, tauroursodeoxycholic acid: T-UDCA, deoxycholic acid: DCA, taurodeoxycholic acid: T-DCA, lithocholic acid: LCA, tauroolithocholic acid: T-LCA) from serum for all groups completed using column-switching high throughput liquid chromatography (HPLC). D: The ratio of total serum CA to DCA from all groups. A two-way ANOVA was used with an uncorrected Fisher's LSD *post hoc* test where significance was noted at $P < 0.05$ (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). For the individual bile acid composition, *indicates genotype differences within sex normalized to WT, and + (* $P < 0.05$, ++ $P < 0.01$, +++ $P < 0.001$, ++++ $P < 0.0001$) indicates sex differences within genotype normalized to males. Each bar is the mean $\pm$ SEM, and $n = 4$ mice per group.

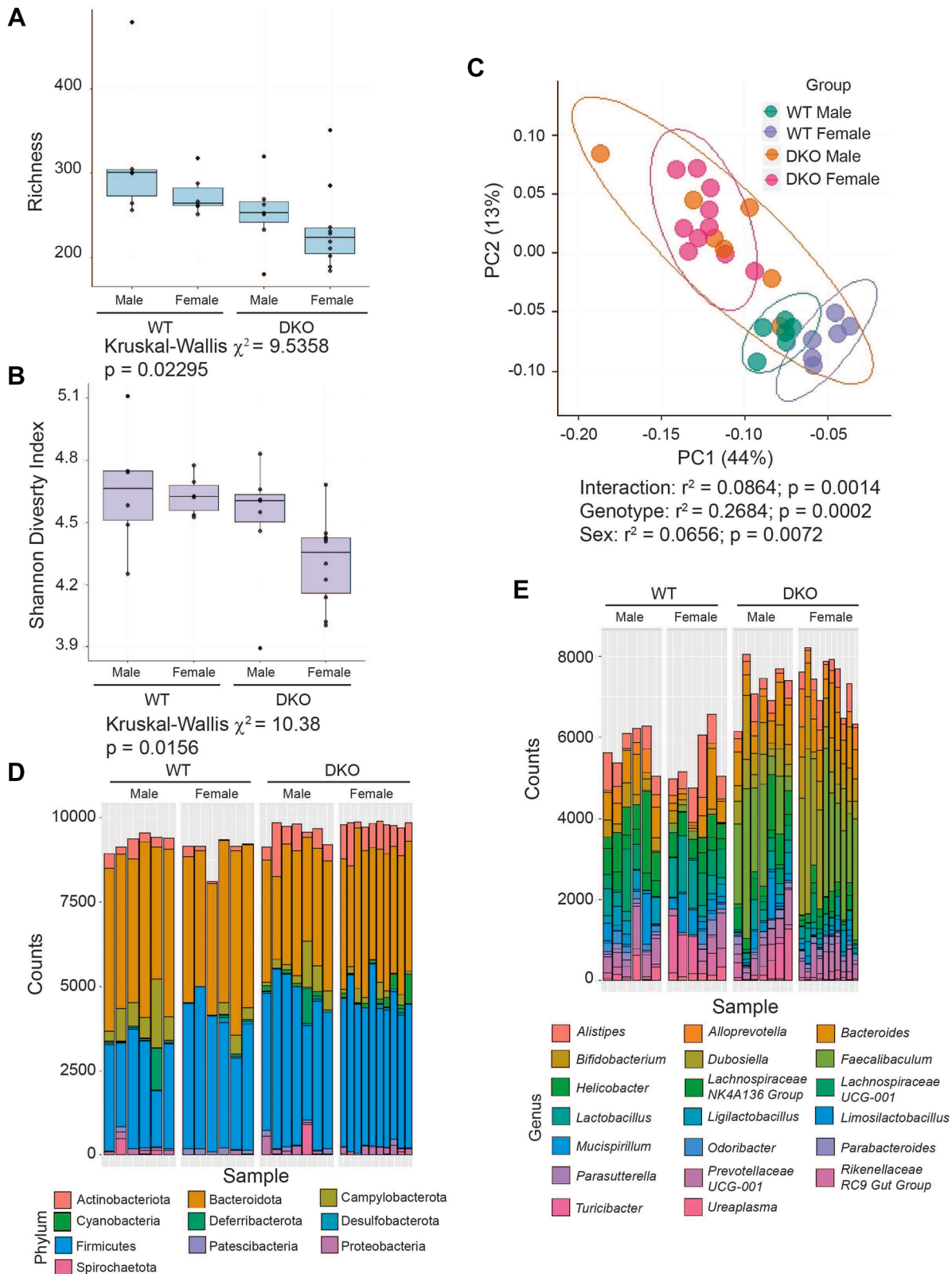


Fig. 3. Sex-dependent microbial diversity in feces is lost in DKO mice. A: Microbial richness and (B) Shannon diversity index for fecal samples from wild-type (WT) and *Exr/Shp* double knockout (DKO) male and female mice at one year of age. Analysis was completed using a Kruskal-Wallis statistical analysis with significance noted at $P < 0.05$. C: Microbial diversity in fecal samples for all treatment groups with statistical ellipses calculated for each group by mean and a covariance matrix. Samples were normalized to relative abundance. Each dot represents one sample. Statistical analysis was determined using a PERMANOVA with 5000 permutations using vegan version 2.6-4 (<https://cran.r-project.org/web/packages/vegan/index.html>). Significance codes: 0 “***” 0.001 “**” 0.01 “*” 0.05 “.”. Microbial composition for D) phyla and E) top 20 genera for fecal samples from each group were made using “phyloseq” version 1.42.0 (29). n = 6–10 per group.

HCC on a standard chow diet (**Fig. 1A**) (8). As expected, 100% of the male DKO mice developed large and aggressive liver tumors (9/10 were HCC and 1/10 displayed HCC and cholangiocarcinoma), resulting in increased liver weights and elevated hepatosomatic index compared to WT mice (**Supplemental Fig. S1A–D**). In contrast, few micronodules were seen in DKO female mice (**Fig. 1B, C**). We next measured the expression of multiple HCC marker genes (**Fig. 1D–K**) and found reduced expression of *Fasn*, *Pepck*, *Crp*, and *Ghul* in DKO male mice (**Fig. 1D, E, H, J**), suggestive of HCC molecular subtype group 4 (31, 32). Since FXR is a known regulator of *Pepck* and *Fasn* gene expression (33–35), knockout of *Fxr* (*Fxr*KO) liver tumor models may exhibit a molecular profile of group 4 subtype, and additional markers may be needed to ascertain it.

As a bile acid overload model, DKO mice have significantly higher total bile acid levels than WT mice (**Fig. 2A**; **Supplemental Fig. S2A**). Also, in line with our prior findings, female DKO mice displayed lower serum and hepatic bile acid concentrations than DKO males (**Fig. 2A**; **Supplemental Fig. S2A**). Intriguingly, BA compositional analysis revealed a sharp increase in serum cholic acid (CA) levels with free-CA being more than taurine-conjugated CA (TCA) in aged DKO males compared to WT and DKO females (**Fig. 2B, C**). Though CA increased in DKO females compared to WT mice, TCA levels were predominant. In aged WT mice, serum BAs were comparable between the sexes. Next, we examined hepatic bile acid composition in WT and DKO and found that, as expected, muricholates and taurocholic acid comprised 80%–90% of

the bile acid pool. In fact, ample TCA concentrations were found in both sexes of DKO mice, indicative of intact taurine conjugation function of the liver (**Supplemental Fig. S2B, C**).

Since CA can be converted to DCA, a known carcinogen, we examined the CA/DCA ratio and found no difference between DKO male and female livers (**Supplemental Fig. S2D**). However, serum CA to DCA ratio was significantly higher in male DKO mice compared with the females (**Fig. 2D**). It is relevant to note that DCA levels (both free and taurine conjugated) are elevated in DKO males and females. Also, TDCA is significantly higher in HCC-bearing DKO males than in WT males. One interpretation of high circulating CA levels despite intact hepatic bile acid conjugation is that a robust gut microbiota-mediated bile acid deconjugation may be occurring in male DKO mice resulting in excess CA. In addition, the overall proportion of secondary bile acids to the total pool were lower in the DKO model, suggesting a compensatory mechanism or a decrease in microbial diversity secondary to chronic exposure to BA overload (**Fig. 2B, C**). Therefore, we examined genes related to bile acid homeostasis and investigated if differences in cecal and fecal microbiome exist between male and female DKO mice.

DKO mice have altered expression of bile acid synthesis and transport genes

Aged DKO mice showed alterations in bile acid synthesis, with DKO females robustly inducing the classical pathway (*Cyp7a1* and *Cyp8b1* genes

TABLE 1. Phyla and genera from fecal samples having significant genotype or sex differences

Phylum	Genus	Genotype <i>P</i> -value	Sex <i>P</i> -value
Actinobacteriota		4.08E-07	0.110585
	^a <i>Bifidobacterium</i>	4.01E-07	0.110845
Firmicutes		0.000211868	0.049272
	^a <i>Faecalibaculum</i>	5.40E-09	0.244417
	<i>Dubosiella</i>	7.59E-31	0.008946
	<i>Turicibacter</i>	6.08E-91	0.841538
	^a <i>Roseburia</i>	1.38E-06	0.624541
	<i>Ureaplasma</i>	1.26E-08	0.606167
	<i>Lachnospiraceae</i> NK4A136 group	0.001357841	0.469894
	<i>Incertae Sedis</i>	1.15E-05	0.010372
	^a <i>Erysipelatoclostridium</i>	2.55E-09	6.47E-05
	<i>Anaerotruncus</i>	0.000186003	0.412304
	[<i>Eubacterium</i>] <i>siraeum</i> group	0.99791048	0.038001
	^a <i>Tyzzerella</i>	0.000114065	3.84E-05
Bacteroidota		2.30E-06	0.125221
	^a <i>Alistipes</i>	0.001665699	0.480394
	<i>Odoribacter</i>	0.000446658	0.446896
	<i>Rikenella</i>	4.53E-05	0.051008
Campylobacterota		0.459523866	0.004246
	<i>Helicobacter</i>	0.459523866	0.004246
Spirochaetota		0.38951808	0.000897
	<i>Brachyspira</i>	0.38951808	0.000897
Deferribacterota		0.598318348	0.001169
	<i>Mucispirillum</i>	0.598318348	0.001169
Cyanobacteria		1.93E-06	0.582775

^aDenotes genera associated with bile salt hydrolase (*bsh*) activity.

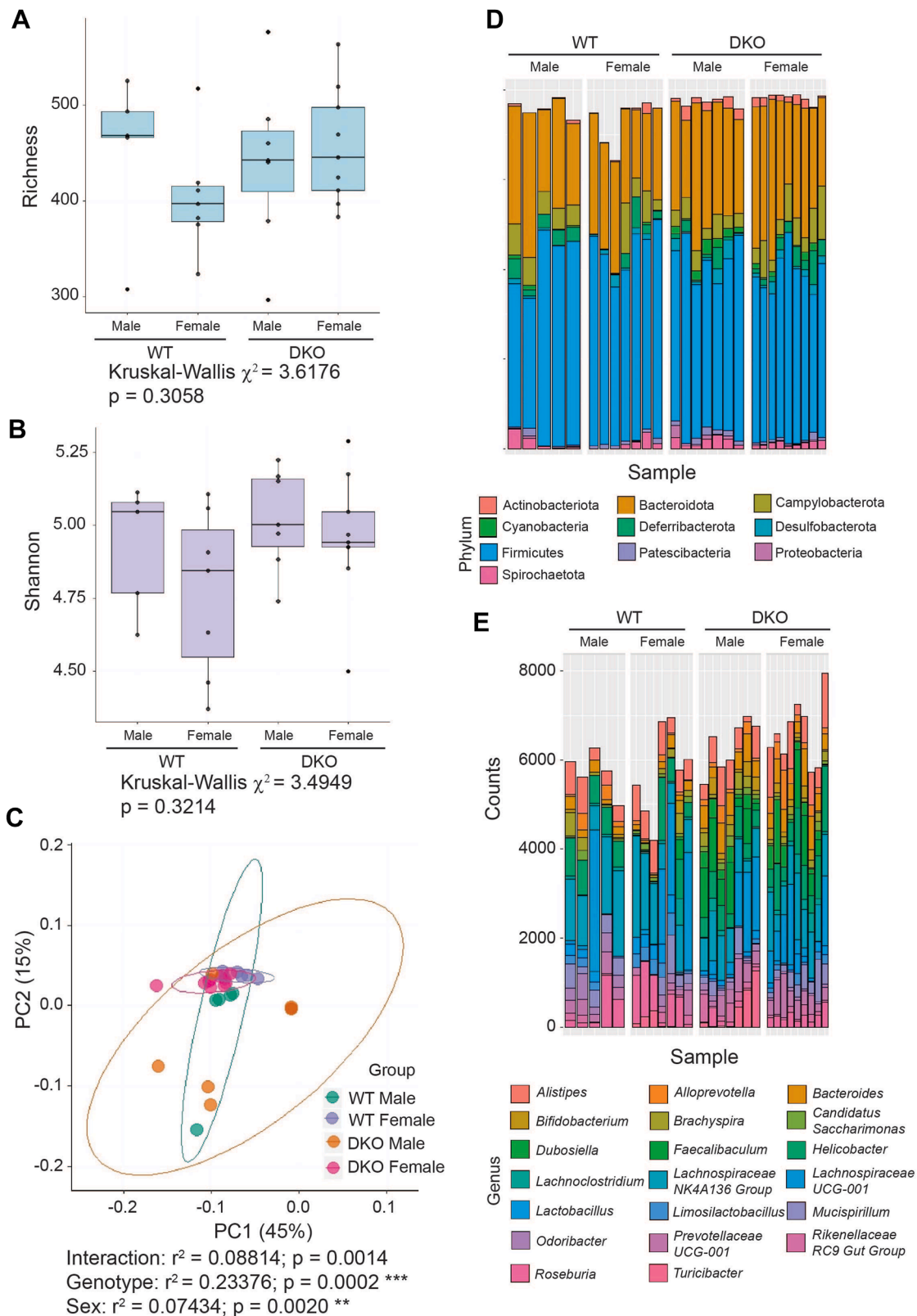


Fig. 4. Male DKO mice display sex-dependent microbial diversity in the cecum. A: Microbial richness and (B) Shannon diversity index for cecal samples from wild-type (WT) and *Fxr/Shp* double knockout (DKO) male and female mice at one year of age. Analysis was completed using a Kruskal-Wallis statistical analysis with significance noted at $P < 0.05$. C: Microbial diversity in cecal samples for all treatment groups with statistical ellipses calculated for each group by mean and a covariance matrix. Samples were normalized to relative abundance. Each dot represents one sample. Statistical analysis was determined using a PERMANOVA with 5000 permutations using vegan version 2.6-4 (<https://cran.r-project.org/web/packages/vegan/index.html>). Significance codes: 0 “***” 0.001 “**” 0.01 “*” 0.05 “.”. Microbial composition for (D) phyla and (E) top 20 genera for fecal samples from each group were made using “phyloseq” version 1.42.0 (29), n = 6–10 per group.

(Supplemental Fig. S3A, B)) and suppression of the alternative pathway (36), especially expression of *Cyp27a1* (Supplemental Fig. S3C) compared to WT females. On the other hand, DKO males with HCC, had reduced expression of all the BA synthesis genes, including that of male-specific *Cyp7b1* ((37), Supplemental Fig. S3D) and muricholate-producing *Cyp2c70* (38) (Supplemental Fig. S3E). This reduction is consistent with lower percentage of total MCA present in DKO males than in the WT mice (Supplemental Fig. S2C).

Next, we investigated the bile acid-CoA: amino acid N-acyltransferase (*Baat*) gene, which is crucial for bile acid conjugation (39). *Baat* expression is higher in females, although its levels are reduced in DKO mice (Supplemental Fig. S3F), ample levels of taurine-conjugated bile acids were found in all four groups of mice. Since *Cyp2a12* mediated hydroxylation of bile acids after enterohepatic circulation (40), we examined and found it to be higher in WT female livers but reduced in DKO females (Supplemental Fig. S3G). Finally, we examined sulfotransferases, which sulfate bile acids in the liver prior to excretion (41). Consistent with their female-specific expression *Sult2a1* was 1000X higher than male mice, but its induction was noted in both DKO males and females (Supplemental Fig. S3H). This is in line with cholestatic DKO liver trying to lower the bile acid burden.

Finally, we examined genes involved in bile acid transport to and from the liver (Supplemental Fig. S4). DKO mice displayed reduced expression of many of the genes encoding for BA import *Slc10a1* (NTCP), *Slc10a1* (OATPIA1), and *Slc10b2* (OATPIB2) (Supplemental Fig. S4A–C) and BA export *Abcb11* (BSEP), *Slc51b* (OSTB), *Abcb1a* (MDR1) (Supplemental Fig. S4D–F). This may be attributed to loss of *Fxr* and *Shp* and subsequent cholestasis. We found several transporter genes prominently expressed in WT females compared to males (*Slc10a1*, *Slc10b2*, *Abcb11*, *Slc51b*), but their expressions were dampened, and sex differences were lost in DKO mice. Other genes that encode transport to bile canaliculi, such as *Abcc2* (MRP2), were lower only in DKO females, while both *Abcc3* and *Abcc4* (MRP3 and 4), which are responsible for bile acid export into systemic circulation, were induced in the DKO model compared to WT (Supplemental Fig. S4G–I).

Overall, the expression of the genes involved in bile acid synthesis, uptake, and export can explain certain aspects of serum and hepatic bile acid profiles seen in DKO mice but not address the high levels of cholic acid present in tumor-bearing DKO males.

Next, we investigated if dysbiosis occurs in DKO animals, and if there are sex differences in the fecal and cecal microbiome. We found sex differences in fecal microbial composition between WT and DKO mice such that female DKO mice had significantly reduced richness and Shannon Diversity Index

TABLE 2. Phyla and genera from cecal samples having significant genotype or sex differences

Phylum	Genus	Genotype <i>P</i> -value	Sex <i>P</i> -value
Campylobacterota		3.53E-41	2.15E-08
	<i>Helicobacter</i>	3.53E-41	2.15E-08
Actinobacteriota		7.70E-11	0.198477
	<i>Bifidobacterium</i>	6.32E-11	0.231093
Proteobacteria		1.62E-06	7.62E-06
	<i>Parasutterella</i>	2.06E-06	0.002132
Cyanobacteria		2.74E-07	0.000582
Firmicutes ^a		0.141	0.823
	<i>Faecalibaculum</i>	3.63E-11	0.686397
	<i>Dubosiella</i>	1.46E-27	0.536518
	<i>Roseburia</i>	0.001645725	0.030139
	<i>Ureaplasma</i>	4.32E-05	0.256573
	<i>Candidatus Arthromitus</i>	1.62E-05	0.007472
	<i>Lachnoclostridium</i>	0.000102808	0.068837
	<i>Anaeroplasm</i>	0.999976433	4.01E-10
	<i>ASF356</i>	1.58E-06	0.126594
	<i>Limosilactobacillus</i>	0.002126431	0.015721
	<i>Erysipelatoclostridium</i>	0.996843205	0.000965
	<i>A2</i>	0.000485963	0.639616
	<i>GCA-900066575</i>	4.02E-05	0.230422
	<i>Butyrivibrio</i>	0.001064723	0.830538
	[<i>Eubacterium</i>] <i>siraeum</i> group	0.999998464	0.00026
	<i>Tyzzerella</i>	0.000143708	0.006533
	<i>UCG-003</i>	0.00358245	0.006832
	<i>Paludicola</i>	0.997455215	0.022679
Bacteroidota ^a		0.243	0.963
	<i>Bacteroides</i>	3.31E-07	0.407755

^aNotes insignificant phyla but contained significant genera.

^bDenotes genera associated with bile salt hydrolase (*bsh*) activity.

(Fig. 3A, *P* = 0.02295, Fig. 3B, *P* = 0.0156). Further, PERMANOVA revealed interactions between sex and genotype for beta diversity (*P* = 0.0014) (Fig. 3C). The phylum and genus level data showed unique sex-specific fecal microbiomes (Fig. 3D, E), with seventy percent (7/10) of the identified phyla and 36% (18/50) of the genera displaying either significant genotype- or sex-differences (Table 1).

Actinobacteriota was one of the top phyla that was enriched in DKO feces irrespective of the sex (Supplemental Fig. S5A). Within Actinobacteriota, the genus *Bifidobacterium* displayed higher abundance in DKO mice than WT (Supplemental Fig. S5D). Firmicutes were abundantly detected in the fecal samples (Supplemental Fig. S5B, Table 1) with higher amounts in WT females than males (Supplemental Fig. S5B). Of note, Firmicutes counts were specifically increased in DKO males (Supplemental Fig. S5B). In our analysis, this phylum only had three genera with significantly altered abundance—*Faecalibaculum*, *Dubosiella*, and *Roseburia*—the first two were enriched in DKO feces while the third one was reduced compared to WT (Supplemental Fig. S5E–I).

The phylum Bacteroidota and genus *Alistipes* were also reduced in both sexes of DKO feces (Table 1, Supplemental Fig. S5C, F). Although *Helicobacter*, which belongs to the Campylobacterota phylum, was lower in DKO feces, it was abundant in WT and showed an enrichment in male versus female WT feces

(Supplemental Fig. S5G). Thus, the fecal microbiome changes in DKO mice are clearly indicative of dysbiosis. Importantly, many of the phyla that are altered harbor bile acid transformation and hence may underlie changes in BA composition seen in DKO animals (17, 42).

Since fecal microbiome analysis revealed primarily genotype differences but not sex differences, we analyzed the cecal content of WT and DKO mice of both sexes to examine microbes that may have been missed by fecal analysis. We found distinct clustering and sex differences in microbial composition between the WT and DKO cecal samples (Fig. 4, Supplemental Fig. S6). Although the richness observed in the WT cecum is lost in the DKO samples (Fig. 4A), we observed no difference in Shannon Diversity between sexes and genotypes (Fig. 4B). We also saw a statistically significant interaction between genotype and sex for beta diversity, and particularly, most of the male DKO mice are separated from the other groups (Fig. 4C).

We found distinct genotype and sex differences in cecal microbiota (Fig. 4D, E) with forty percent (4/10) identified phyla and 32% (21/64) identified genera had either significant genotype or sex differences (Table 2). In the phylum Campylobacterota, which includes the genus *Helicobacter*, we found intriguing sex differences such that aged WT female cecum had lower counts than WT males, whereas these counts were higher in DKO females than DKO males (Supplemental Fig. S6A, C). Actinobacteriota was another phylum that had significant genotype differences (Supplemental Fig. S6B), with more enrichment in DKO mice. *Bifidobacterium*, known for bile acid metabolism, was higher in DKO than WT cecum (Supplemental Fig. S6D). Although Firmicutes at the phylum level had comparable abundance between all the cecal samples (Table 2), DKO mice displayed enriched *Faecalibaculum* and *Dubosiella* compared to WT, which had virtually no abundance (Supplemental

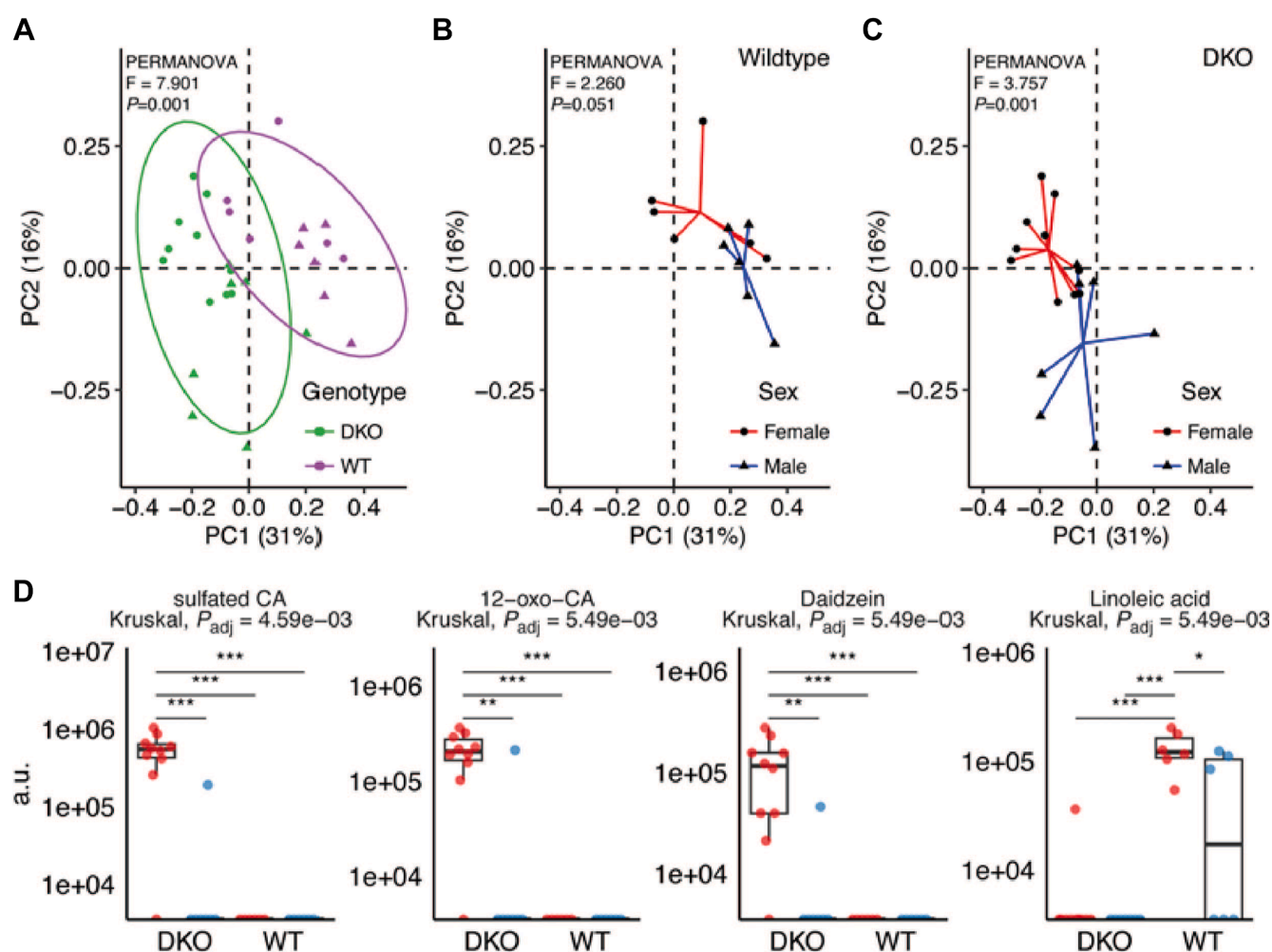


Fig. 5. Female DKO mice have increased excretion of sulfated CA and 12-oxo-CA. A: Principal coordinate analysis of fecal metabolomes based on Bray-Curtis dissimilarity, showing significant differences between *Fxr*^{-/-} *Shp*^{-/-} (DKO) and wild-type mice, as determined by PERMANOVA. Subsets of (B) WT or (C) DKO mice show that differences between sexes approached significance for WT mice but were highly significant for DKO mice. D: Top annotated metabolites differing between sexes within genotypes, as determined by Kruskal-Wallis test followed by Benjamini-Hochburg P value correction, with points colored by sex of the mouse. Comparisons within each metabolite were performed using post-hoc Dunn tests. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

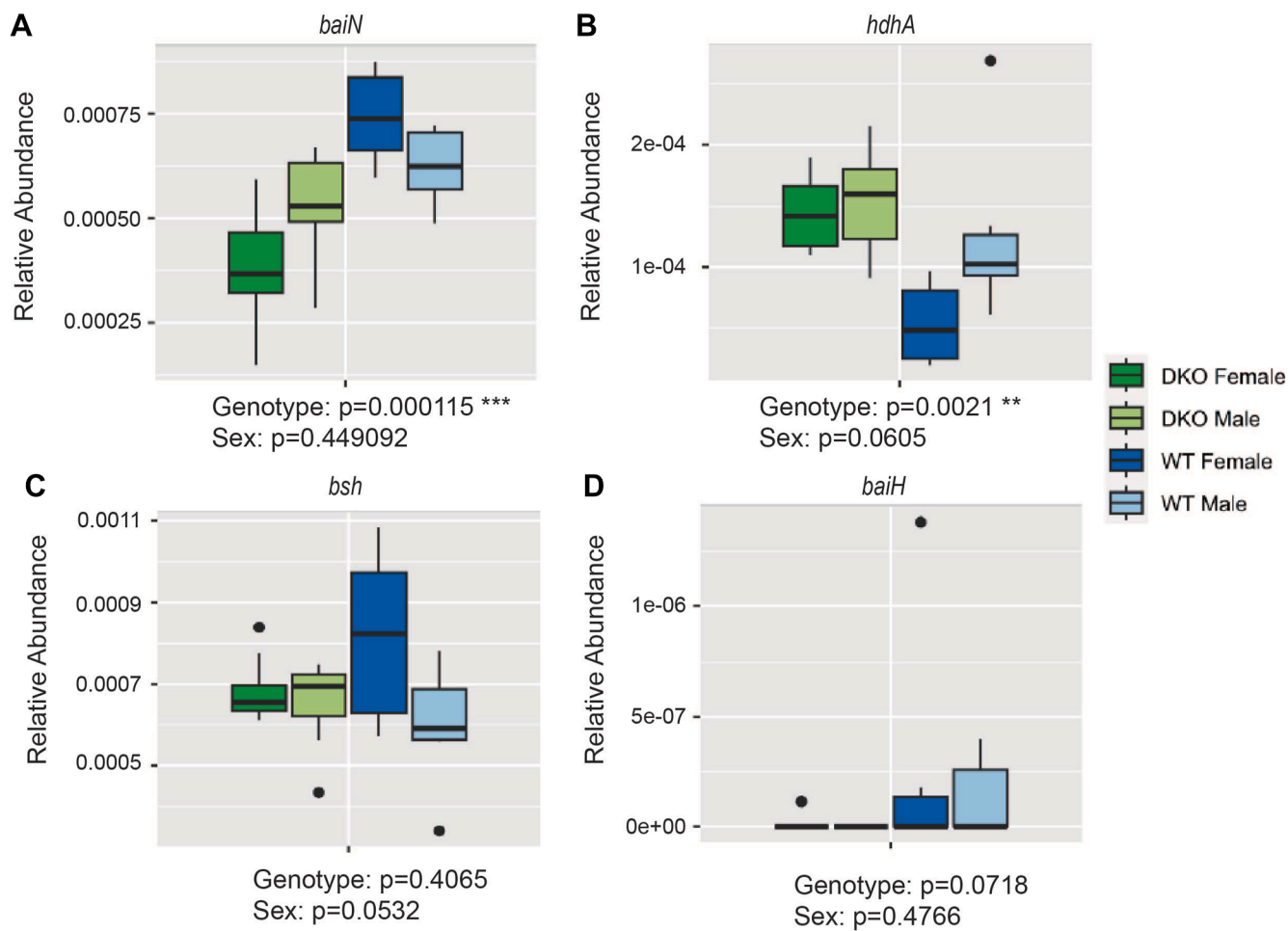


Fig. 6. DKO male and female mice have altered predictive function of microbial genes associated with secondary bile acid synthesis in the feces. Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) imputed relative abundance of select microbial genes (A) *bai*, N, (B) *hdhA*, (C) *bsh*, and (D) *baiH* from fecal samples of one-year-old male and female wild-type (WT) and *Fxr/Shp* double knockout (DKO) mice. Statistical analysis was determined using a multivariate analysis using vegan version 2.6-4 (<https://cran.r-project.org/web/packages/vegan/index.html>). Significance codes: 0 “***” 0.001 “**” 0.01 “*” 0.05 “.”.

Fig. S6E, G). Conversely, *Roseburia* with bile salt hydrolase activity was reduced in male and female DKO mice compared with wild-type mice (Supplemental Fig. S6H). On the other hand, both sexes of DKO mice had increased counts of *Bacteroides*, known bile acid metabolizers (Supplemental Fig. S6F), although at the phylum Bacteroidota level this difference was absent (Table 2).

Taken together, our findings indicate dysbiosis in DKO mice. In fact, many of the alterations occurred in the genera *Bifidobacterium*, *Alistipes*, and *Bacteroides* that are known to have bile acid modification ability (17, 42), which in turn may contribute to changes in BA composition and subsequently to HCC.

DKO female mice exhibit increased levels of fecal sulfated and 12-oxo-CA

LC-MS/MS untargeted metabolomics was used to determine if there are differences in fecal excretion of metabolites between the different cohorts of mice, as

we found the fecal and cecal microbiome were altered in DKO mice. We detected varying levels of fatty acids, bile acids, phospholipids, carbohydrates, and terpenoids. Metabolite differences between WT and DKO feces are evident in the heatmap (Supplemental Fig. S7). More importantly, we found specific-sex differences in the fecal metabolites between WT, as well as DKO males and females (Fig. 5A–C). Some of the more variable metabolites between genotypes include linoleic acid, which is higher in WT animals, whereas sulfated CA was altered in DKO animals. To visualize the sex differences in the metabolome, we calculated the Bray-Curtis dissimilarities between samples and then visualized the variation with PCoA plots. Particularly, in DKO female feces, we found elevated levels of sulfated-CA and 12 oxo-CA than any of the other groups, which is consistent with lower circulating CA levels in these animals compared to DKO males (Fig. 5D). We also noticed more daidzein, a natural isoflavone, in the DKO feces, and more fecal linoleic

acid in WT animals. Both daidzein and linoleic acid have been speculated to protect against HCC, and additional studies would be warranted to evaluate them. Nonetheless, these data revealed that increased excretion of CA metabolites occurs in DKO females. To examine if microbial action may be responsible for this CA difference, we performed further computational analysis of the microbiome data.

We performed functional imputations using PICRUSt—software that leverages microbial abundance files (*i.e.*, ASV tables) to predict microbial gene abundances—for WT and DKO microbial communities. We then extracted the subset of gene abundances from these functional profiles that corresponded to genes involved in bile acid metabolism. We found reduced relative abundance in DKO mice for *baiN* based on genotype (3-dehydro-bile acid Δ 4,6-reductase) in the fecal ($P = 0.000115$, Fig. 6A) and cecal samples ($P = 0.00247$, Fig. 7B), which is relevant for 7- α -hydroxylation and secondary bile acid synthesis (43). However, *bsh* (bile salt hydrolase), known for deconjugating bile acids (44, 45), and *baiH* (7 β -hydroxy-3-oxochol-24-oyl-CoA 4-desaturase), known for the dehydroxylation of bile acids (46–48), remained

unaltered in the DKO mice in the feces and cecum (Fig. 6C, D, Fig. 7A, D). Moreover, the DKO fecal microbiome shows an increase in *hdhA* (genotype: $P = 0.0021$) (7 α -hydroxysteroid dehydrogenase) in the feces (Fig. 6B), which is involved in ursodeoxycholic acid production (21, 49), and consistently DKO mice display higher circulating UDCA levels (Fig. 2C). Cecal samples also indicate higher *hdhA* predictive function in the DKO male mice than WT mice (Fig. 7C). Thus, PICRUSt analysis provides insight into how microbial changes may contribute to the bile acid changes that we see in the DKO mice. These predictions will need to be verified in the future.

Finally, we examined whether a combination of specific BAs and microbes correlates with HCC incidence. We chose to examine CA and DCA (a known hepatocarcinogen) and test with the abundance of fecal *Faecalibaculum*, which was enriched in the DKO feces and *Bifidobacterium*, which has *bsh* activity and is linked to HCC. We found that the ratio of unconjugated CA to total bile acid positively correlated with the counts of *Faecalibaculum* but not with *Bifidobacterium* (Fig. 8A, B). Conversely, unconjugated DCA negatively correlated with total counts of *Faecalibaculum* but not

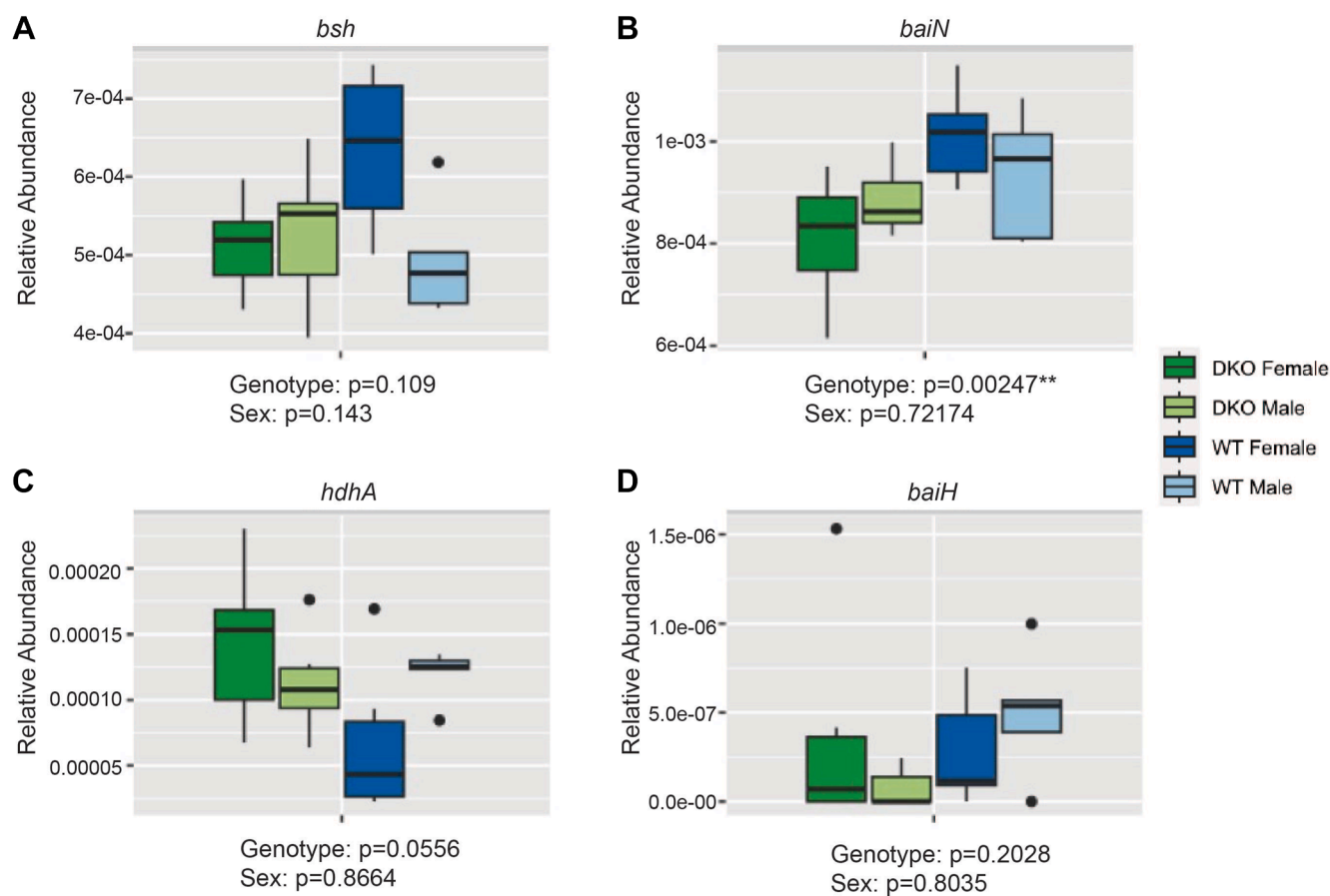


Fig. 7. DKO mice have altered predictive function of microbial genes associated with secondary bile acid synthesis in the cecum, PICRUSt imputed relative abundance of microbial genes. A: *bsh*, (B) *baiN*, (C) *hdhA*, and (D) *baiH* from cecal samples of one-year-old male and female wild-type (WT) and *Fxr/Shp* double knockout (DKO) mice. Statistical analysis was determined using a multivariate analysis using vegan version 2.6–4 (<https://cran.r-project.org/web/packages/vegan/index.html>). Significance codes: 0 “***” 0.001 “**” 0.01 “*” 0.05 “.”.

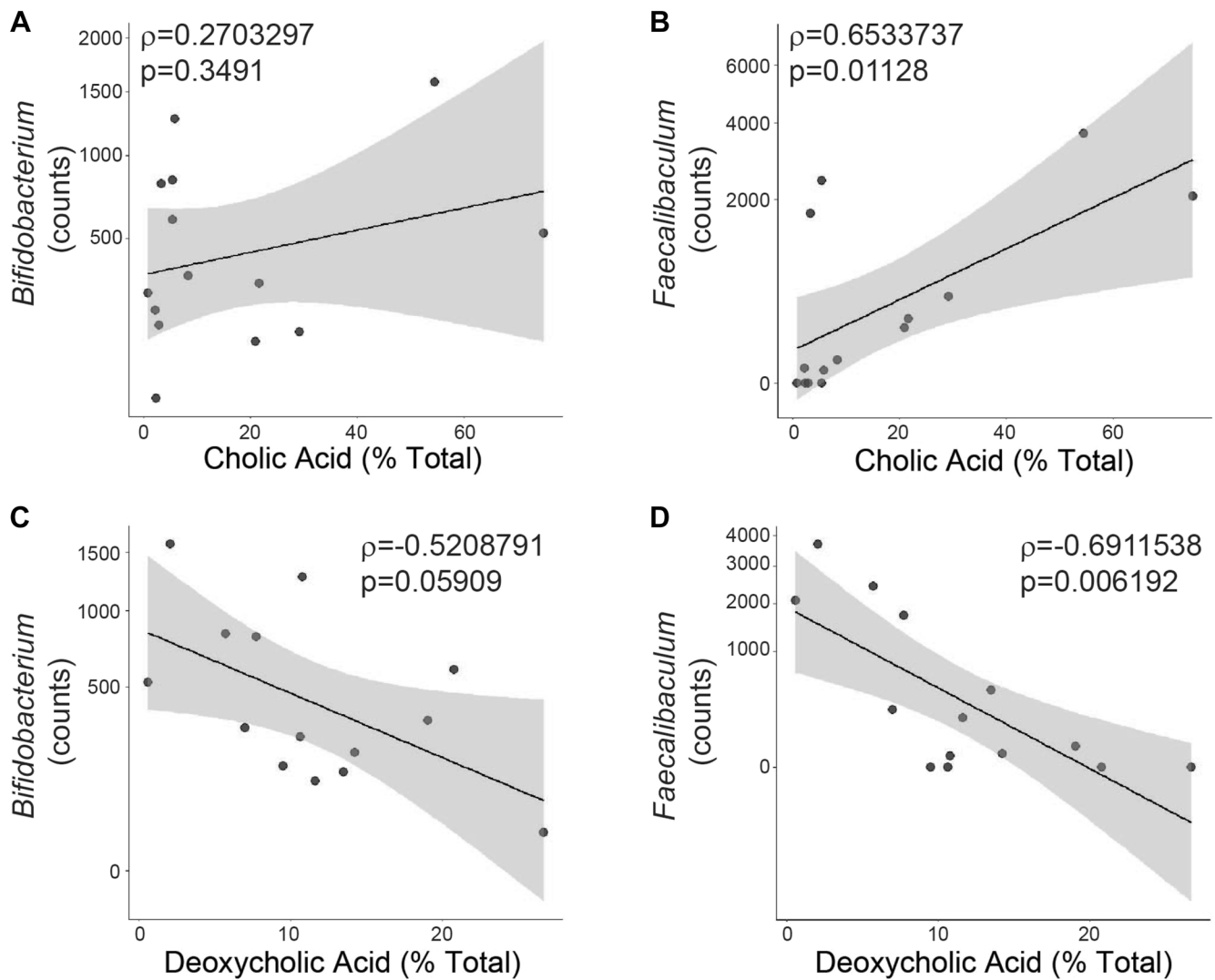


Fig. 8. Fecal *Faecalibaculum* correlates with the proportions of unconjugated CA and DCA during HCC. Correlations for male and female wild-type and *Fxr/Shp* double knockout mice at one year of age for percent of (A, B) cholic acid (CA) and (C, D) deoxycholic acid (DCA) in the total serum bile acid composition with fecal microbial genera (A, C) *Bifidobacterium* and (B, D) *Faecalibaculum*. Spearman's rank correlation test was used for statistical analysis, and the y-axis of each graph is log-transformed.

with *Bifidobacterium* (Fig. 8C, D). Thus, unconjugated serum CA and fecal *Faecalibaculum*, both of which are abundant in tumor-bearing DKO males, may be leveraged as potential biomarkers for HCC in the future.

DISCUSSION

These data demonstrate that the DKO mouse model of HCC has altered bile acid composition and dysbiosis. Although loss of FXR and SHP can also impact inflammation and apoptosis (50–54) in hepatic carcinogenesis, we did not investigate this aspect, which is a limitation. Instead, we focused on bile acid homeostasis. By utilizing this DKO model that develops HCC in a sexually dimorphic pattern, we identified distinct profiles of circulating bile acids and cecal and fecal microbial genera.

In line with our findings, several human and mouse studies have reported high bile acids and sex disparity during HCC development (8, 55). Most of these studies focused on secondary bile acids, specifically DCA (18, 56), which has been associated with NAFLD progression to cancer in patients and murine models (18–21). However, more recently, an increase in primary bile acids has been associated with HCC development, notably in patients with viral load of hepatitis B and C who develop HCC (57). Our results are consistent with this data, as we found high proportions of circulating levels of primary bile acid (mostly CA) rather than DCA in DKO male mice that develop liver cancer. Although DCA concentrations increased in the DKO mice, their fraction in the total bile acid pool was lower compared to WT mice. To understand why primary bile acid (CA) proportionally increased in DKO males, we examined fecal and cecal microbial composition and fecal metabolites.

We note dysbiosis in DKO mice and a decrease in microbial diversity. However, *Bifidobacterium*, known to deconjugate bile acids was higher, and some of its species are linked to HCC (21, 38, 50). We also found high abundance of *Faecalibaculum*, which are linked to higher serum bile acids, including CA (58). The beneficial effect of *Faecalibaculum* is context-dependent. For example, *F. rodentium* and its human homolog, *Holdemanella bififormis*, are linked to a protective role in intestinal cancer (59) but not for patients with liver fibrosis and non-alcoholic fatty liver disease (NAFLD) (19, 60–63). Both diseases are risk factors for HCC and correlate with the increase in these microbes. A limitation of our study is that we are unable to resolve to the species level. Additional metagenomic analysis would be needed to address this.

Nonetheless, untargeted fecal metabolomics revealed changes in fatty acids, phosphatidylcholine, amino acids, shikimates, and alkaloids in addition to bile acids between the different cohorts. When we focused on sex differences between DKO males and females, excretion of sulfated and 12-oxo-CA stood out. So does this imply that female DKO mice are able to employ mechanisms to modify CA for excretion, whereas the DKO male mice do not? Despite similar expression of the *Sult2a1* gene in both sexes of DKO mice, only DKO females excreted more sulfated CA. It is known that the colonic microbiome harbors sulfatase function, and (41) they may possibly be higher in DKO males. Future studies challenging DKO mice with CA-enriched diet will enable us to delineate some of these sex-specific discrepancies in CA excretion. Besides bile acids, fatty acids, and amino acid metabolites are linked to liver fibrosis and HCC (64–67), and they may also contribute to the sex difference in HCC incidence in the DKO mice and warrant additional research to test if their excretion corresponds with HCC progression.

Finally, fecal *Faecalibaculum* as a readout for liver cancer should be tested with other models of HCC as a prognostic marker. Although we know DKO mice have high CA prior to tumor onset, we do not know when the enrichment of *Faecalibaculum* occurs. Temporal studies to measure fecal counts of *Faecalibaculum* with primers will be valuable to assess when during HCC progression *Faecalibaculum* counts increase. Several studies have examined the increase of secondary bile acids as prognostics for HCC patients (4–6, 68–70), but it is now known that HCC patients have elevated levels of primary bile acids as early as four years prior to diagnosis (6). Taken together, we posit that monitoring a change in *Faecalibaculum*, or the human homolog *Holdemanella*, along with serum CA concentrations, may be beneficial and has the potential to be a biomarker for HCC.

Data Availability

All data are contained within the manuscript with raw data from untargeted metabolomics submitted to GNPS (Global Natural Products Social Molecular

Networking Database, University of California, San Diego) and PacBio dataset of cecal and fecal samples will be deposited into a publicly accessible repository, the location and identifying information (i.e., accession numbers) will be provided prior to publication. 

Supplemental Data

This article contains [supplemental data](#).

Author Contributions

A. E. D., D. V. G., R. A. Q., C. A. G., and S. A. writing – review & editing; A. E. D., R. A. Q., and S. A. writing – original draft; A. E. D., D. V. G., R. A. Q., and C. A. G. methodology; A. E. D., D. V. G., R. A. Q., and S. A. investigation; A. E. D., D. V. G., R. A. Q., and C. A. G. formal analysis; A. E. D., D. V. G., and C. A. G. data curation; A. E. D. and S. A. conceptualization; D. V. G., C. A. G., and S. A. visualization; R. A. Q. and S. A. supervision; R. A. Q. and S. A. resources; R. A. Q. and S. A. project administration; S. A. funding acquisition.

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Conflict of Interest

The authors declare that they do not have any conflicts of interest with the content of this article.

Abbreviations

CA, cholic acid; DCA, deoxycholic acid; DKO, double knockout; FXR, farnesoid X receptor; HCC, Hepatocellular carcinoma; HPLC, high-performance liquid chromatography; MRM, multiple-reaction monitoring; TCA, taurine-conjugated CA.

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