Supplementary data to:

Gut microbiota inhibit Asbt-dependent intestinal bile acid reabsorption via Gata4

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SUPPLEMENTAL MATERIALS AND METHODS

Gallbladder cannulation

Collection of bile was performed as described before[1, 2]. Mice were anesthetized with a mixture of Hypnorm (fentanyl/fluanisone; 1 ml/kg) and diazepam (10 mg/kg). After ligation of the common bile duct close to the duodenum, the gallbladder was cannulated using silastic tubing (Dow Corning, Midland, MI; internal diameter 0.5; external diameter 0.94 mm). After a 5 minute equilibration period, bile was collected for 20 minutes in preweighed vials. During the 20 minute bile collection period, animals were placed in a humidified incubator to ensure maintenance of body temperature. Bile flow was determined gravimetrically, assuming a density of 1 g/mL for bile. Bile was stored at –20 °C until analysis.

Bile acid analysis and kinetics

Bile acid composition was determined in bile, feces and plasma. Feces of individually-housed animals was collected over a 24-hour period, starting on day 4. Fecal samples were dried at room temperature, weighed and homogenized. Bile acid and neutral sterol composition was determined in an aliquot of feces and in 5 µL bile by gas liquid chromatography (GC) as described previously (3-5) modified as follows. Fecal BAs were solubilized from 50mg feces in methanol/0.1N sodium hydroxide (3:1) at 80 °C for 2 hrs. As an internal standard 15 nmol of 5ß-Cholanic acid 7α,12α diol was added. Next, BAs were extracted using C-18 columns and eluted with 75% methanol followed evaporation at 65C under a stream of nitrogen. Subsequently BAs were deconjugated enzymatically by adding choloyl glycine hydrolase at 37 °C for 15hrs in sodium acteate buffer pH5.6. Next, BAs were extracted again using C-18 columns as described above. After drying the samples under nitrogen the bile acids were methylated using acetylchloride/methanol at 55 °C. Subsequently a trimethylsilyl -derivative was made with N,O-bis/trimethylsilyltrifluoroacetuylamide, pyridine and trimethylsilane. BA were diluted in hexane and analyzed by GC (Agilent 6890, Amstelveen, the Netherlands)

using a CPSil 19 capilliary column (25mx0.25mmx0.2µm) (Chrompack, Middelburg, The Netherlands)

The total amount of bile acids or neutral sterols was calculated as the sum of the individually quantified bile acids or neutral sterols.

Bile acid concentration in plasma was measured in 25 µL of homogenized plasma. An internal standard containing D4-cholate, D4-chenodeoxycholate, D4-glycocholate, D4-taurocholate, D4-glycochenodeoxycholate and D4-taurochenodeoxycholate was added. Subsequently, samples were mixed and centrifuged at 15900g. The supernatant was removed, evaporated under vacuum at 40°C and reconstituted in 100 µl of 50% methanol. Bile acid profile was measured using liquid chromatography tandem MS (LC-MS/MS) as described[6]. In a separate experiment, 300 µg [^{13}C]-cholate (in 0.5% $NaHCO_3$ in PBS, pH = 7.4) was intravenously administered at day three of antibiotic treatment. Subsequently, retro-orbital blood samples (75 µl) were obtained at 12, 24, 36, 48 and 60 h after injection of [^{13}C]-cholate in control and antibiotic-treated mice. [^{13}C]-cholate was measured on a Agilent 7890A GC connected to a Agilent 5975C MSD (Agilent, Amstelveen, Holland). Gas-liquid chromatographic separation was performed on a 10 m × 0.100 mm column with a film thickness of 0.1 µm (DB-5MS; Agilent). Isotope ratios were determined in the selected ion monitoring mode on m/z 623.4 (M0) and 625.3 (M2) for CA.

Calculation of the kinetics of cholic acid decay in blood were adapted from pharmacokinetic algorithms[7]. In this experiment the most appropriate equation for the fractional contribution of [^{13}C]-cholate in blood at time point t was given by: $f(t) = f_1\ e^{-k1t} + f_2\ e^{-k2t} - f_3\ e^{-k3t}$, as described previously [8]. In which f represent the fractional contributions at time point 0 and k the fractional elimination rates. The equation describes three different kinetic phases of the administered [^{13}C]-cholate. The exponential curve with subscript 3 describes the entrance of the label, which is often ignored. The exponential curve with subscript 1 describes the initial fast elimination of the label probably due to a non-steady state period introduced by the injection of the label. The exponential curve with subscript 2 describes the elimination of the

label during steady state and therefore this curve is used to describe the kinetics of cholic acid synthesis and disposal in the body. For each individual mouse, an optimal fit for the curve was generated using SAAM II software (SAAM Institute, Inc. Seattle, WA, USA).

Biliary cholesterol and phospholipids

Biliary lipids were extracted according to Bligh and Dyer[9]. Cholesterol and phospholipids in bile were determined as described[10]. Biliary secretion rates were calculated by multiplication of the respective concentration with bile flow (µL/min) and normalized for bodyweight, expressed in nmol/min/100g.

RNA isolation and PCR procedures

Total RNA was isolated using TRI Reagent (Invitrogen) and quantified with a NanoDrop ND-100 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). cDNA synthesis was performed from 1 µg of total RNA using reagents from Invitrogen. Gene expression was measured using quantitative polymerase chain reaction (qPCR) performed with a 7900HT FAST system using FAST PCR master mix, Taqman probes and MicroAmp FAST optical 96-well reaction plates (Applied Biosystems Europe, Nieuwekerk ad IJssel, The Netherlands). Primer and probe sequences are deposited online (www.rtprimerdb.org). Relative expression levels were normalized to *36b4*.

Western blot

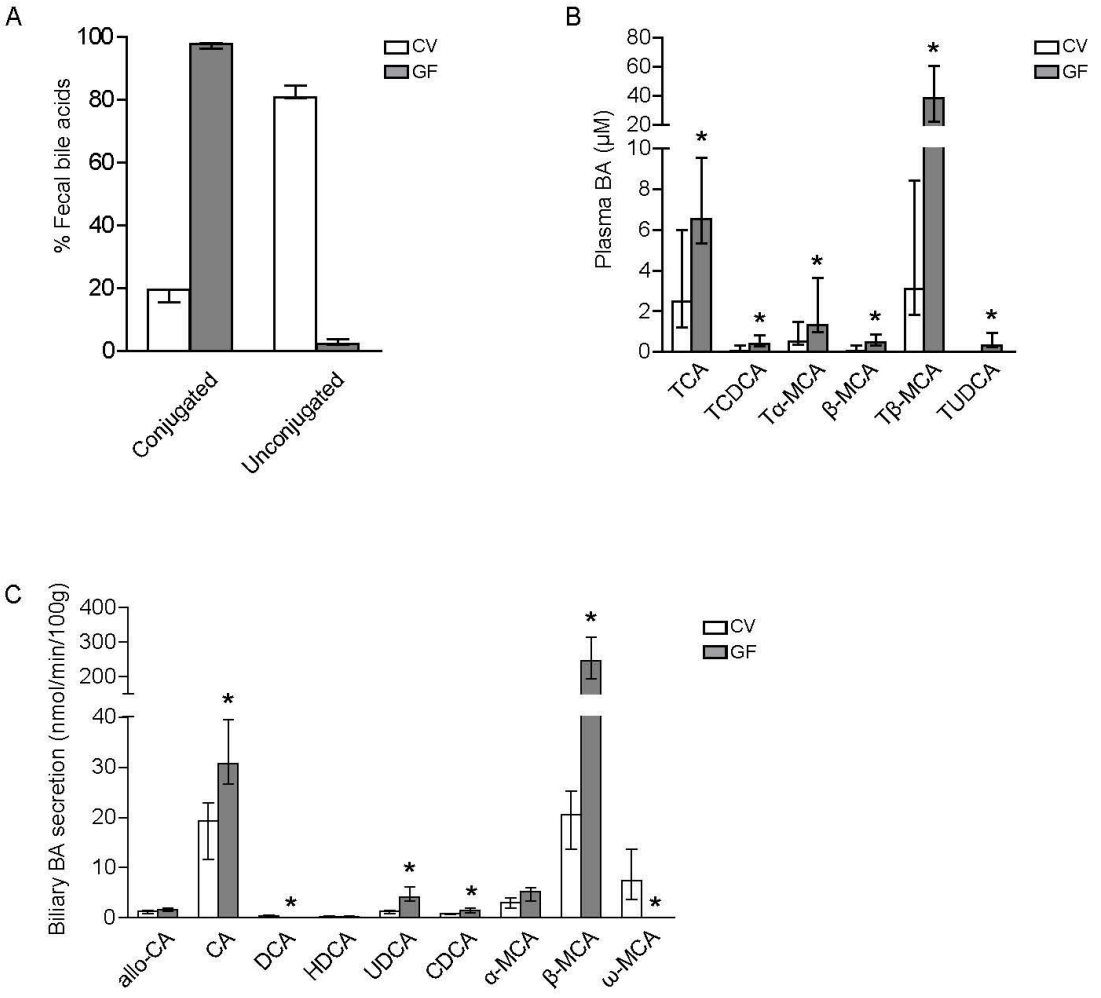
Western blotting of ASBT and FGF15 protein was carried out on homogenates of scraped ileal mucosa. Protein concentrations were determined with the bicinchoninic acid (BCA) assay (Pierce Biotechnology, Inc., Rockford, IL, USA). Equal amounts of protein were separated by SDS-PAGE electrophoresis and blotted onto nitrocellulose. ASBT was visualized using a rabbit anti-mouse ASBT antibody, kindly provided by prof. P.A. Dawson (Wake Forest University School of Medicine, Winston-Salem, NC, USA), and FGF15 using a commercial antibody (P-20; Sc-16816; Santa Cruz Biotechnology, Inc., Dallas, TX, USA),

followed by the appropriate HRP-conjugated secondary antibody. HRP was detected using chemiluminescence (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands).

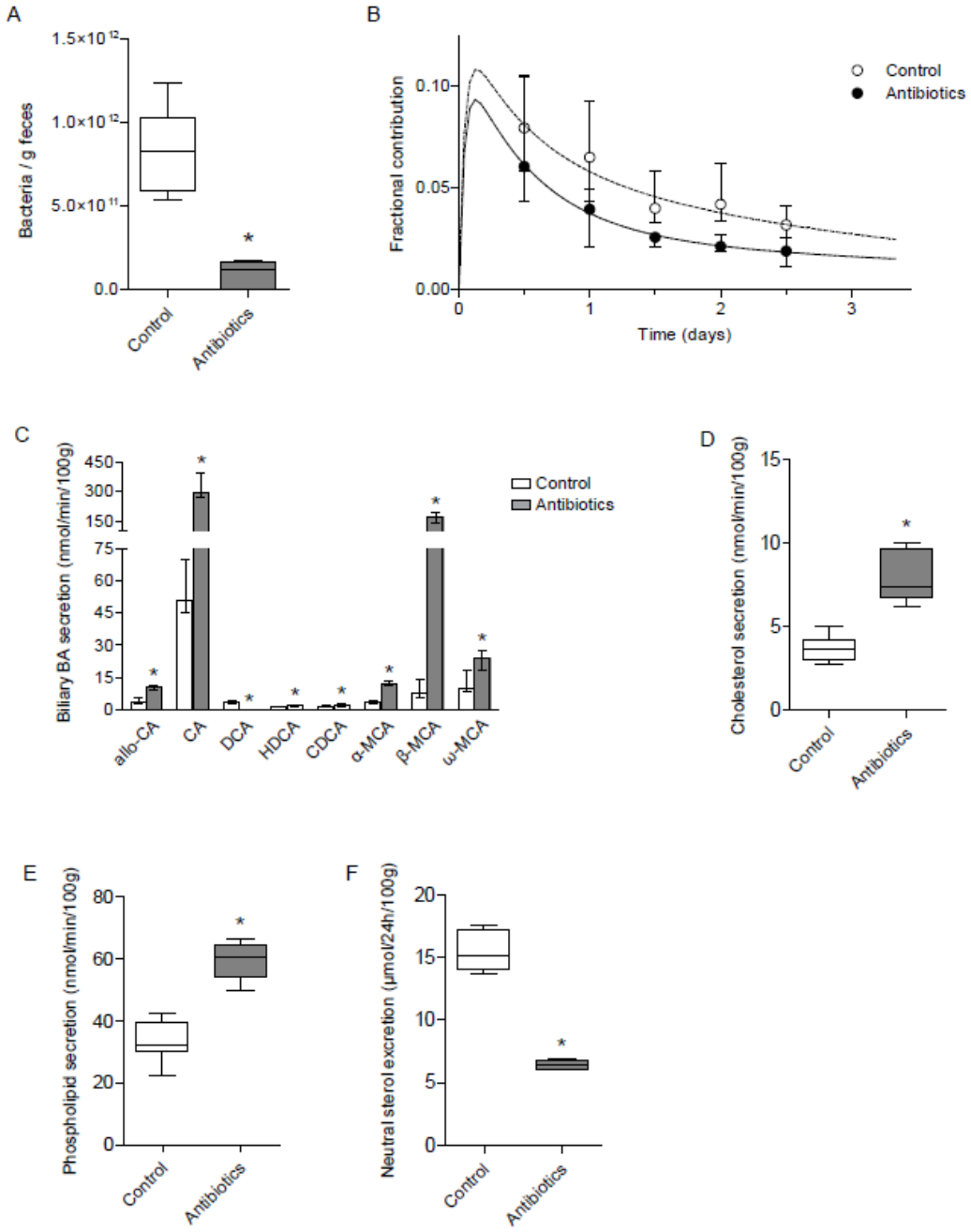
Statistics

All values are presented as Tukey's Box-and-Whiskers plot using median with 25^{th} to 75^{th} percentile intervals (P_{25}-P_{75}) or bar charts with median +/- range. Plots were created using the GraphPad Prism 5 software package. Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, USA). Differences between the groups were analyzed by the non-parametric Mann Whitney U Test with $P < 0.05$ considered statistically significant.

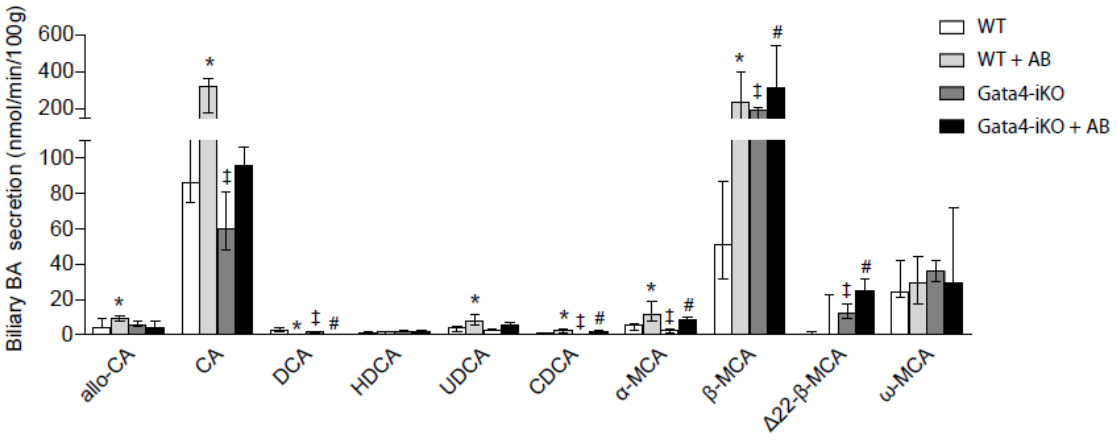
Supplemental figure 1



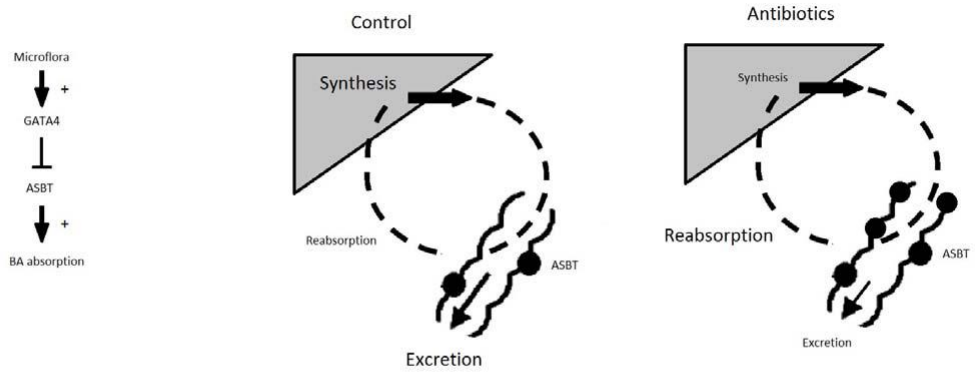
Supplemental figure 1. Bile acid homeostasis in germfree and conventional mice. (A) Percentage of conjugated and unconjugated bile acids in feces of germfree mice (GF) and conventional mice (CV). (B) Composition of bile acids in plasma (μM) and (C) bile acids secreted in bile (nmol/min/100g bodyweight) of germfree mice and conventional mice. Median ± range; n = 6-9/group; * $p < 0.05$.



Supplemental figure 2. (A) Bacterial DNA concentrations in feces (bacteria/g feces) of antibiotic-treated and control mice. (B) Fractional contribution of ^{13}C-cholic acid in plasma of antibiotic-treated and control mice (C) Composition of bile acids secreted in bile (nmol/min/100g bodyweight) of antibiotic-treated and control mice. Biliary secretion of (D) cholesterol (nmol/min/100g bodyweight) and (E) phospholipids (nmol/min/100g bodyweight). (F) Fecal neutral sterol excretion (µmol/24h/100g bodyweight) in antibiotic-treated and control mice. Median ± range; n = 8/group; * $p < 0.05$.



Supplemental figure 3. Composition of bile acids secreted in bile (nmol/min/100g bodyweight) of Gata4-iKO and wildtype mice treated with or without antibiotics. Median ± range; n = 6-7/group; * $p < 0.05$ WT + AB compared to WT; ‡ $p < 0.05$ Gata4-iKO compared to WT; # $p < 0.05$ Gata4-iKO + AB compared to Gata4-iKO.



Supplemental figure 4. Schematic representation of the changes in bile acid homeostasis upon antibiotic treatment. Antibiotic treatment increases Asbt expression and shifts its location to the more proximal parts of the intestine. Bile acid reabsorption is enhanced, whereas fecal bile acid excretion and bile acid synthesis are decreased.

REFERENCES

[1] Kuipers F, van Ree JM, Hofker MH, Wolters H, In't Veld G, Havinga R et al. Altered lipid metabolism in apolipoprotein E-deficient mice does not affect cholesterol balance across the liver. Hepatology 1996; 24:241-247.

[2] Kok T, Hulzebos CV, Wolters H, Havinga R, Agellon LB, Stellaard F et al. Enterohepatic circulation of bile salts in farnesoid X receptor-deficient mice: efficient intestinal bile salt absorption in the absence of ileal bile acid-binding protein. J Biol Chem 2003; 278:41930-41937.

[3] Hulzebos C V, Renfurm L, Bandsma RH, Verkade HJ, Boer T, Boverhof R et al. Measurement of parameters of cholic acid kinetics in plasma using a microscale stable isotope dilution technique: application to rodents and humans. J Lipid Res 2001; 42:1923-1929.

[4] Hulzebos C V, Wolters H, Plosch T, Kramer W, Stengelin S, Stellaard F et al. Cyclosporin a and enterohepatic circulation of bile salts in rats: decreased cholate synthesis but increased intestinal reabsorption. J Pharmacol Exp Ther 2003; 304:356-363.

[5] van der Veen J N, van Dijk TH, Vrins CL, van Meer H, Havinga R, Bijsterveld K et al. Activation of the liver X receptor stimulates trans-intestinal excretion of plasma cholesterol. J Biol Chem 2009; 284:19211-19219.

[6] Alnouti Y, Csanaky IL, Klaassen CD. Quantitative-profiling of bile acids and their conjugates in mouse liver, bile, plasma, and urine using LC-MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci 2008; 873:209-217.

[7] Rowland M, Tozer TN. 1995. *Clinical Pharmokinetics, Concepts and Applications*, 3rd edition, Baltimore, Williams & Wilkins.

[8] Out C, Dikkers A, Laskewitz A, Boverhof R, Ley CV, Kema IP et al. Prednisolone increases enterohepatic cycling of bile acids by induction of Asbt and promotes reverse cholesterol transport. J Hepatol 2014.

[9] Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959; 37:911-917.

[10] Kuipers F, Havinga R, Bosschieter H, Toorop GP, Hindriks FR, Vonk RJ. Enterohepatic circulation in the rat. Gastroenterology 1985; 88:403-411.