SUPPLEMENTAL INFORMATION

Intestinal farnesoid X receptor signaling promotes non-alcoholic fatty liver disease

Supplemental methods

Preparation and cultures of primary mouse hepatocytes

Primary hepatocytes from 6-week-old C57BL/6N mice were obtained by collagenase 1 (Invitrogen, Carlsbad, CA) perfusion. The cells were purified by 45% Percoll (Sigma, St. Louis, MO) density centrifugation and cultured in DMEM (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum and 1% Insulin-Transferrin-Selenium-Ethanolamine (ITS -X) (Invitrogen, Carlsbad, CA). The viability of hepatocytes was determined using trypan blue dye exclusion, and those with higher than 85% viability were used. The medium was changed to DMEM with 1% fetal bovine serum after culturing for 4 hours. After starvation for 4 hours, the cells were exposed to C2:0 ceramide. At the prescribed time points, cells were harvested and subjected to qPCR analysis and measurement of triglyceride content.

RNA analysis

The intestine mucosa was gently scraped and liver flash frozen in liquid nitrogen and both stored at -80°C until RNA was prepared. RNA was extracted from frozen intestine and liver using TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized from 1 μg total RNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). qPCR primers were designed with qPrimerDepot, and the sequences are shown in the Supplemental table 1. Messenger RNA levels were normalized to 18S ribosomal RNA and expressed as fold change relative to the control group.

Western blot analysis

Liver whole-cell or nuclear extracts were prepared. Membranes were incubated with antibodies against FXR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), SREBP1 (BD Biosciences, San Jose, CA), and CIDEA (Abcam, Cambridge, MA) signals obtained were normalized to β-ACTIN (Abcam) for whole cell extract and LAMIN A/C (Santa Cruz) for nuclear extracts. For quantitation of the wetern blot bands, the blots were scanned and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).

16S rRNA gene sequencing of the intestinal microbiome

The bacteria in feces and cecum content were extracted using PowerSoil DNA Isolation Kit (Mo Bio laboratory, Inc., Carlsbad, CA). The PCR products (approximately 1000 bps) were purified using the AgencourtAMPure technology (Beckman Coulter, Brea, CA) as described in 454 Technical Bulletin #2011-002, Short Fragment Removal Procedure. After purification, the products were quantified by both Qubit (Lifetech, Carlsbad, CA) and qPCR, using the KAPA Biosystems Library Quantification Kit (KapaBiosystems, Woburn, MA), pooled based on molar amounts, run on a 1% agarose gel and extracted. After purification with a QIAquick PCR Purification kit (Qiagen,Valencia, CA), the quality and quantity were assessed using a DNA

7500LabChip on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and Qubit quantification. The sequencing was performed using a quarter PTP plate on a 454 Life Sciences Genome Sequencer FLX+ (Roche Diagnostics, Indianapolis, IN). 16S rRNA gene sequencing analysis was performed as previously described (1). Weighted UniFrac analysis to assess changes in the bacterial abundance was performed on the Galaxy web-based platform (2-4).

Metagenomic data analysis

After quality filtering and deduplication, each sample contains on average 11 thousand reads. The mothur software package was used to preprocess the sequencing data and the RDP multi-classifier to assign each sequence to a taxonomic rank. Preprocessing consisted of filtering reads for an average quality of 20, removing duplicated sequences and splitting into samples by barcodes while allowing for one mismatch in the barcode. To account for differences in total reads per sample, classifications were converted to percent of total reads. This approach then permitted accurate comparisons within and between groups. If the "study" effect is significant, the studies are kept separate in the problem as a block effect. If not, it is removed and combined with data from two studies. Finally, Šidák correction was employed for p-value correction.

Metabolomics analysis

For serum lipidomics analysis, 25 µl serum were extracted by 4-fold cold chloroform: methanol (2:1) solution containing 2 µM LPC (17:0), PC (17:0), SM (17:0) and CER (17:0) (Avanti Polar Lipids, Alabaster, AL) as internal standards. The samples were vortexed for 30 sec and then allowed to stand for 5 min at room temperature. The mixture was centrifuged at 13,000 rpm for 5 min and then the lower organic phase was collected and evaporated at room temperature under vacuum and the residue was dissolved in chloroform: methanol (1:1), followed by diluting with isopropanol: acetonitrile: H_2O (2:1:1) containing 2 µM PC (17:0) prior to UPLC-MS analysis. For tissue lipidomics analysis, about 50 mg accurately weighted tissues were homogenized with 700 µL methanol: H_2O (4:3) solution and then extracted using 800 µL chloroform containing 2 µM LPC (17:0), SM (17:0) and CER (17:0) as internal standards. The homogenate was incubated at 37°C for 20 min followed by centrifuged for 20 min at 13,000 rpm. The lower organic phase was transferred to a new tube and dried under vacuum. The residue was suspended with 100 µL chloroform: methanol (1:1) solution and then diluted with isopropanol: acetonitrile: H_2O (2:1:1) solution containing 2 µM PC (17:0) before injection. For lipidomics discovery, the samples were analyzed by UPLC-ESI-QTOFMS using a Water Acquity CSH 1.7um C18 column (2.1x100 mm) under the following conditions: UPLC: A-acetonitrile/water (60/40), B-isopropanol/acetonitrile (90/10), both A and B contain 10mM Ammonium acetate and 0.1% formic acid. Gradient: initial 60% A to 57% A at 2 min, to 50% A at 2.1min*, to 46%A at 12 min, to 30% A at 12.1 min*, to 1% A at 18 min before returning to initial conditions at 18.5 min with equilibration for 2 additional minutes (*indicates ballistic gradient). Flow rate was 0.4 ml/min. Column temperature was maintained at 55°C. MS, under the same conditions as described above, except the run time was 18 min.

NMR-based metabolomics experiments

Methanol, K2HPO4, NaH2PO4 (all in analytical grade), Sodium 3-trimethylsilyl [2,2,3,3-d4] propionate (TSP-d4) and D2O (99.9% in D) were purchased from Sigma-Aldrich (St. Louis, MO). Phosphate buffer (0.1 M K2HPO4/NaH2PO4 and PH 7.4) was prepared with K2HPO4 and

NaH2PO4 for their good solubility and low-temperature stability. Liver (~50 mg) were extracted three times with 0.6 mL 600 µL of precooled methanol-water mixture (2/1, v/v) using the PreCellys Tissue Homogenizer (Bertin Technologies, Rockville, MD). After centrifugation at 11180 x g for 10 min at 4 °C, the combined supernatants were dried. Each of the aqueous extracts was separately reconstituted into 600 µL phosphate buffer containing 50% D_2O and 0.005% TSP-d4 (chemical shift reference). Following centrifugation, 550 µL of each extract was transferred into 5 mm NMR tube. Cecal content samples were directly extracted using an optimized procedure described previously (5). Briefly, samples (~50 mg) were mixed with 600 µL precooled phosphate buffer, vortexed for 30s and subjected to three consecutive freeze-thaws followed by homogenization using the Precellys Tissue Homogenizer. After centrifugation (11,180 x g, 4 °C) for 10 min, the supernatants (550 µL) were transferred into 5 mm NMR tubes for NMR analysis.

^{1}H NMR spectroscopy

^{1}H NMR spectra of aqueous liver and fecal extracts were recorded at 298 K on a Bruker Avance III 850 MHz spectrometer (operating at 850.23 MHz for 1H) equipped with a Bruker inverse cryogenic probe (Bruker Biospin, Germany). Typical one-dimensional NMR spectrum was acquired for each of all samples employing the first increment of NOESY pulse sequence (NOESYPR1D). To suppress the water signal, a weak continuous wave irradiation was applied to the water peak during recycle delay (2 s) and mixing time (100 ms). The 90° pulse length was adjusted to approximately 10 µs for each sample and 64 transients were collected into 32 k data points for each spectrum with spectral width of 20 ppm. To facilitate NMR signal assignments, a range of 2D NMR spectra were acquired and processed as described previously (Dai et al., 2010; Ding et al., 2009) for selected samples including 1H-1H correlation spectroscopy (COSY), ^{1}H-^{1}H total correlation spectroscopy (TOCSY), ^{1}H-^{13}C heteronuclear single quantum correlation (HSQC), and ^{1}H-^{13}C heteronuclear multiple bond correlation spectra (HMBC).

Spectral data processing and multivariate data analysis

All free induction decays (FID) were multiplied by an exponential function with a 1 Hz line-broadening factor prior to Fourier transformation. ^{1}H NMR spectra were corrected manually for phase and baseline distortions and spectral region δ 0.5-9.5 was integrated into regions with equal width of 0.004 ppm (2.4 Hz) using AMIX software package (V3.8, Bruker-Biospin, Germany). Region δ 4.45-5.20 was discarded by imperfect water saturation. Regions δ 1.15-1.23 and δ 3.62-3.69 were also removed for ethanol contaminations in the cecal contents during mice dissection process. Each bucketed region was then normalized to the total sum of the spectral integrals to compensate for the overall concentration differences prior to statistical data analysis.
Multivariate data analysis was carried out with SIMCAP+ software (version 13.0, Umetrics, Sweden). Principal Component Analysis (PCA) was initially carried out on the NMR data to generate an overview and to assess data quality. Orthogonal Projection to Latent Structures with Discriminant Analysis (OPLS-DA) was subsequently conducted on the NMR data. The OPLS-DA models were validated using a 7-fold cross validation method and the quality of the model was described by the parameters R2X and Q2 values (Figure 3 and 4, Supplemental Table2). To facilitate interpretation of the results, back-transformation (6) of the loadings generated from the OPLS-DA was performed prior to generating the loadings plots, which were color-coded with the Pearson linear correlation coefficients of variables (or metabolites) using an in-house developed script for MATLAB (The Mathworks Inc.; Natwick, MA). The color-coded

correlation coefficient indicates the significance of the metabolite contribution to the class separation, with a "hot" color (e.g., red) being more significant than a "cold" color (e.g., blue). In this study, a cutoff value of $|r| > 0.811$ ($r > 0.755$ and $r < -0.755$) was chosen for correlation coefficient as significant based on the discrimination significance ($p \leqslant 0.05$).

Bile salt hydrolase activity

Fecal proteins were prepared from the feces sample (0.5 g) in pH 7.4 phosphate buffered saline (PBS, 5.0 mL) using sonication. Bile salt hydrolase (BSH) activity was measured based on the generation of CDCA from TCDCA in the feces. Briefly, incubation was carried out in 3 mM sodium acetate buffer, pH 5.2, containing 0.1 mg/ml fecal protein and 50 µM TCDCA-d5 in a final volume of 200 µl. After a 20 min incubation at 37 °C, the reaction was stopped by plunging the samples into dry ice. 100 µl, of acetonitrile, was directly added to the 100 µl reaction mix. After centrifuging at 14,000 × *g* for 20 min, 5 µl of the supernatant was transferred to an auto sampler vial subjected to analysis by a UPLC system coupled with a XEVO triple quadrupole tandem mass spectrometer (Waters Corp., Milford, MA).

Mitochondrial isolation and functional studies

For intestinal mitochondria, the mucosa of ileum was gently scraped, was washed 2X with PBS, minced in ice-cold mitochondrial homogenization buffer (225mM mannitol, 75mM sucrose, 5mM MOPS, 0.5mM EGTA and 2mM taurine (pH 7.25)) containing 0.2% BSA, and homogenized in a loose fitting homogenizer. Homogenates were centrifuged at 500xg for 10 min at 4°C. The supernatant was then centrifuged at 10,000xg for 10 min at 4°C. The final mitochondrial pellet was resuspended in mitochondrial isolation buffer containing 0.2% BSA at a concentration of 0.5 mg/ml before functional assessment. Oxygen consumption of isolated mitochondria was measured in a chamber connected to a Clark-type O_2 electrode (Instech) and O_2 monitor (Model 5300, YSI Inc) at 25°C. Mitochondria were incubated in respiration buffer (120mM KCl, 5mM MOPS, 0.1mM EGTA, 5mM KH2PO4 and 0.2% BSA) with substrates for either complex I (5 mM glutamate and 5 mM malate), or complex II (5 mM succinate and 1µM rotenone). State 3 (maximal) respiration activity was measured after addition of 1 mM ADP. ADP independent respiration activity (State 4) was monitored after addition of 2µM oligomycin. The respiratory control ratio was determined by the state 3/state 4 respiration rates.

Synthesis of β muricholic acid (βMCA, 9) and taurine β muricholic acid (TβMCA, 10)

β-Muricholic acid, **9** was prepared as illustrated in the Scheme 1 by following a published procedure (7). In general, esterification of chenodeoxycholic acid **1** with methanol under acidic condition provided ester **2** in quantitative yield. Protections of the hydroxyl group in 3-position with ethyl chloroformate gave carbonate **3** in quantitative yield. Oxidation of the 6-hydroxyl group with potassium chromate gave ketone **4**. Bromination with 47% HBr solution followed by reduction with $NaBH_4$ gave bromohydrin **6**, in moderate yield. Reductive dehydrobromination with zinc metal gave olefin **7** on oxidation with osmium tetroxide followed by hydrolysis gave β-muricholic acid (βMCA, **9**) in overall yield of 20%. βMCA (**9**) was reacted with taurine, triethylamine, and EDDQ in DMF. Upon work up, hygroscopic triethylammonium salt of TβMCA **10** was obtained in 44% yield. ^{1}H NMR ($CDCl_3$) 0.66 (s, 3H, 18-CH_3), 0.91 (s, 3H, 19-CH_3), 0.93 (d, 3H, J=6.5Hz, 21-CH_3), 2.98 (t, 2H, CH_2SO_3H, *J*= 5.5Hz), 3.4-3.52 (m, 1H, C_3-H), 3.68-3.75 (m, 2H, NH-CH_2), 3.83-3.87 (m, 1H, C_7-H).

Supplemental references

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5. Wu, J., An, Y., Yao, J., Wang, Y., and Tang, H. 2010. An optimised sample preparation method for NMR-based faecal metabonomic analysis. *Analyst* 135:1023-1030.
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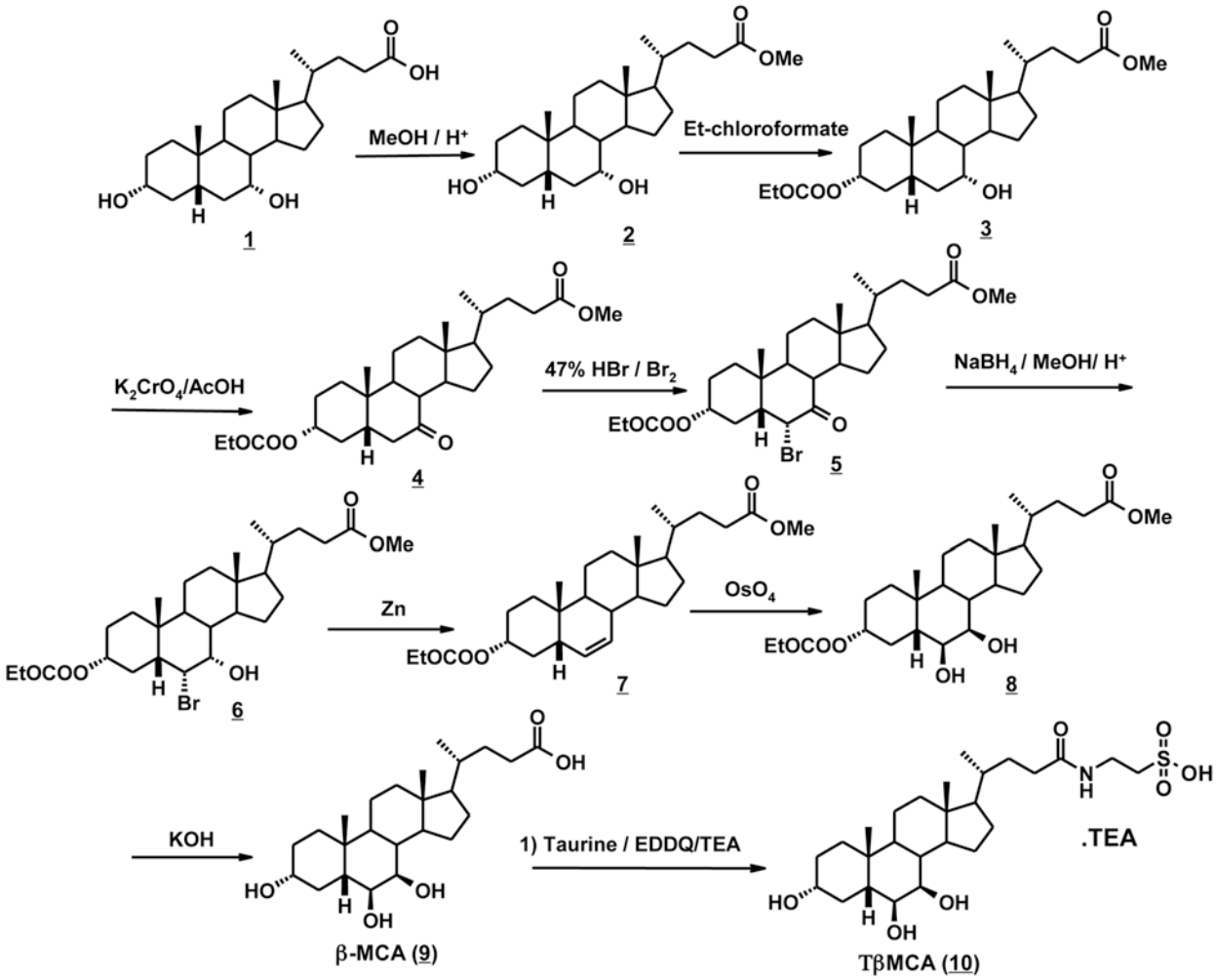
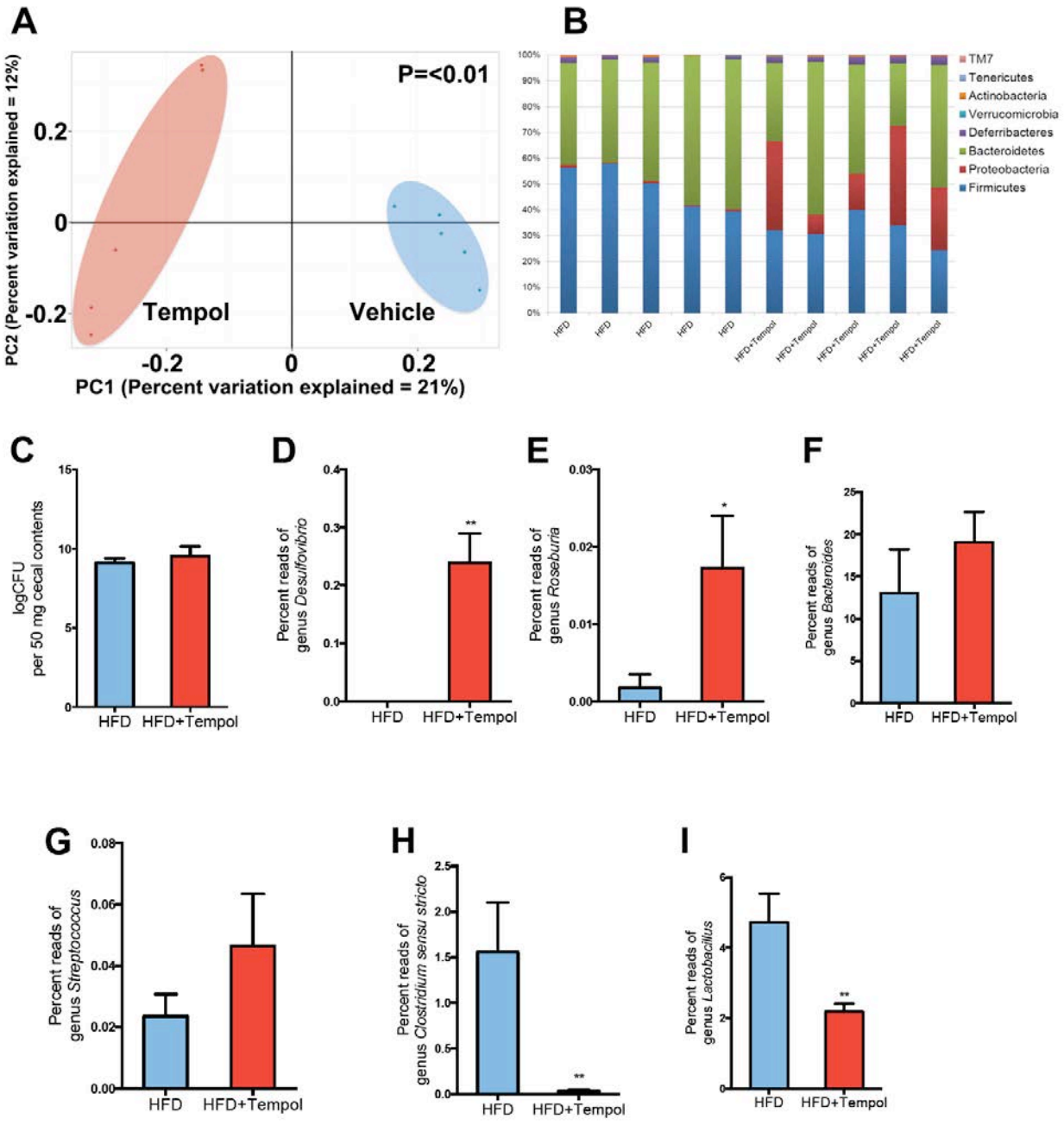
7. Iida, T., Momose, T., Tamura, T., Matsumoto, T., Chang, F., Goto, J., and Nambara, T. 1989. Potential bile acid metabolites. 14. Hyocholic and muricholic acid stereoisomers. *J Lipid Res* 30:1267-1279.



Scheme 1: Synthesis of β-MCA (9) and TβMCA (10)

Supplemental figures



Supplemental Figure 1. Tempol treatment modifies the gut microbiota in the HFD-induced NAFLD model.

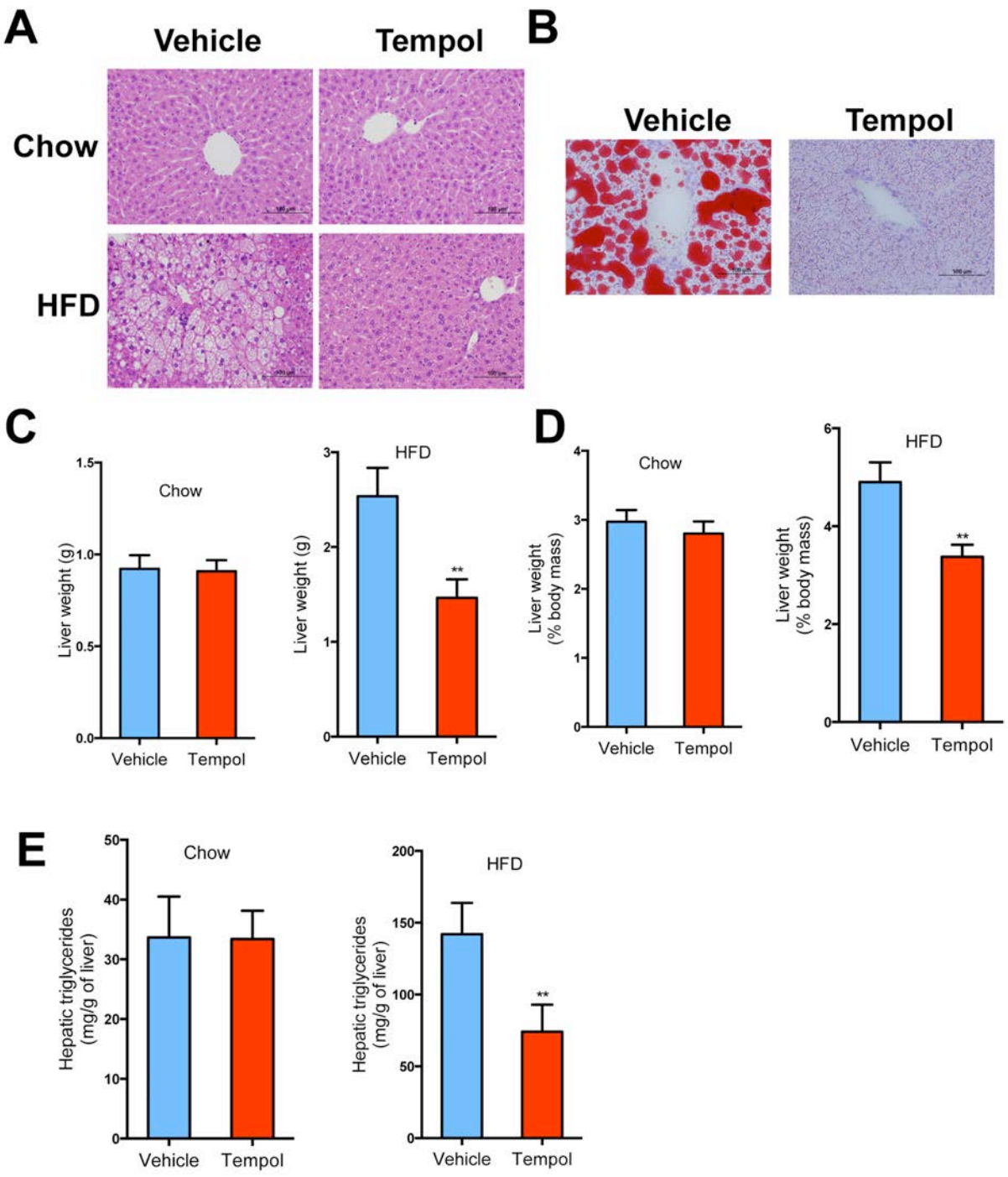
(A) Principal coordinates analysis of sample data with p-value generated from weighted Unifrac distances. Blue circles represent vehicle cecal communities and red squares represent tempol cecal communities after 16 weeks of HFD feeding. n=5 mice per group.

(B) 16S rRNA gene sequencing analysis at the phylum levels of cecum content after 16 weeks of tempol treatment in HFD-fed mice. The vertical values are from one mouse. n=5 mice per group.

(C) qPCR analysis at total bacterial counts of cecum content after tempol treatment on a HFD for 16 weeks. n=5 mice per group.

(D)-(I) 16S rRNA gene sequencing analysis of genus levels of cecum content after 16 weeks of tempol treatment in HFD-fed mice. n=5 mice per group.

(C-I) Data are presented as mean±SD. Two-tailed Student's t-test. $^{**}P<0.01$ and $^{*}P<0.05$ compared to vehicle-treated mice.



Supplemental Figure 2. Tempol treatment reduces the development of NAFLD.

(A) Representative H&E staining of liver sections of vehicle- and tempol-treated mice on a chow diet (upper panel) and on a HFD (lower panel) for 16 weeks. n=5 mice per group.

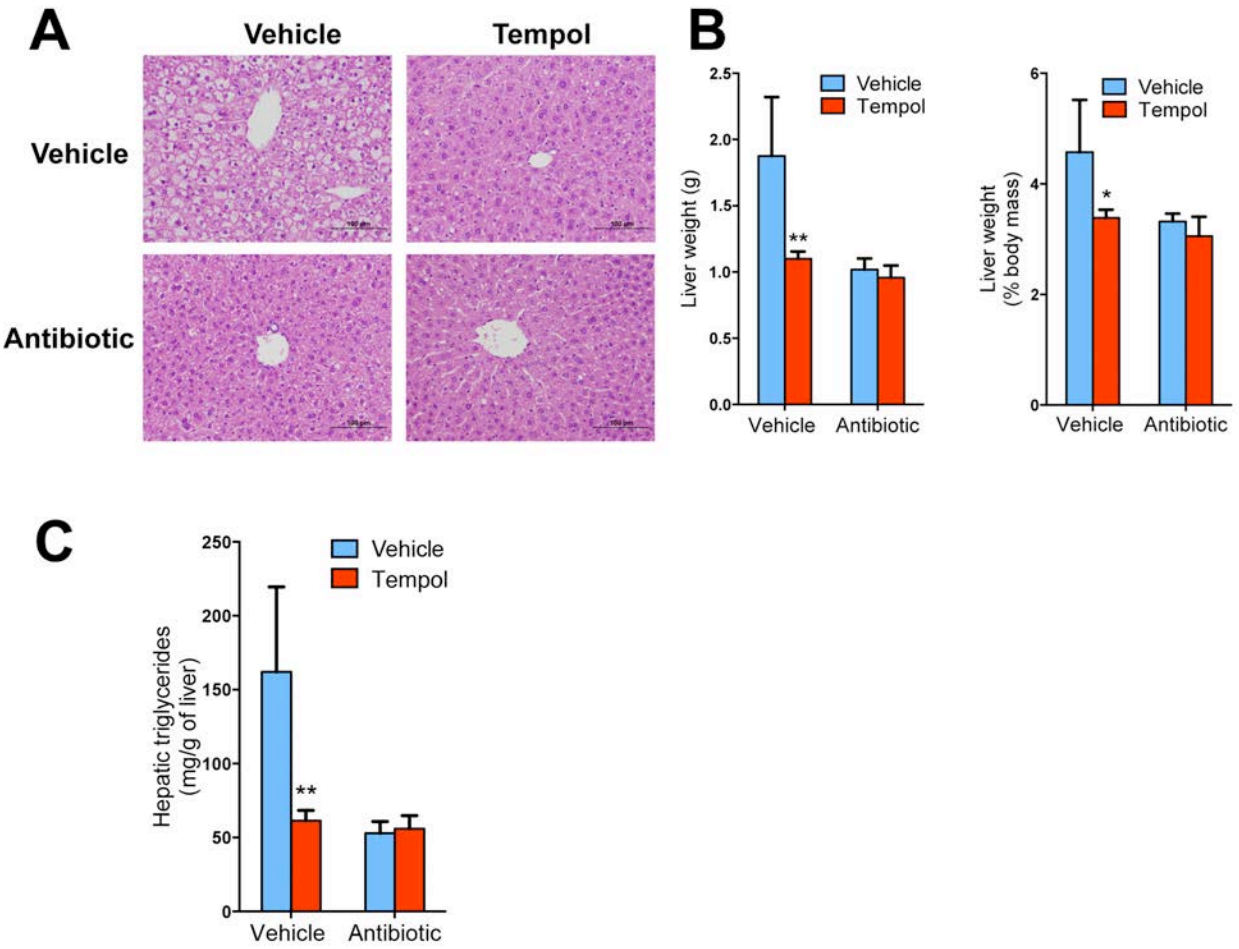
(B) Representative Oil Red O staining of liver sections of vehicle- and tempol-treated mice on a HFD for 16 weeks. Lipids stain positive (red color) with Oil Red O. n=5 mice per group.

(C) Liver weights of vehicle- and tempol-treated mice on a chow diet (left panel) and on a HFD (right panel) for 16 weeks. n=5 mice per group.

(D) Liver weight to body weight ratios of vehicle- and tempol-treated mice on a chow diet (left panel) and on a HFD (right panel) for 16 weeks. n=5 mice per group.

(E) Liver triglyceride contents of vehicle- and tempol-treated mice on a chow diet (left panel) and on a HFD (right panel) for 16 weeks. n=5 mice per group.

(B-E) Data are presented as mean ± SD. Two-tailed Student's t-test. $^{**}P<0.01$ compared to vehicle-treated mice.



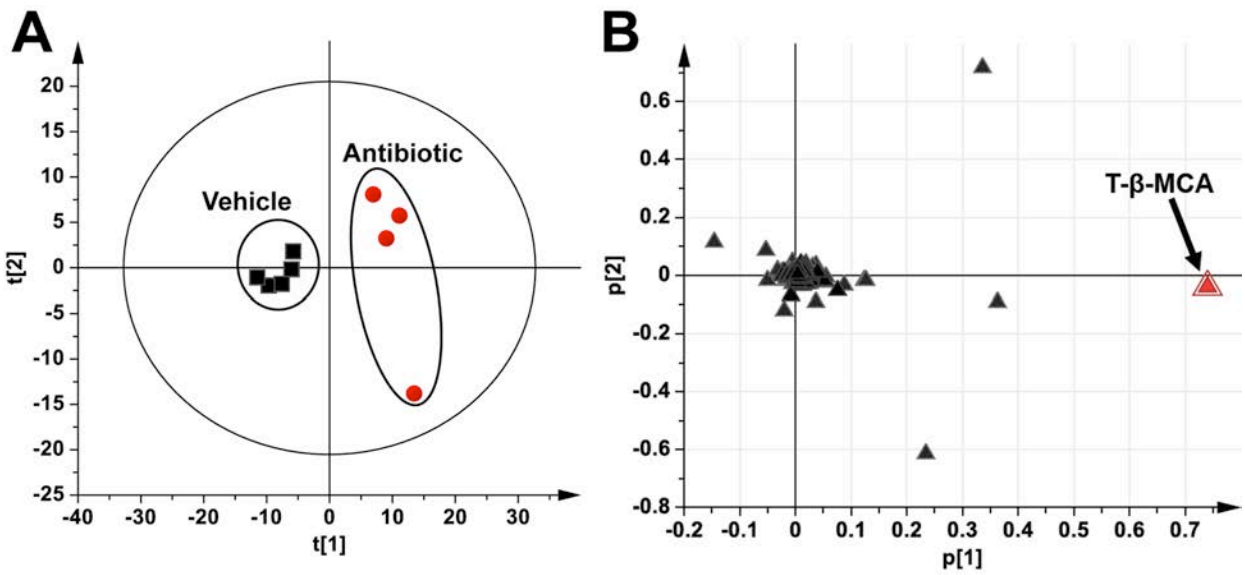
Supplemental Figure 3. Inhibition of gut microbiota is crucial for tempol treatment improved NAFLD.

(A) Representative H&E staining of liver sections from tempol treatment in vehicle and antibiotic-depleted mice fed a HFD for 16 weeks. n=5 mice per group.

(B) Liver weights and liver weight to body weight ratios from tempol treatment in vehicle and antibiotic-depleted mice fed a HFD for 16 weeks. n=5 mice per group.

(C) Liver triglyceride contents from tempol treatment in vehicle and antibiotic-depleted mice fed a HFD for 16 weeks. n=5 mice per group.

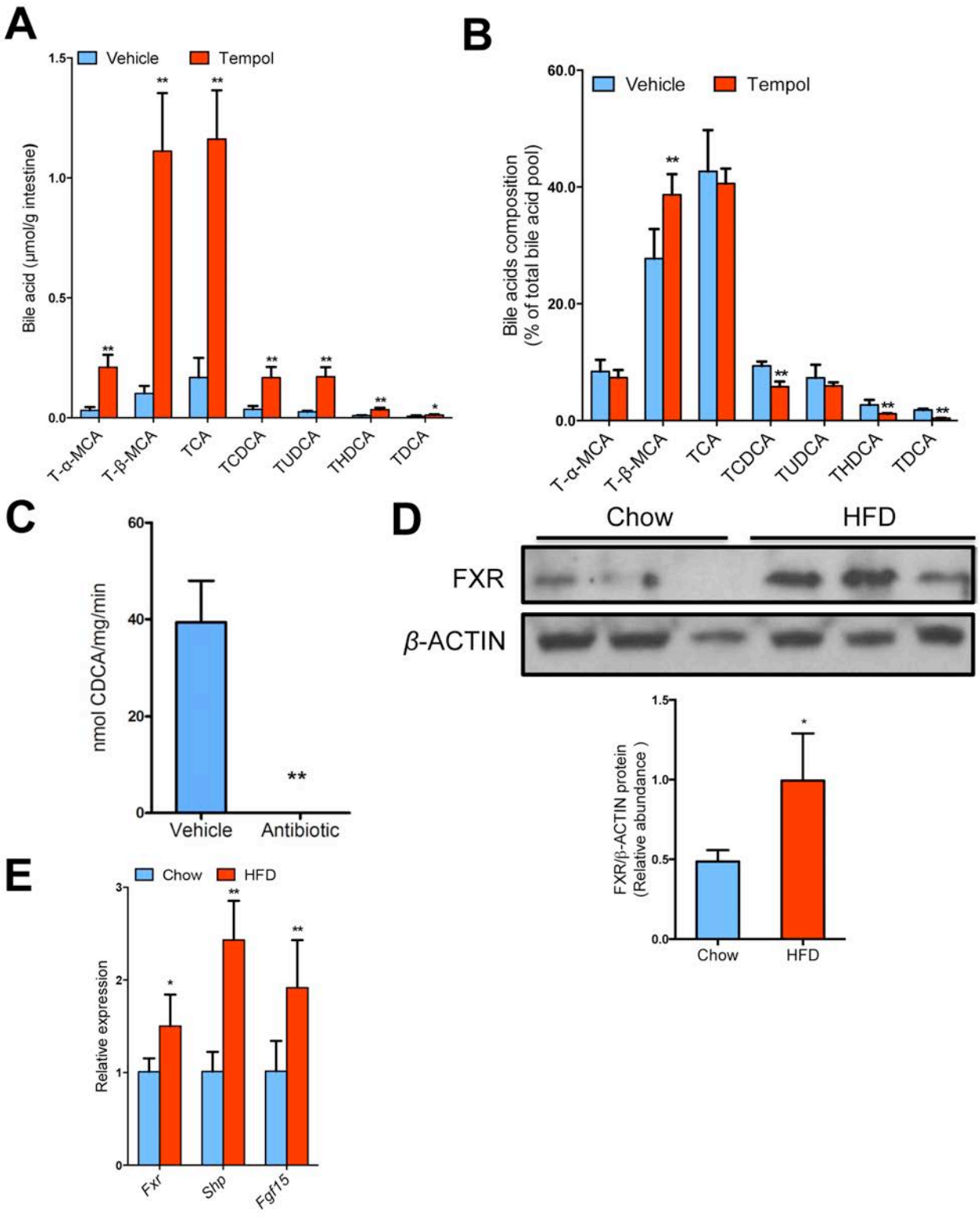
(D) (B and C) Data are presented as mean ± SD. One-way ANOVA with Tukey's correction. **P*<0.05 and ***P*<0.01 compared to vehicle-treated mice.



Supplemental Figure 4. Antibiotic treatment altered bile acid composition.

(A) Scores scatter plot of a PCA model of ileum ions in vehicle- and antibiotic-treated mice after 7 weeks of HFD feeding. n=4-5 mice per group.

(B) Loadings scatter plot of a PCA model of ileum ions in vehicle- and antibiotic-treated mice after 7 weeks of HFD. The p[1] and p[2] values represent the contributing weights of each ion to principal components 1 and 2. The identities of two ions with the highest loading values are annotated in the plot. All the data were obtained in negative mode (ESI^-). n=4-5 mice per group.


A
Vehicle
Tempol
Bile acid (μmol/g intestine)
1.5
1.0
0.5
0.0
T-α-MCA
T-β-MCA
TCA
TCDCA
TUDCA
THDCA
TDCA
B
Vehicle
Tempol
Bile acids composition
(% of total bile acid pool)
60.0
40.0
20.0
0.0
T-α-MCA
T-β-MCA
TCA
TCDCA
TUDCA
THDCA
TDCA
C
nmol CDCA/mg/min
60
40
20
0
Vehicle
Antibiotic
D
Chow
HFD
FXR
β-ACTIN
FXR/β-ACTIN protein
(Relative abundance)
1.5
1.0
0.5
0.0
Chow
HFD
E
Chow
HFD
Relative expression
3
2
1
0
Fxr
Shp
Fgf15

Supplemental Figure 5. Gut microbiota regulated bile acid composition.

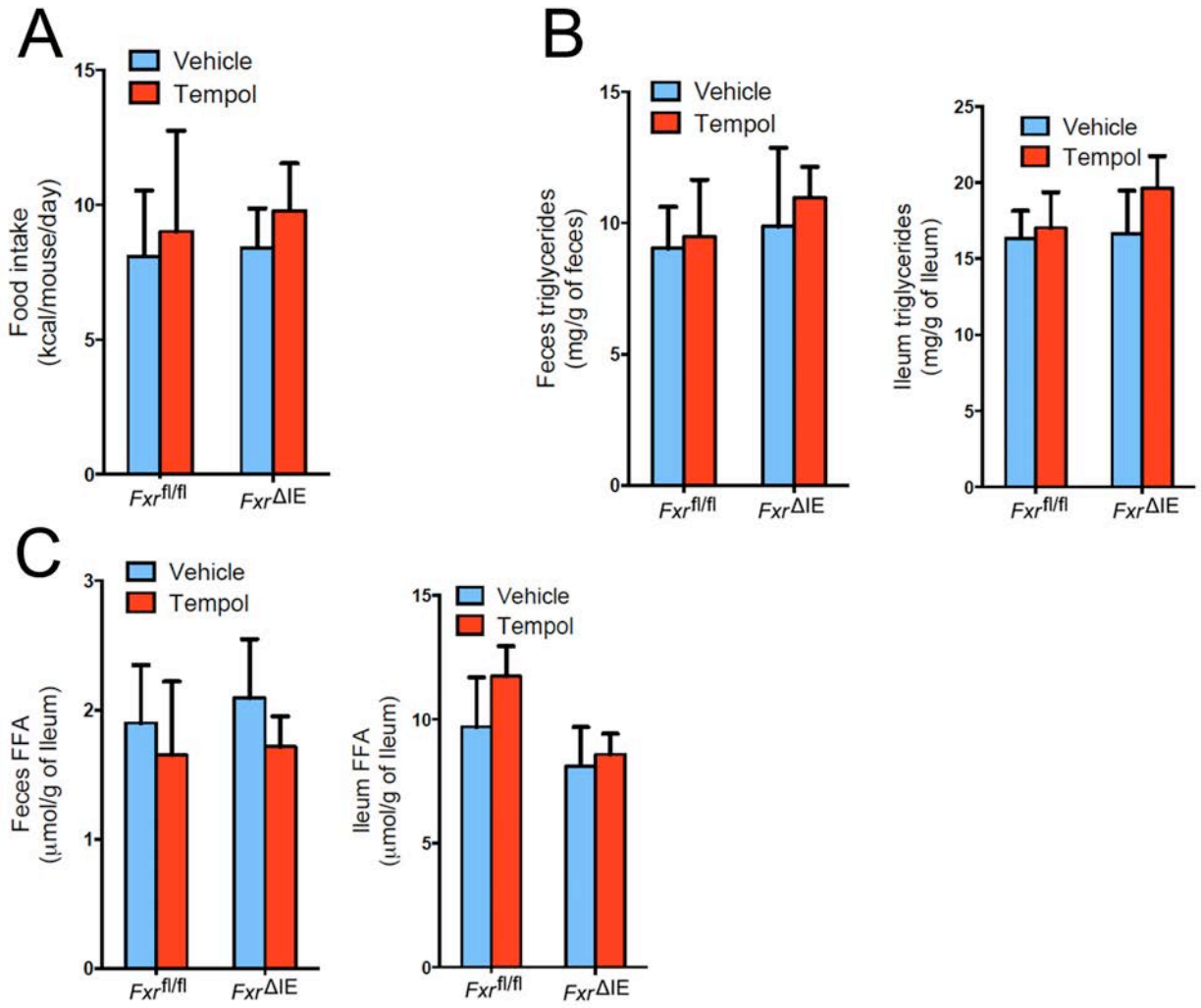
(A) The absolute quantities of individual taurine-conjugated bile acids in the ileum after 16 weeks tempol treatment in HFD-fed mice. Data are mean ± SD. Two-tailed Student's t-test. *P<0.05, **P<0.01 compared to vehicle treated mice. n=5 mice per group.

(B) The proportion of individual taurine-conjugated bile acids, expressed as percent of the total bile acid pool, in the ileum from vehicle and tempol group fed a HFD for 16 weeks. Data are mean ± SD. Two-tailed Student's t-test. **P<0.01 compared to vehicle treated mice. n=5 mice per group.

(C) Fecal BSH enzyme activity after 7 weeks antibiotic treatment in HFD-fed mice. n=4-5 mice per group. Data are presented as mean ± SD. Two-tailed Student's t-test. **P<0.01 compared to vehicle treated mice.

(D) Western blot analysis of ileum FXR expression after 12 weeks of HFD feeding (upper panel) and quantitation of FXR expression (lower panel). Data are presented as mean ± SD. Two-tailed Student's t-test. *P<0.05 compared to chow diet treatment. n=3 mice per group.

(E) mRNA levels of FXR target genes in the ileum after 12 weeks of HFD feeding. The expression was normalized to 18S RNA. n=5 mice per group. Data are presented as mean ± SD. Two-tailed Student's t-test. *P<0.05, ***P*<0.01 compared to chow diet treatment.



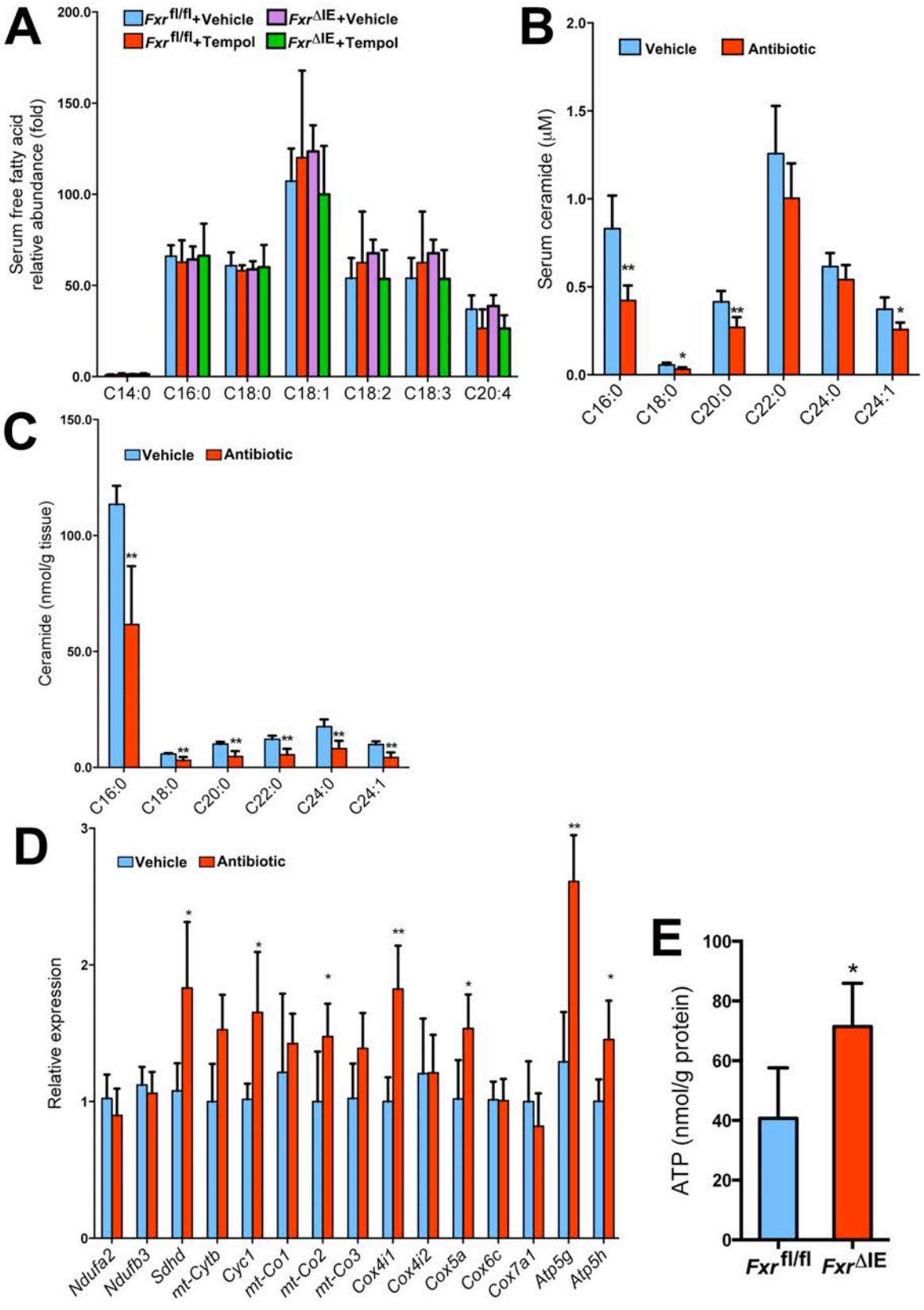
Supplemental Figure 6. Inhibition of intestinal FXR had no effects on fat absorption.

(A) Food intake of vehicle- and tempol-treated *Fxr*$^{fl/fl}$ and *Fxr*$^{\Delta IE}$ mice fed a HFD for 16 weeks. n=5 mice per group.

(B) Feces and ileum triglyceride levels vehicle- and tempol-treated *Fxr*$^{fl/fl}$ and *Fxr*$^{\Delta IE}$ mice fed a HFD for 16 weeks. n=5 mice per group.

(C) Feces and ileum FFA levels vehicle- and tempol-treated *Fxr*$^{fl/fl}$ and *Fxr*$^{\Delta IE}$ mice fed a HFD for 16 weeks. n=5 mice per group.

(D) All data are presented as mean ± SD. No significant differences were detected among the groups.


A
Fxr fl/fl +Vehicle
Fxr ΔIE +Vehicle
Fxr fl/fl +Tempol
Fxr ΔIE +Tempol
Serum free fatty acid relative abundance (fold)
200.0
150.0
100.0
50.0
0.0
C14:0
C16:0
C18:0
C18:1
C18:2
C18:3
C20:4
B
Vehicle
Antibiotic
Serum ceramide (μM)
2.0
1.5
1.0
0.5
0.0
C16:0
C18:0
C20:0
C22:0
C24:0
C24:1
C
Vehicle
Antibiotic
Ceramide (nmol/g tissue)
150.0
100.0
50.0
0.0
C16:0
C18:0
C20:0
C22:0
C24:0
C24:1
D
Vehicle
Antibiotic
Relative expression
3
2
1
0
Ndufa2
Ndufb3
Sdhd
mt-Cytb
Cyc1
mt-Co1
mt-Co2
mt-Co3
Cox4i1
Cox4i2
Cox5a
Cox6c
Cox7a1
Atp5g
Atp5h
E
ATP (nmol/g protein)
100
80
60
40
20
0
Fxr fl/fl
Fxr ΔIE

Supplemental Figure 7. Inhibition of intestinal FXR improved intestine mitochondrial function.

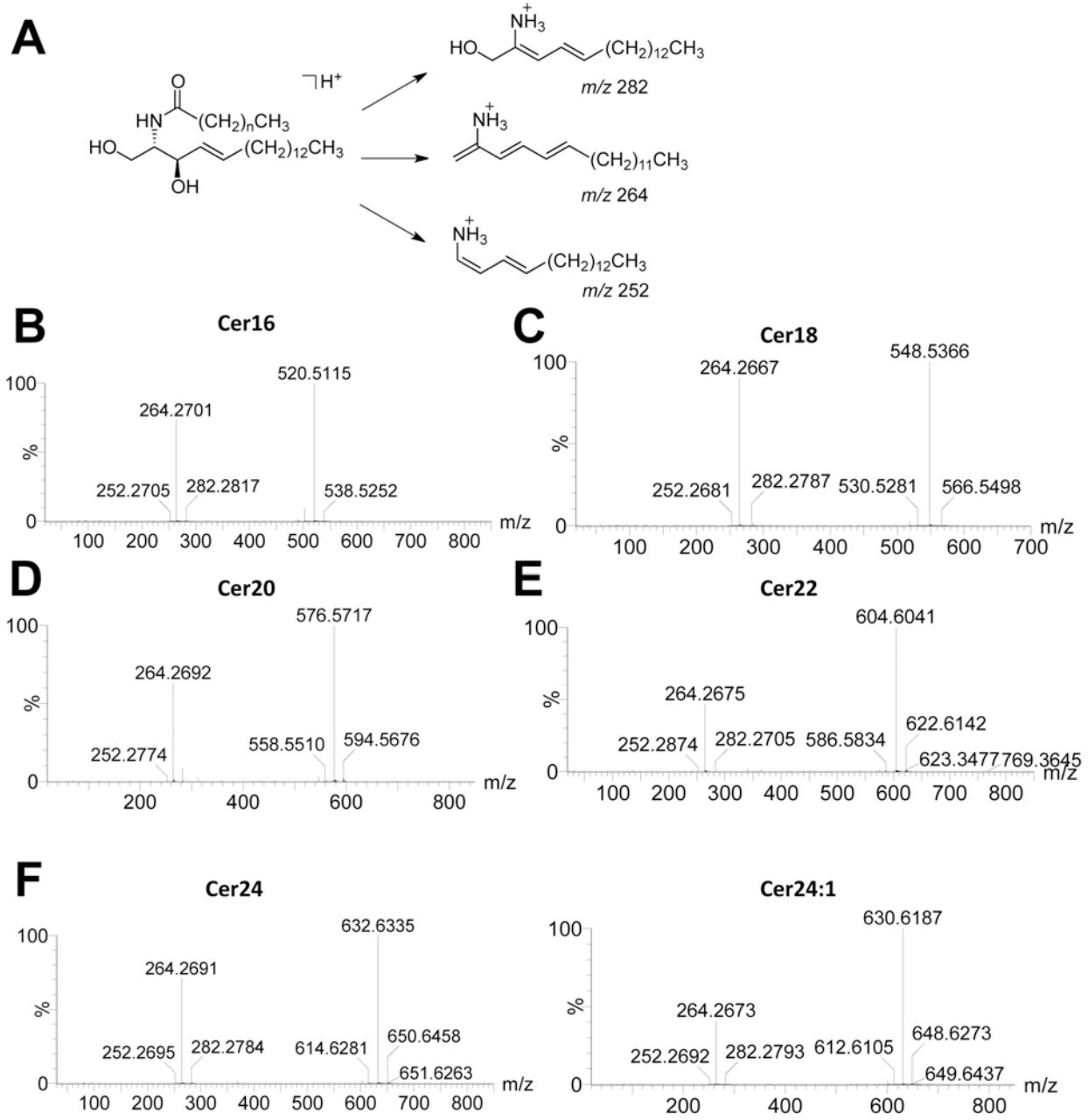
(A) Serum free fatty acids in vehicle- and tempol-treated $Fxr^{fl/fl}$ mice and $Fxr^{\Delta IE}$ mice fed a HFD for 16 weeks. n=4-5 mice per group. Data are presented as mean ± SD. One-way ANOVA with Tukey's correction. No significant differences were detected among the groups.

(B) Serum ceramides profiles after 7 weeks of antibiotic treatment in HFD-fed mice. n=5 mice per group.

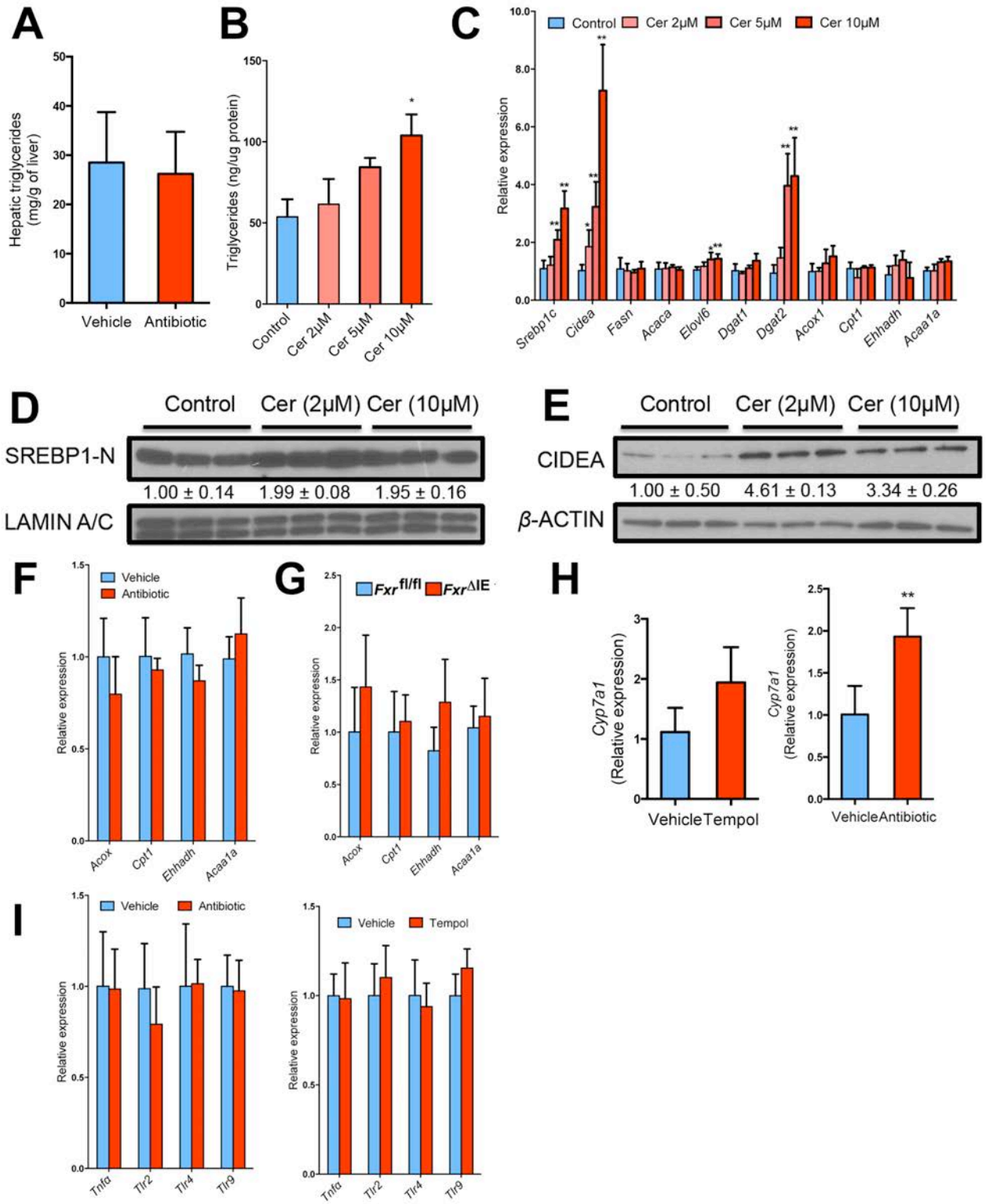
(C) Ileum ceramides profiles after 7 weeks antibiotic treatment in HFD-fed mice. n=5 mice per group.

(D) mRNA levels of mitochondrial oxidative phosphorylation-related genes from ileum mucosa after 7 weeks of antibiotic treatment of HFD-fed mice. Expression was normalized to 18S RNA. n=5 mice per group. (B-D) Data are presented as mean ± SD. Two-tailed Student's t-test. *P<0.05, **P<0.01 compared to vehicle treated mice.

(E) ATP levels in the ileum mucosa of $Fxr^{fl/fl}$ mice and $Fxr^{\Delta IE}$ mice fed a HFD for 14 weeks. n=5 mice per group. Data are presented as mean ± SD. Two-tailed Student's t-test. *P<0.05 compared to $Fxr^{fl/fl}$ mice.



Supplemental Figure 8. Lipidomics analysis of ceramides.
(A)-(F) Tandem MS and chemical structures of ceramides.


A
Hepatic triglycerides (mg/g of liver)
Vehicle
Antibiotic
B
Triglycerides (ng/ug protein)
Control
Cer 2μM
Cer 5μM
Cer 10μM
C
Control
Cer 2μM
Cer 5μM
Cer 10μM
Relative expression
Srebp1c
Cidea
Fasn
Acaca
Elovl6
Dgat1
Dgat2
Acox1
Cpt1
Ehhadh
Acaa1a
D
Control
Cer (2μM)
Cer (10μM)
SREBP1-N
1.00 ± 0.14
1.99 ± 0.08
1.95 ± 0.16
LAMIN A/C
E
Control
Cer (2μM)
Cer (10μM)
CIDEA
1.00 ± 0.50
4.61 ± 0.13
3.34 ± 0.26
β-ACTIN
F
Vehicle
Antibiotic
Relative expression
Acox
Cpt1
Ehhadh
Acaa1a
G
Fxrfl/fl
FxrΔIE
Relative expression
Acox
Cpt1
Ehhadh
Acaa1a
H
Cyp7a1 (Relative expression)
Vehicle
Tempol
Cyp7a1 (Relative expression)
Vehicle
Antibiotic
I
Vehicle
Antibiotic
Relative expression
Tnfα
Tlr2
Tlr4
Tlr9
Vehicle
Tempol
Relative expression
Tnfα
Tlr2
Tlr4
Tlr9

Supplemental Figure 9. Ceramide regulated SREBP-1c-CIDEA pathway in liver.

(A) Liver triglyceride contents after 3 days of antibiotic treatment in HFD-fed mice. n=5 mice per group. Data are presented as mean ± SD. Two-tailed Student's t-test. *P<0.05, **P<0.01 compared to vehicle-treated mice.

(B) Primary hepatocyte triglyceride contents after 24 hours treatment with different doses of ceramide (n=4). Three independent experiments were performed.

(C) mRNA levels of fatty acid synthesis, triglyceride synthesis, and fatty acid catabolism related genes in primary hepatocytes after 16 hours treatment with different doses of ceramide (n=5). Four independent experiments were performed. (B and C) Data are presented as mean ± SD. Two-tailed Student's t-test. *P<0.05, **P<0.01 compared with control.

(D) Western blot analysis of nuclear SREBP1-N expression from primary hepatocytes after 24 hours ceramide treatment (n=3).

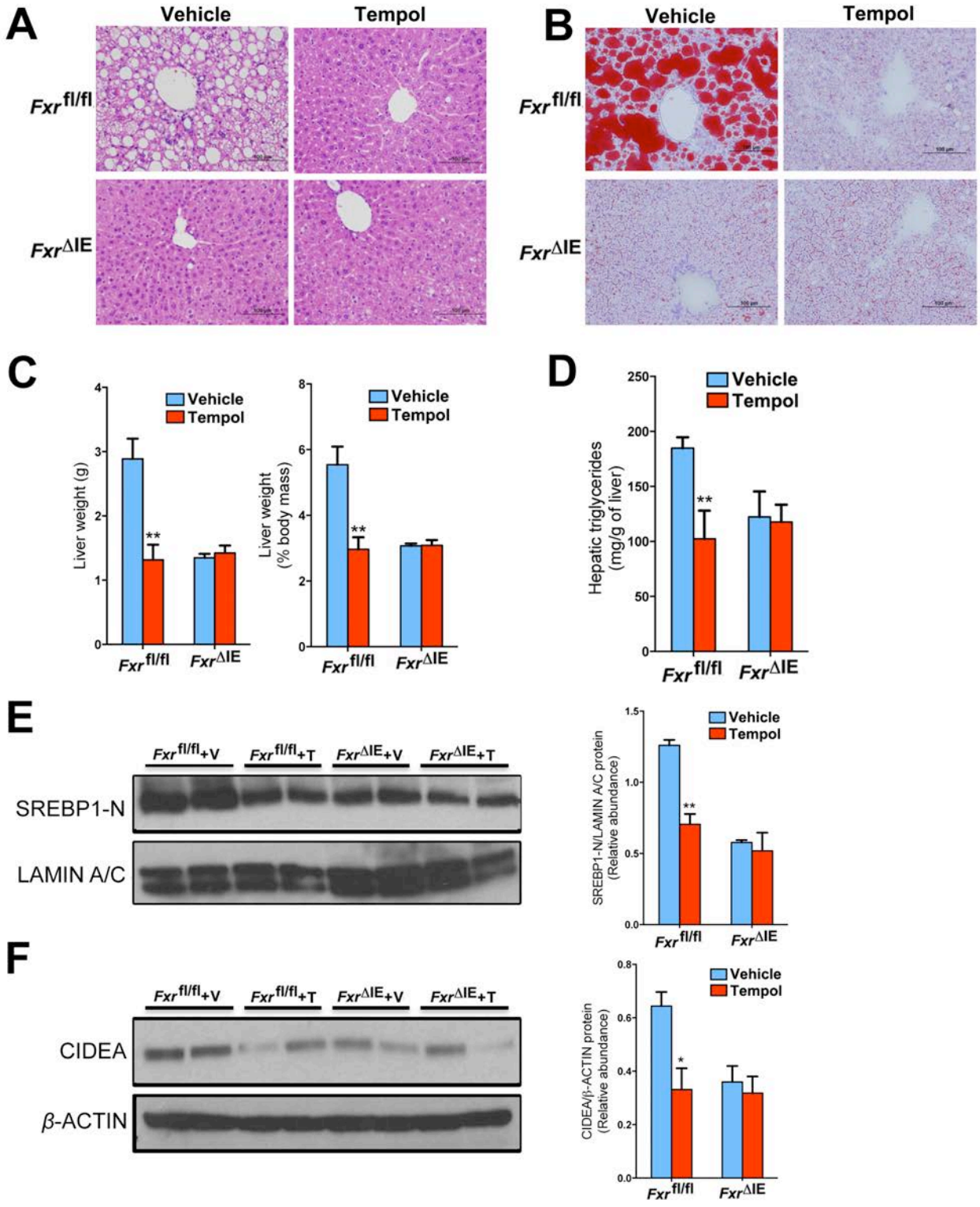
(E) Western blot analysis of CIDEA expression from primary hepatocytes after 24 hours ceramide treatment (n=3).

(F) mRNA levels of fatty acid oxidation-related genes in the liver of mice fed a HFD for 7 weeks. n=4-5 mice per group.

(G) mRNA levels of fatty acid oxidation related genes in the livers from $Fxr^{fl/fl}$ mice and $Fxr^{\Delta IE}$ mice fed a HFD for 14 weeks. n=5 mice per group. (F and G) The expression was normalized to 18S RNA. Data are presented as mean ± SD. Two-tailed Student's t-test. No significant differences were detected between the groups.

(H) mRNA levels of Cyp7a1 in the livers of HFD-fed mice. n=5 mice per group.

(I) mRNA levels of inflammation related genes in the livers of HFD-fed mice. n=5 mice per group. (H and I) The expression was normalized to 18S RNA. Data are presented as mean ± SD. Two-tailed Student's t-test. **P<0.01 compared to vehicle treated mice.



Supplemental Figure 10. Tempol improves NAFLD via the inhibition of intestinal FXR.
(A) Representative H&E staining of vehicle and tempol-treated *Fxr*$^{fl/fl}$ mice and *Fxr*$^{\Delta IE}$ mice on a HFD for 16 weeks. n=4-5 mice per group.

(B) Oil red O staining of liver sections from vehicle and tempol-treated $Fxr^{fl/fl}$ mice and $Fxr^{\Delta IE}$ mice on a HFD for 16 weeks. n=4-5 mice per group. Lipids stain positive (red color) with oil red O.

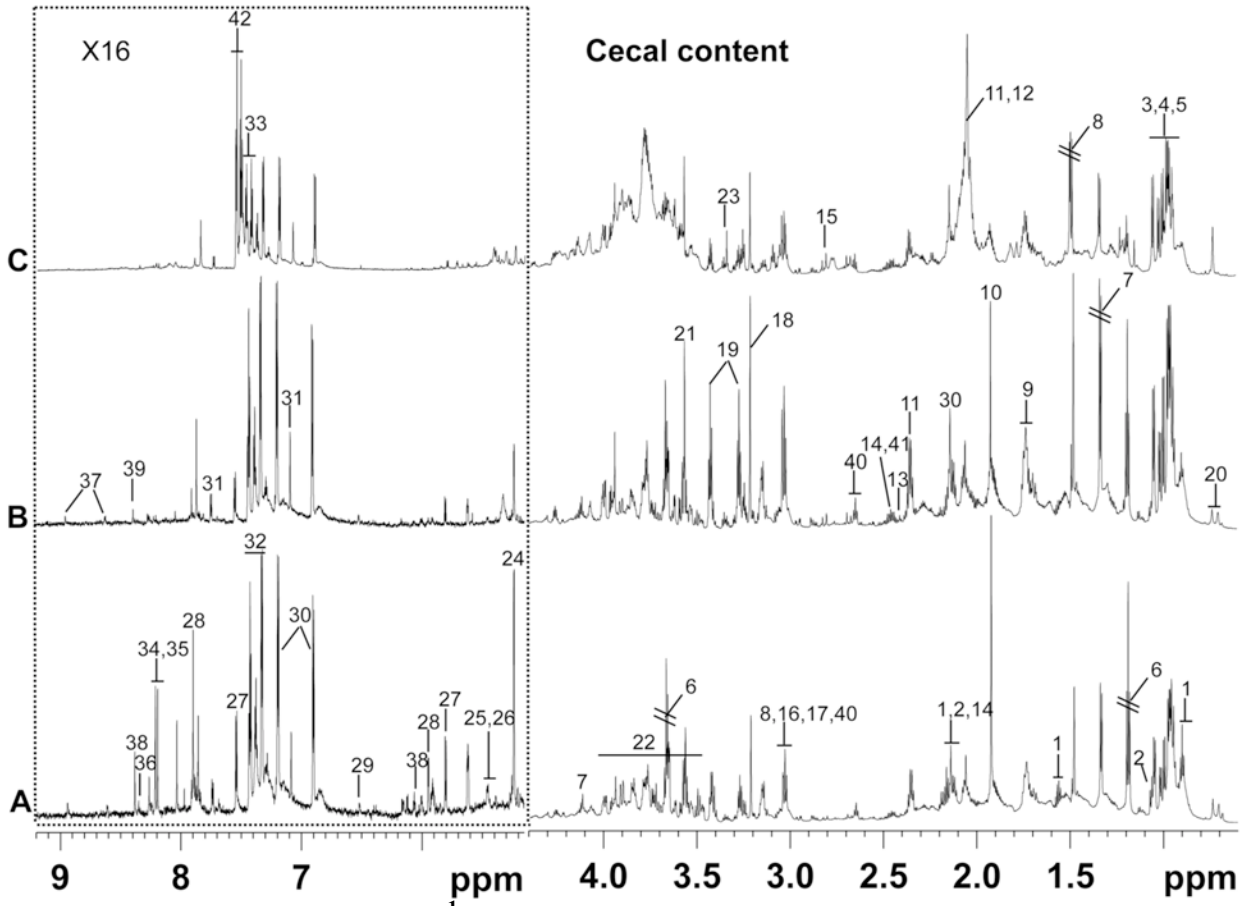
(C) Liver weights and liver weight to body weight ratios of vehicle and tempol-treated $Fxr^{fl/fl}$ mice and $Fxr^{\Delta IE}$ mice on a HFD for 16 weeks. n=4-5 mice per group.

(D) Liver triglyceride contents of vehicle and tempol-treated $Fxr^{fl/fl}$ mice and $Fxr^{\Delta IE}$ mice on a HFD for 16 weeks. n=4-5 mice per group.

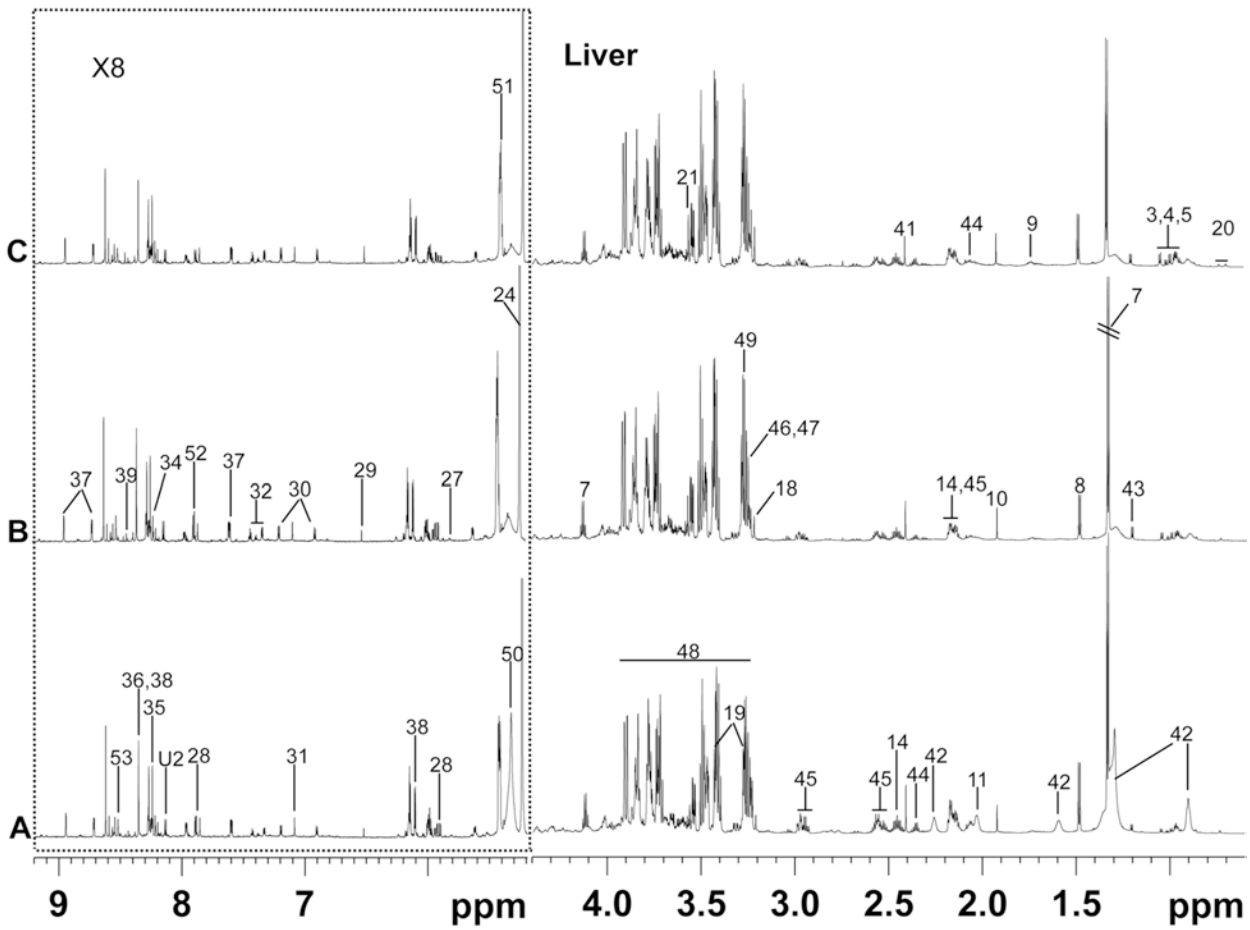
(E) Western blot analysis of liver SREBP1-N expression after 16 weeks tempol treatment on HFD-fed mice (left panel) and quantitation of SREBP1-N expression (right panel). Results are representative of twice.

(F) Western blot analysis of liver CIDEA expression after 16 weeks tempol treatment on HFD-fed mice (left panel) and quantitation of CIDEA expression (right panel). Results are representative of twice.

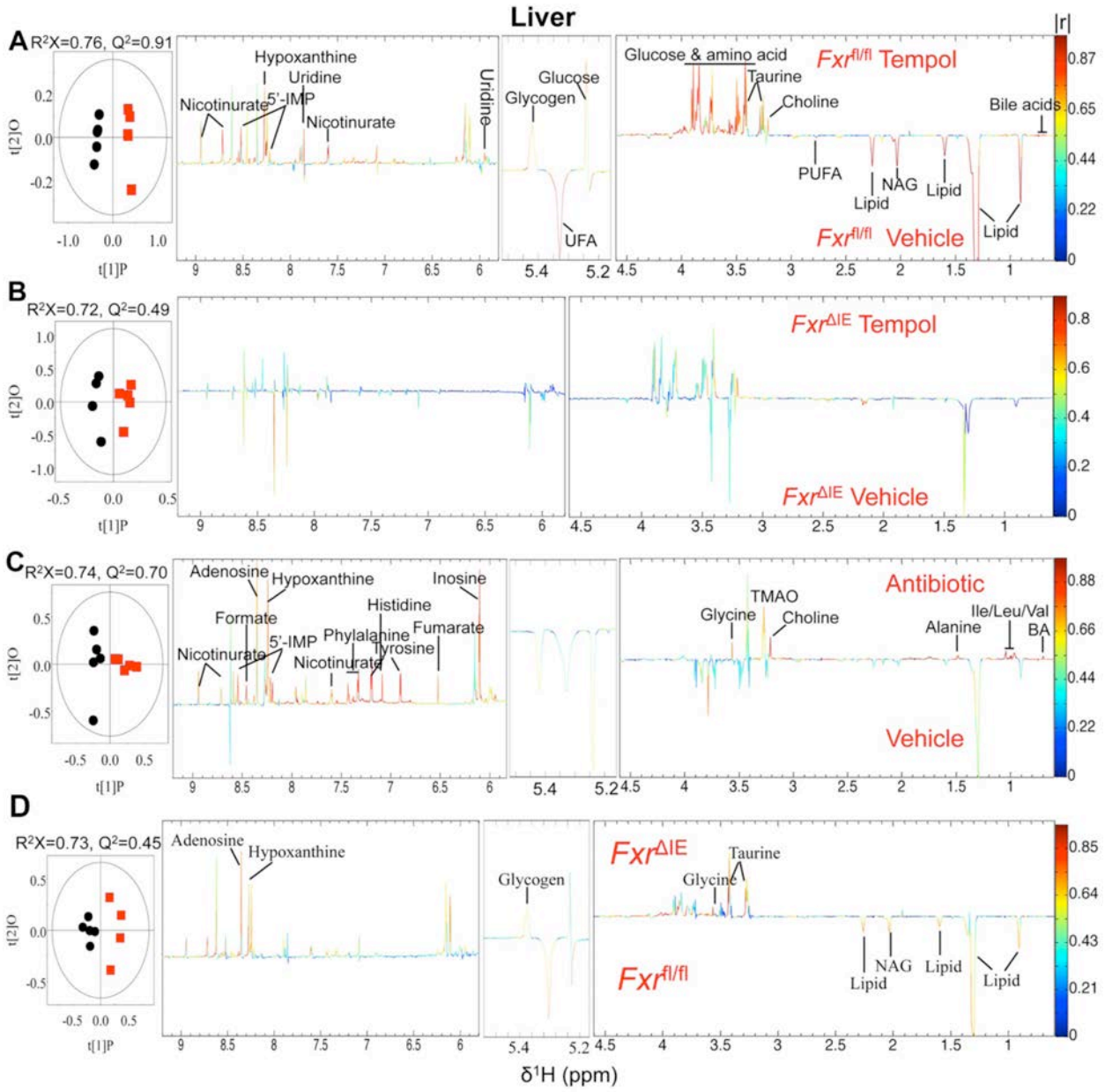
(G) All data are presented as mean ± SD. One-way ANOVA with Tukey's correction. $*P<0.05$, $**P<0.01$ compared to vehicle-treated mice of the same genotype.



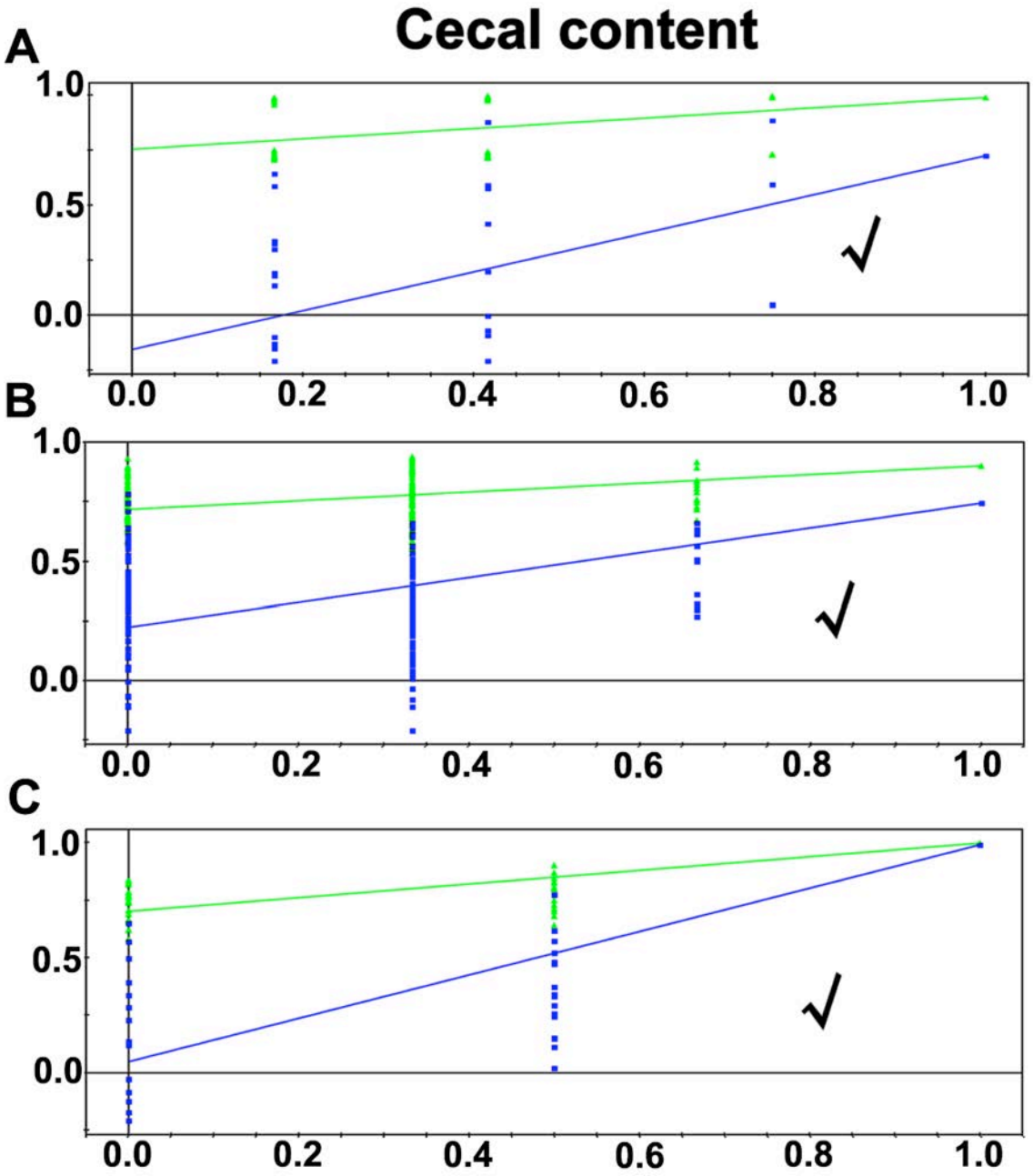
Supplemental Figure 11. Typical ^{1}H-NMR spectra of cecal content extracts.
(A)-(C) Representative 850 MHz ^{1}H NMR spectra of cecal content aqueous extracts from vehicle **(A)**, tempol **(B)** and antibiotic treatment group **(C)** fed a HFD. The regions of δ 5.1-9.2 in the spectra were vertically expanded 16 times compared with the region of δ 0.6-4.4. Keys: 1, n-butyrate; 2, propionate; 3, isoleucine; 4, leucine; 5,valine; 6,ethanol; 7, lactate; 8, alanine; 9, lysine; 10, acetate; 11, N-acetyl-glycoprotein; 12, L-proline; 13, pyruvate; 14, glutamine; 15, trimethylamine; 16, creatine; 17, α-ketoglutarate; 18, choline; 19, taurine; 20, bile acids; 21, glycine; 22, oligosaccharides; 23, methanol; 24, α-glucose; 25, raffinose; 26, stachyose; 27,uracil; 28, uridine; 29,fumarate; 30,tyrosine; 31, histidine; 32, phenylalanine; 33, urocanate; 34, adenine; 35, hypoxanthine; 36, adenosine; 37, nicotinurate; 38, inosine; 39, formate; 40, methionine; 41, succinate; 42, lipid; 43, D-3-hydroxybutyrate; 44, glutamate; 45, glutathione; 46,phosphocholine(PC); 47, glycerophosphocholine (GPC); 48, glucose & amino acids; 49, TMAO; 50, unsaturated fatty acid (UFA); 51, glycogen; 52, xanthine; 53, inosine 5'-monophosphate (5'-IMP); 54, U1; 55, U2; See also Supplemental Table 2.



Supplemental Figure 12. Typical ^{1}H-NMR spectra of liver extracts.
(A)-(C) Representative 850 MHz ^{1}H NMR spectra of liver aqueous extracts from vehicle **(A)**, tempol **(B)**, and antibiotic treated group **(C)** fed a HFD. The regions of δ 5.15-9.20 in the spectra were vertically expanded 8 times compared with the region of δ 0.6-4.4. For identification of the peak numbers, refer to keys in Supplemental Figure11.

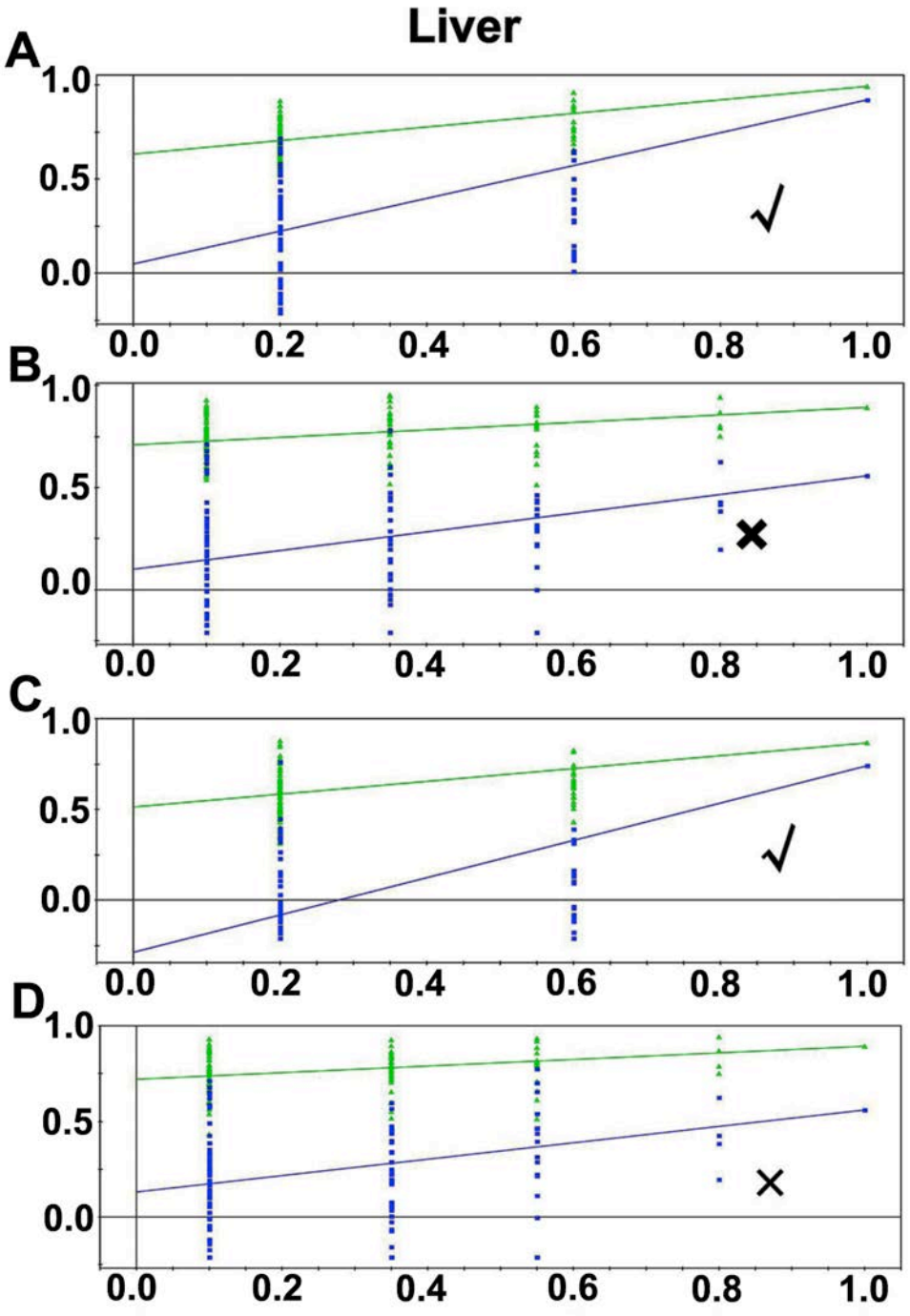


Supplemental Figure 13. NMR metabolomics analysis of liver extracts.
(A)-(D) O-PLS-DA scores (left) and correlation coefficient-coded loadings plots for the models (right) from NMR spectra of liver aqueous extracts. The discriminations of metabolic profile of cecal contents between vehicle-treated *Fxr*$^{fl/fl}$ mice and tempol-treated *Fxr*$^{fl/fl}$ mice fed a HFD for 16 weeks **(A)**, vehicle-treated *Fxr*$^{\Delta IE}$ mice and tempol-treated *Fxr*$^{\Delta IE}$ mice fed a HFD for 16 weeks **(B)**, vehicle-treated mice and antibiotic-treated mice fed a HFD for 7 weeks **(C)**, and *Fxr*$^{fl/fl}$ mice and *Fxr*$^{\Delta IE}$ mice fed a HFD for 14 weeks **(D)**. The correlation coefficient values indicating the significant changed metabolites were shown in Supplemental Table 3. (|r| cutoff value is 0.755, n = 5, $P < 0.05$; CV-ANOVA: $P = 1.43\times10^{-4}$, 0.57, 3.41×10^{-3} and 0.36, respectively).



Supplemental Figure 14. The quality cross-validation of PLS-DA models for ^{1}H NMR data of cecal content aqueous extracts.

(A)-(C) Cross-validation with permutations test plots (200 permutations) for the PLS-DA models constructed from ^{1}H NMR data of cecal content aqueous extracts between vehicle-treated *Fxr*$^{fl/fl}$ mice and tempol-treated *Fxr*$^{fl/fl}$ mice fed a HFD for 16 weeks **(A)**, vehicle-treated *Fxr*$^{\Delta IE}$ mice and tempol-treated *Fxr*$^{\Delta IE}$ mice fed a HFD for 16 weeks **(B)**, and vehicle-treated mice and antibiotic-treated mice fed a HFD for 7 weeks **(C)**. n=4-5 mice per group.



Supplemental Figure 15. Quality cross-validation of PLS-DA models for ^{1}H NMR data of liver extracts.

(A)-(D) Cross-validation with permutations test plots (200 permutations) for the PLS-DA models constructed from ^{1}H NMR data of liver aqueous extracts between vehicle-treated *Fxr*$^{fl/fl}$ mice and tempol-treated *Fxr*$^{fl/fl}$ mice fed a HFD for 16 weeks **(A)**, vehicle-treated *Fxr*$^{\Delta IE}$ mice and tempol-treated *Fxr*$^{\Delta IE}$ mice fed a HFD for 16 weeks **(B)**, vehicle-treated mice and antibiotic-treated mice fed a HFD for 7 weeks **(C)**, and *Fxr*$^{fl/fl}$ mice and *Fxr*$^{\Delta IE}$ mice fed a HFD for 14 weeks (D). n=4-5 mice per group.

SUPPLEMENTAL TABLES

Supplemental Table 1. Primers used for qPCR

Mouse primers	Sequence
18S FWD	5'- ATTGGAGCTGGAATTACCGC -3'
18S REV	5'- CGGCTACCACATCCAAGGAA -3'
Fxr FWD	5'- TGGGCTCCGAATCCTCTTAGA -3'
Fxr REV	5'- TGGTCCTCAAATAAGATCCTTGG -3'
Shp FWD	5'- TCTGCAGGTCGTCCGACTATTC -3'
Shp REV	5'- AGGCAGTGGCTGTGAGATGC -3'
Cyp7a1 FWD	5'- AACAACCTGCCAGTACTAGATAGC -3'
Cyp7a1 REV	5'- GTGTAGAGTGAAGTCCTCCTTAGC -3'
Fgf15 FWD	5'- GCCATCAAGGACGTCAGCA -3'
Fgf15 REV	5'- CTTCCTCCGAGTAGCGAATCAG -3'
Pparα FWD	5'- CCCAAGGGAGGAATAGCTTCT -3'
*Pparα*REV	5'- CTCTGCGATGCGGTTCCAA -3'
Ndufa2 FWD	5'- TTGCGTGAGATTCGCGTTCA-3'
Ndufa2 REV	5'- ATTCGCGGATCAGAATGGGC-3'
Ndufb3 FWD	5'- ACAGACAGTGGAAAATTGAAGGG-3'
Ndufb3 REV	5'- GCCCATGTATCTCCAAGCCT-3'
Sdhd FWD	5'- TGGTCAGACCCGCTTATGTG -3'
Sdhd REV	5'- GGTCCAGTGGAGAGATGCAG -3'
mt-Cytb FWD	5'- ACCAATCTCCCAAACCATCA -3'
mt-Cytb REV	5'- TCCAGAGACTTGGGGATCTAAC -3'

Cyc1 FWD	5'- GAGGCCGAAAGGTGATGCT -3'
Cyc1 REV	5'- GCACTCACGGCAGAATGAAG -3'
mt-Co1 FWD	5'- CTCGCCTAATTTATTCCACTTCA -3'
mt-Co1 REV	5'- GGGGCTAGGGGTAGGGTTAT -3'
mt-Co2 FWD	5'- ACCTGGTGAACTACGACTGCTAGA -3'
mt-Co2 REV	5'- TGCTTGATTTAGTCGGCCTGGGAT -3'
mt-Co3 FWD	5'- GCAGGATTCTTCTGAGCGTTCT - 3'
mt-Co3 REV	5'- GTCAGCAGCCTCCTAGATCATGT - 3'
Cox4i1 FWD	5'- GAGCCTGATTGGCAAGAGAG - 3'
Cox4i1 REV	5'- GATCAGCGTAAGTGGGGAAA - 3'
Cox4i2 FWD	5'- GGCTGCGTCTTCTTCTTCAT - 3'
Cox4i2 REV	5'- ATGGGGTTGCTCTTCATGTC - 3'
Cox5a FWD	5'- GCCGCTGTCTGTTCCATTC -3'
Cox5a REV	5'- GCATCAATGTCTGGCTTGTTGAA -3'
Cox6c FWD	5'- GCGTCTGCGGGTTCATATTG -3'
Cox6c REV	5'- TCTGCATACGCCTTCTTTCTTG -3'
Cox7a1 FWD	5'- GCTCTGGTCCGGTCTTTTAGC-3'
Cox7a1 REV	5'- GTACTGGGAGGTCATTGTCGG-3'
Atp5g FWD	5'- CCAGAGGCCCCATCTAAGC -3'
Atp5g REV	5'- CCCCAGAATGGCATAGGAGAAG -3'
Atp5h FWD	5'- GCTGGGCGTAAACTTGCTCTA -3'
Atp5h REV	5'- CAGACAGACTAGCCAACCTGG -3'
Srebp1c FWD	5'- GGAGCCATGGATTGCACATT-3'
Srebp1c REV	5'- GCTTCCAGAGAGGAGGCCAG -3'

Cidea FWD	5'- TGACATTCATGGGATTGCAGAC -3'
Cidea REV	5'- GGCCAGTTGTGATGACTAAGAC -3'
Fsp27 FWD	5'- TGACATTCATGGGATTGCAGAC-3'
Fsp27 REV	5'- GGCCAGTTGTGATGACTAAGAC-3'
Pparg2 FWD	5'- TCTGGGAGATTCTCCTGTTGA -3'
Pparg2 REV	5'- GGTGGGCCAGAATGGCATCT -3'
Fas FWD	5'- AAGTTGCCCGAGTCAGAGAACC -3'
Fas REV	5'- ATCCATAGAGCCCAGCCTTCCATC -3'
Acaca FWD	5'- ATGGGCGGAATGGTCTCTTTC -3'
Acaca REV	5'- TGGGGACCTTGTCTTCATCAT -3'
Elovl6 FWD	5'- GAAAAGCAGTTCAACGAGAACG -3'
Elovl6 REV	5'- AGATGCCGACCACCAAAGATA -3'
Dgat1 FWD	5'- GACGGCTACTGGGATCTGA -3'
Dgat1 REV	5'- TCACCACACACCAATTCAGG -3'
Dgat2 FWD	5'- CGCAGCGAAAACAAGAATAA-3'
Dgat2 REV	5'- GAAGATGTCTTGGAGGGCTG-3'
Cpt1 FWD	5'- TCTTCACTGAGTTCCGATGGG -3'
Cpt1 REV	5'- ACGCCAGAGATGCCTTTTCC -3'
Acox FWD	5'- CCGCCACCTTCAATCCAGAG -3'
Acox REV	5'- CAAGTTCTCGATTTCTCGACGG -3'
Acaa1a FWD	5'- TCTCCAGGACGTGAGGCTAAA-3'
Acaa1a REV	5'- CGCTCAGAAATTGGGCGATG -3'
Tnfα FWD	5'- CCACCACGCTCTTCTGTCTAC - 3'
Tnfα REV	5'- AGGGTCTGGGCCATAGAACT - 3'

Tlr2 FWD	5'- CATCACCGGTCAGAAAACAA - 3'
Tlr2 REV	5'- ACCAAGATCCAGAAGAGCCA - 3'
Tlr4 FWD	5'- ATGGCATGGCTTACACCACC - 3'
Tlr4 REV	5'- GAGGCCAATTTTGTCTCCACA - 3'
Tlr9 FWD	5'- ATGGTTCTCCGTCGAAGGACT - 3'
Tlr9 REV	5'- GAGGCTTCAGCTCACAGGG - 3'
Sptlc1 FWD	5'- CGAGGGTTCTATGGCACATT- 3'
Sptlc1 REV	5'- GGTGGAGAAGCCATACGAGT - 3'
Sptlc2 FWD	5'- TCACCTCCATGAAGTGCATC - 3'
Sptlc2 REV	5'- CAGGCGTCTCCTGAAATACC - 3'
Sptlc3 FWD	5'- ACACAATCCTAAGACCCAGCA - 3'
Sptlc3 REV	5'- AGACTGGCTTATCCTCAGCATA - 3'
Degs1 FWD	5'- AATGGGTCTACACGGACCAG - 3'
Degs1 REV	5'- TGGTCAGGTTTCATCAAGGAC - 3'
Cers2 FWD	5'- AAGTGGGAAACGGAGTAGCG- 3'
Cers2 REV	5'- ACAGGCAGCCATAGTCGTTC - 3'
Cers4 FWD	5'- GGATTAGCTGATCTCCGCAC - 3'
Cers4 REV	5'- CCAGTATGTCTCCTGCCACA - 3'
Cers5 FWD	5'- CTTCTCCGTGAGGATGCTGT- 3'
Cers5 REV	5'- GTGTCATTGGGTTCCACCTT - 3'
Cers6 FWD	5'- AAGCCAATGGACCACAAACT - 3'
Cers6 REV	5'- TGCTTGGAGAGCCCTTCTAAT - 3'
Smpd1 FWD	5'- GTTACCAGCTGATGCCCTTC- 3'
Smpd1 REV	5'- AGCAGGATCTGTGGAGTTG - 3'

Smpd2 FWD	5'-CTCCAGCCATGAAGCTCAAC - 3'
Smpd2 REV	5'- TTCAGAAAGTCTCCCAAGCG - 3'
Smpd3 FWD	5'- CCTGACCAGTGCCATTCTTT - 3'
Smpd3 REV	5'- AGAAACCCGGTCCTCGTACT - 3'
Sgms1 FWD	5'- GAACACGCGGAGAAGCTAGT- 3'
Sgms1 REV	5'- AACATTCGTTCGCCTCATTT - 3'
Sgms2 FWD	5'-CTGTGCGGAGACTTCCTCTT - 3'
Sgms2 REV	5'- CACCAGAAGTGACGAGGTGA - 3'
Acer1 FWD	5'- TATCAGCACCTTCTTGACGTTC- 3'
Acer1 REV	5'- GCCGAAGATCATCATCCCTAATC- 3'
Acer3 FWD	5'- GGTGACCTTGTTCGTCGCT - 3'
Acer3 REV	5'- TGAATTGCACCAAAAATTGGAGG - 3'

Supplemental Table 2. ^{1}H NMR chemical shifts for metabolites assigned in liver and cecal content extracts from the mice fed a HFD.

key	metabolites	moieties	δ ^{1}H (ppm) and multiplicity[a]	Samples[b]
1	n-butyrate	CH_3, CH_2, CH_2	0.91(t), 1.56(m), 2.16(t)	C
2	Propionate	CH_3, CH_2	1.06(t), 2.18(q)	C
3	Isoleucine	α CH, β CH, γ CH_3, δ CH_3	3.65(d), 1.95(m), 0.99(t), 1.02(d)	L, C
4	Leucine	α CH, β CH_2, γ CH_3, δ CH_3	0.94(d), 3.72(t), 1.96(m), 0.91(d)	L, C
5	Valine	α CH, β CH, γ CH_3	3.6(d), 2.26(m), 0.98(d), 1.04(d)	L, C
6	Ethanol	CH3, CH2	1.18(t), 3.65(q)	C
7	Lactate	α CH, β CH_3	4.11(q), 1.32(d)	L, C
8	Alanine	α CH, β CH_3	3.77(q), 1.48(d)	L, C
9	Lysine	α CH, β CH_2, γ CH_2, δ CH_2	3.76(t), 1.89(m), 1.72(m), 3.01(t)	L, C
10	Acetate	CH_3	1.91(s)	L, C
11	N-acetyl-glycoprotein	CH_3	2.03(s)	L, C
12	L-proline	CH_2, CH_2, CH	2.05(m), 2.34(m), 3.4(m)	C
13	Pyruvate	CH_3	2.38(s)	C
14	Glutamine	α CH, β CH_2, γ CH_2	2.15(m), 2.44(m), 3.77(m)	L, C
15	Trimethylamine	CH_3	2.88(s)	C
16	Creatine	CH_3, CH_2	3.03(s), 3.93(s)	C
17	α -ketoglutarate	γ CH_2, β CH_2	2.45(t), 3.01(t)	C
18	Choline	$N(CH_3)_3$, OCH_2, NCH_2	3.2(s), 4.05(t), 3.51(t)	L, C
19	Taurine	S-CH_2, N-CH_2	3.26(t), 3.40(t)	L, C
20	Bile acids	CH_3	0.73(m)	L, C
21	Glycine	CH_3	3.57(s)	L, C
22	Oligosaccharides	α CH resonances	3.3-3.9	C
23	Methanol	CH_3	3.36 (s)	C

24	α -Glucose	1-CH	5.23(d)	L, C
25	Raffinose	1-CH	5.41(d)	C
26	Stachyose	1-CH	5.41(d)	C
27	Uracil	1-CH, 2-CH	5.81(d), 7.54(d)	L, C
28	Uridine	11-CH, 7-CH, 12-CH, 6-CH, 5-CH, 4-CH, CH_2	7.88(d), 5.92(d), 5.9(d), 4.36(m), 4.24(t)	L, C
29	Fumarate	CH	6.53(s)	L, C
30	Tyrosine	CH, CH	6.89(dd), 7.18(dd)	L, C
31	Histidine	2-CH, 4-CH, CH_2	7.75(t), 7.08(d), 6.05(d)	L, C
32	Phenylalanine	Ring-CH	7.40(m), 7.33(m), 7.35(m)	L, C
33	Urocanate	CHCOOH, CH(ring), 5CH	6.40(d), 7.31(d), 7.43(s)	C
34	Adenine	2CH, 6CH	8.19(s), 8.21(s)	L, C
35	Hypoxanthine	1-CH, 2-CH	8.20(s), 8.21(s)	L, C
36	Adenosine	14-CH	8.32(s)	L, C
37	Nicotinurate	2-CH, 6-CH, 4-CH, 5-CH	8.93(s),8.62(d),8.25(d),7.60(dd),	L, C
38	Inosine	14-CH, 1-CH, 8-CH, 4'-CH, 5'-CH, CH_2(1/2)	8.34(s), 6.09(d), 8.22(s), 4.76(t), 4.47(m)	L, C
39	Formate	CH	8.45(s)	L, C
40	Methionine	δ CH_3, β CH_2, γ CH_2	2.14(s), 2.16(m), 2.65(t)	C
41	Succinate	CH_3	2.41(s)	L, C
42	Lipid	CH_3, $(CH_2)_n$, CH_2-C=C, CH_2-C=O,C-CH_2-C=,-CH=CH-	0.89(m), 1.27(m), 2.0(m), 2.3(m), 2.78(m), 5.3(m)	L
43	D-3-hydroxybutyrate	CH, CH_2, γ CH_3, CH_2	4.16(dt),2.41(dd),1.20(d),2.31(dd)	L
44	Glutamate	α CH, β CH_2, γ CH_2	2.08(m), 2.34(m), 3.75(m)	L
45	Glutathione	CH_2, CH_2, S-CH_2, N-CH, CH	2.16(m), 2.55(m), 2.95(dd), 3.78(m), 4.56(q)	L
46	Phosphocholine(PC)	$N(CH_3)_3$, OCH_2, NCH_2	3.22(s), 4.21(t), 3.61(t)	L
47	Glycerophosphocholine	$N(CH_3)_3$, OCH_2, NCH_2	3.22(s), 4.32(t), 3.68(t)	L

48	Glucose & amino acids	α CH resonances	3.3-3.9	L
49	TMAO	CH_3	3.27(s)	L
50	Unsaturated fatty acid	CH=CH	2.73, 5.3	L
51	Glycogen	1-CH	5.38-5.45(m)	L
52	Xanthine	CH	7.89 (s)	L
53	Inosine 5'-monophosphate (5'-IMP)	H14, H8, H1, H2, H3	8.56(s), 8.19(s), 6.12(d), 4.52 (dd), 4.37 (dt)	L
54	U1	CH	7.44(t),7.46(d)	C
55	U2	CH, CH, CH	8.07(s), 8.15(s), 8.73 (s)	L

[a] Key: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublet.

[b] Liver (L), and cecal content (C) aqueous extracts.

Supplemental Table 3. Significantly changed metabolites in the liver and cecal content of mice exposed to tempol and antibiotic compared with controls fed a HFD.

Metabolite	Cecal content			Liver			
	Fxr$^{fl/fl}$+Vehicle Vs *Fxr*$^{fl/fl}$+Tempol	*Fxr*$^{\Delta IE}$+Vehicle Vs *Fxr*$^{\Delta IE}$+Tempol	Vehicle Vs Antibiotic	*Fxr*$^{fl/fl}$+Vehicle Vs *Fxr*$^{fl/fl}$+Tempol	*Fxr*$^{\Delta IE}$+Vehicle Vs *Fxr*$^{\Delta IE}$+Tempol	Vehicle Vs Antibiotic	*Fxr*$^{fl/fl}$ Vs *Fxr*$^{\Delta IE}$
	R^2X=0.70	R^2X=0.67	R^2X=0.86	R^2X=0.76	R^2X=0.72	R^2X=0.74	R^2X=0.73
	Q^2=0.49	Q2=0.53	Q^2=0.98	Q^2=0.91	Q^2=0.49	Q^2=0.70	Q^2=0.45
Lipid	—	—	—	-0.88	—	—	-0.67
Choline	—	—	—	+0.73	—	+0.81	—
TMAO	—	—	—	—	—	+0.72	—
n-butyrate	-0.82	—	-0.87[a]	—	—	—	—
Propionate	-0.78	—	-0.82	—	—	—	—
Acetate	-0.81	—	-0.84	—	—	—	—
Taurine	+0.69	—	-0.79	+0.71	—	—	+0.79
Oligosaccharide	+0.85	—	+0.88	—	—	—	—
UFA	—	—	—	-0.88	—	—	-0.69
Glucose	+0.81	—	-0.86	+0.73	—	—	—
Glycogen	—	—	—	+0.77	—	—	+0.62
Lactate	—	+0.73	-0.85	—	—	—	—
Alanine	—	+0.64	-0.83	—	—	+0.76	—
Creatine	—	+0.73	—	—	—	—	—
Fumarate	—	—	—	—	—	+0.74	—
Isoleucine	—	—	-0.80	—	—	+0.81	—
Leucine	—	—	-0.78	—	—	+0.80	—
Valine	—	—	-0.82	—	—	+0.83	—

Bile acids	—	-0.73	-0.81	+0.76	—	+0.79	—
Tyrosine	—	+0.59	—	—	—	+0.85	—
Phenylalanine	—	+0.57	—	—	—	+0.82	—
Histidine	—	—	—	—	—	+0.81	—
Glutamine	—	—	-0.85	—	—	—	—
Uridine	—	—	-0.86	+0.72	—	—	—
Hypoxanthine	—	—	-0.81	+0.79	—	+0.81	+0.63
Fromate	—	—	-0.83	—	—	+0.78	—
5'-IMP	—	—	—	+0.80	—	+0.82	—
Nicotinurate	—	—	—	+0.78	—	+0.77	—
Inosine	—	—	—	+0.78	—	+0.79	—
Adenosine	—	—	—	+0.78	—	+0.81	+0.78

[a] Correlation coefficient values obtained from OPLS-DA of treatment groups;

+ and – indicate a significant increase and decrease of metabolite levels in the treatment groups compared to the control mice; — no change or not detected.

Supplemental Table 4. Cross-validation with permutation test and CV-ANOVA for PLS-DA and OPLS-DA models from NMR spectra of liver and cecal content extracts in mice fed a HFD.

Samples	Cecal content		Liver	
Pair-wise comparison	OLPS-DA CV-ANOVA	PLS-DA Permutation test	OLPS-DA CV-ANOVA	PLS-DA Permutation test
Tempol Vs WT Vehicle	*	√	***	√
Tempol Vs KO Vehicle	*	√	NS	×
Antibiotic Vs WT Vehicle	***	√	**	√
KO Vehicle Vs WT Vehicle	—	—	NS	×

*p < 0.05, **p < 0.01, ***p < 0.001; NS, no significance; √ pass; × fail; — not determined.