

1 **Title:** Ursodeoxycholic acid (UDCA) mitigates the host inflammatory response during
2 *Clostridioides difficile* infection by altering gut bile acids which attenuates NF- κ B
3 signaling via bile acid activated receptors

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1 **Importance**

2 The clinical utility of ursodiol for prevention of recurrent CDI is currently in Phase 4
3 clinical trials. However, the mechanism by which ursodiol exerts its impacts on *C.*
4 *difficile* pathogenesis is poorly understood. Herein, we demonstrated that ursodiol
5 pretreatment attenuates CDI pathogenesis early in the course of disease in mice, which
6 coincides with alterations in the cecal and colonic inflammatory transcriptome, bile acid
7 activated receptors nuclear farnesoid X receptor (FXR), and transmembrane G protein-
8 coupled membrane receptor 5 (TGR5), which are able to modulate the innate immune
9 response through signaling pathways such as NF- κ B. Ursodiol attenuated an overly
10 robust inflammatory response that is detrimental to the host during CDI, and thus
11 remains a viable non-antibiotic treatment and/or prevention strategy against CDI.
12 Likewise, modulation of the host innate immune response via bile acid activated
13 receptors, FXR and TGR5, represents a new potential treatment strategy for patients
14 with CDI.

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1 **Abstract**

2 *Clostridioides difficile* infection (CDI) is associated with increasing morbidity and
3 mortality posing an urgent threat to public health. Recurrence of CDI after successful
4 treatment with antibiotics is high, thus necessitating discovery of novel therapeutics
5 against this enteric pathogen. Administration of the secondary bile acid ursodeoxycholic
6 acid (UDCA, ursodiol) inhibits the life cycle of various strains of *C. difficile in vitro*,
7 suggesting the FDA approved formulation of UDCA, known as ursodiol, may be able to
8 restore colonization resistance against *C. difficile in vivo*. However, the mechanism(s)
9 by which ursodiol is able to restore colonization resistance against *C. difficile* remains
10 unknown. Here, we confirmed that ursodiol inhibits *C. difficile* R20291 spore
11 germination and outgrowth, growth, and toxin activity in a dose dependent manner *in*
12 *vitro*. In a murine model of CDI, exogenous administration of ursodiol resulted in
13 significant alterations in the bile acid metabolome with little to no changes in gut
14 microbial community structure. Ursodiol pretreatment resulted in attenuation of CDI
15 pathogenesis early in the course of disease, which coincided with alterations in the
16 cecal and colonic inflammatory transcriptome, bile acid activated receptors nuclear
17 farnesoid X receptor (FXR), and transmembrane G protein-coupled membrane receptor
18 5 (TGR5), which are able to modulate the innate immune response through signaling
19 pathways such as NF- κ B. Although ursodiol pretreatment did not result in a consistent
20 decrease in the *C. difficile* life cycle *in vivo*, it was able to attenuate an overly robust
21 inflammatory response that is detrimental to the host during CDI. Ursodiol remains a
22 viable non-antibiotic treatment and/or prevention strategy against CDI. Likewise,

1 modulation of the host innate immune response via bile acid activated receptors, FXR
2 and TGR5, represents a new potential treatment strategy for patients with CDI.

3 **Abbreviations**

4 α MCA – α -Muricholic acid; β MCA – β -Muricholic acid; ω MCA – ω -Muricholic acid; CA –
5 Cholic acid; CDCA – Chenodeoxycholic acid; DCA – Deoxycholic acid; GCDCA –
6 Glycochenodeoxycholic acid; GDCA – Glycodeoxycholic acid; GLCA – Glycolithocholic
7 acid; GUDCA – Glycoursodeoxycholic acid; HCA – Hyodeoxycholic acid; iDCA –
8 Isodeoxycholic acid; iLCA – Isolithocholic acid; LCA – Lithocholic acid; TCA –
9 Taurocholic acid; TCDCA – Taurochenodeoxycholic acid; TDCA – Taurodeoxycholic
10 acid; THCA – Taurohyodeoxycholic acid; TUDCA – Tauroursodeoxycholic acid;
11 T β MCA– Tauro- β -muricholic acid; T ω MCA –Tauro ω -muricholic acid; UDCA
12 Ursodeoxycholic acid.

13 **Introduction**

14 *Clostridioides difficile* is a leading nosocomial enteric pathogen that causes significant
15 human morbidity and mortality, resulting in 29,000 deaths per year and costing over
16 \$4.8 billion in healthcare expenses annually in the United States.¹⁻⁵ A major risk factor
17 for *C. difficile* infection (CDI) is the use of antibiotics.^{6,7} Antibiotics lead to significant and
18 enduring shifts in the gut microbiota and metabolome⁸⁻¹⁰, resulting in loss of colonization
19 resistance against *C. difficile*. Although the exact mechanisms of colonization resistance
20 against *C. difficile* remain unclear, there is increasing evidence that microbial derived
21 secondary bile acids play an important role.¹⁰⁻¹³

22 Antibiotics, either vancomycin or fidaxomicin, are recommended for an initial
23 episode of CDI.¹⁴ Unfortunately antibiotic treatment can further disrupt the gut microbial

1 community and recurrence of CDI after cessation of antibiotics is high, occurring in 20-
2 30% of patients.^{3,15-18} Even fidaxomicin, a bactericidal antibiotic against *C. difficile*,
3 alters the intestinal microbial community structure, just to a lesser extent than
4 vancomycin.¹⁹ Fecal microbiota transplantation (FMT) is a highly effective treatment for
5 recurrent CDI, however the long-term ramifications of this conditionally FDA approved
6 procedure are unknown.^{20,21} A potential mechanism by which FMT leads to resolution of
7 CDI in a mouse model^{22,23} and in humans¹³ is by restoring microbial derived secondary
8 bile acids.

9 Secondary bile acids, such as deoxycholic acid (DCA), lithocholic acid (LCA) and
10 ursodeoxycholic acid (UDCA, ursodiol), are produced as a collaborative effort by the
11 host (production of primary bile acids) and the gut microbiota (biochemical modification
12 of host primary bile acids into secondary bile acids).^{24,25} Our group and others have
13 demonstrated that numerous microbial derived secondary bile acids, including UDCA,
14 are able to inhibit spore germination, growth, and toxin activity of various *C. difficile*
15 strains *in vitro*.^{26,27} We also know that secondary bile acids are associated with
16 colonization resistance against *C. difficile* in mice and humans.^{10,13,28} Furthermore, oral
17 UDCA administration successfully prevented recurrence of *C. difficile* ileal pouchitis in a
18 single patient,²⁹ suggesting that exogenous administration of the secondary bile acid
19 UDCA may be able to restore colonization resistance against *C. difficile in vivo*.²⁹

20 UDCA is available in an FDA approved formulation known as ursodiol.³⁰ Ursodiol
21 is used to treat a variety of hepatic and biliary diseases due to its myriad of beneficial
22 effects, including: anticholestatic, antifibrotic, antiproliferative, and anti-inflammatory
23 properties.³¹⁻⁴⁰ Additionally, off-label use of ursodiol is currently in Phase 4 clinical trials

1 for the prevention of recurrent CDI.⁴¹ It is well established that ursodiol alters the
2 hepatic, biliary, and serum bile acid pools.^{31,42} However, it remains unclear if and how
3 exogenously administered ursodiol alters the indigenous gut microbial community
4 structure and/or bile acid metabolome, which contributes to colonization resistance
5 against *C. difficile*.

6 Based on our previous work with UDCA,²⁶ we hypothesized that ursodiol would
7 *directly* inhibit different stages of the *C. difficile* life cycle, and ameliorate disease when
8 administered to mice in a model of CDI. Due to their detergent like properties, bile acids
9 can also directly alter the gut microbial composition, subsequently altering bile acid
10 metabolism and host physiology.⁴³ Gastrointestinal crosstalk between bile acids and the
11 gut microbiota is well recognized (reviewed in Wahlström et al.)⁴³, but the impact of
12 ursodiol on these interactions remains unknown. Similarly, the central role bile acids
13 play in regulation of the innate immune response is growing (reviewed in Fiorucci et
14 al.).⁴⁴ Because of this, we also investigated the *indirect* effects or the extent to which the
15 impact of ursodiol on CDI correlated with changes in the gut microbiota, bile acid
16 metabolome, and the host inflammatory response.

17 Here we show that ursodiol is able to significantly decrease *C. difficile* spore
18 germination, growth, and toxin activity in a dose-dependent manner *in vitro*, but not to
19 the same level *in vivo*. In *C. difficile* infected mice, ursodiol pretreatment resulted in
20 attenuation of CDI pathogenesis early in the course of disease, which coincided with
21 alterations in gut bile acids, the host inflammatory transcriptome, and gene expression
22 of bile acid activated receptors nuclear farnesoid X receptor (FXR) and transmembrane
23 G protein-coupled membrane receptor 5 (TGR5), which are able to modulate the innate

1 immune response through signaling pathways such as NF- κ B. Although ursodiol
2 pretreatment did not restore colonization resistance against *C. difficile*, it attenuated an
3 overly robust host inflammatory response that is detrimental to the host during CDI.
4 Together, these results suggest that ursodiol remains a viable non-antibiotic treatment
5 and/or prevention strategy against CDI by acting on the FXR-FGR axis via NF- κ B
6 signaling. Targeting the bile acid activated receptors, FXR and TGR5, represents a
7 novel approach for the development of therapies for the prevention and treatment of
8 CDI.

9 **Materials and Methods**

10 **Spore preparation.** *C. difficile* R20291 spores were prepared as described
11 previously.^{22,26,45} Briefly *C. difficile* R20291 were grown at 37°C anaerobically overnight
12 in 2 ml Columbia broth and then added to 40 ml culture of Clospore media in which it
13 was allowed to sporulate for 5–7 days. Spores were harvested by centrifugation, and
14 subjected to 3–5 washes with sterile cold water to ensure spore purity. Spore stocks
15 were stored at 4°C in sterile water until use.

16 ***C. difficile in vitro* spore germination and outgrowth assay.** The spore germination
17 and outgrowth assay performed was modified from Carlson et al., Theriot et al., and
18 Thanissery et al.^{22,26,46} Purified spores were enumerated and tested for purity before
19 use. The spore stock was resuspended in ultrapure water to achieve an initial spore
20 concentration of approximately 10⁶ spores/ml. The spore suspension was subjected to
21 heat treatment (65°C for 20 min) to eliminate any vegetative cells. The spores were then
22 plated on both brain heart infusion (BHI) with 100 mg/liter L-cysteine, and BHI media
23 supplemented with 0.1% TCA, and further incubated at 37°C for 24 hr. We observed a

1 lawn of *C. difficile* colonies on BHI media supplemented with 0.1% TCA, and no visible
2 growth on BHI media alone, which indicates that the spore suspension was devoid of
3 vegetative cells. Bile acid solutions containing four to five concentrations were used to
4 study a dose response [Ursodiol U.S.P (0.0076, 0.076, 0.76, and 7.64 mM, Spectrum
5 Chemical, CAS 128-13-2)]. Bile acids were dissolved in ethanol, passed into the
6 anaerobic chamber (Coy labs), and added to BHI broth with 0.1% TCA.
7 Chendeoxycholate (CDCA, Fisher Scientific, 50328656) at 0.04% is a known inhibitor of
8 TCA mediated spore germination, and was used as a negative control. TCA at 0.1%
9 made in water, and water and ethanol were used as positive controls. *C. difficile*
10 R20291 spores were added to BHI broth supplemented with and without TCA 0.1% and
11 bile acids at concentrations listed above, and allowed to incubate for 30 min at 37°C
12 anaerobically. Bacterial enumeration of the samples was performed on both BHI agar
13 (vegetative cells only) and BHI agar supplemented with 0.1% TCA (germinated spores
14 and vegetative cells). Percent germination was calculated as [(CFUs on BHI
15 supplemented with TCA + SBAs) / (CFUs on BHI supplemented with TCA alone)] × 100.
16 All measurements were performed in triplicate for each isolate and expressed as
17 percent germination.

18 ***C. difficile in vitro* growth kinetics assay.** The growth kinetic assay was modified
19 from Thanissery et al. and performed over a 24 hr period.²⁶ *C. difficile* R20291 was
20 cultured overnight at 37°C in BHI plus 100 mg/liter L-cysteine broth in an anaerobic
21 chamber. After 14 hr of growth, *C. difficile* R20291 was subcultured 1:10 and 1:5 into
22 BHI plus 100 mg/liter L-cysteine and allowed to grow for 3 hr anaerobically at 37°C. The
23 culture was then diluted in fresh BHI to a starting optical density at 600 nm (OD₆₀₀) of

1 0.01 within a conical tube at a final volume of 50 ml. Five concentrations of filter-
2 sterilized ursodiol (0.0076, 0.076, 0.76, 3.82, 7.64 mM dissolved in ethanol) were added
3 to each culture and anaerobically incubated for 72 hr at 37°C. The optical density was
4 monitored every 4 hours for 12 hr and then once at 24 hr. Cultures were inverted prior
5 to obtaining the optical density on a cell density meter (WPA Biowave). Due to optical
6 density interference from gelatin formation at 7.64 mM ursodiol, this concentration was
7 only assessed for viability.

8 To assess *C. difficile* R20291 viability, culture aliquots (100 µL) were taken at
9 each time point and enumerated on TBHI plates to obtain total colony forming units
10 (CFU)/mL, which represents total vegetative cells and spores of *C. difficile* R20291. At
11 each time point, a second culture aliquot (100 µL) was heat-treated at 65°C for 20 min
12 in order to eliminate all vegetative cells. This heat-treated culture aliquot was then
13 enumerated on TBHI plates to obtain total CFU/ml, which represents total spores only.

14 ***C. difficile* in vitro Vero cell cytotoxicity assay.** This protocol is modified from
15 Winston et al.⁴⁷ Vero cells were grown and maintained in DMEM media (Gibco
16 Laboratories, 11965-092) with 10% fetal bovine serum (Gibco Laboratories, 16140-071)
17 and 1% Penicillin streptomycin solution (Gibco Laboratories, 15070-063). Cells were
18 incubated with 0.25% trypsin (Gibco Laboratories, 25200-056) washed with 1X DMEM
19 media and harvested by centrifugation 1,000 RPM for 5 min. Cells were plated at 1 x
20 10⁴ cells per well in a 96-well flat bottom microtiter plate (Corning, 3596) and incubated
21 overnight at 37°C / 5% CO₂. Culture supernatants from the anaerobic growth assay
22 were defrosted on ice and three 200 µL aliquots from each treatment and time point
23 were then centrifuged at 1,750 RPM for 5 min to pellet vegetative *C. difficile*.

1 Supernatants were collected from each sample and 10-fold dilutions, to a maximum of
2 10^{-6} , were performed. Sample dilutions were incubated 1:1 with PBS (for all dilutions) or
3 antitoxin (performed for 10^{-1} and 10^{-4} dilutions only, TechLabs, T5000) for 40 min at
4 room temperature. Following incubation, these admixtures were added to the Vero cells
5 and plates were incubated overnight at 37°C / 5% CO_2 . Vero cells were viewed under
6 200X magnification for rounding after overnight incubation. The cytotoxic titer was
7 defined as the reciprocal of the highest dilution that produced rounding in 80% of Vero
8 cells for each sample. Vero cells treated with purified *C. difficile* toxins (A and B) and
9 antitoxin (List Biological Labs, 152C and 155C; TechLabs, T5000) were used as
10 controls.

11 **Ethics statement.** The Institutional Animal Care and Use Committee (IACUC) at North
12 Carolina State University College of Veterinary Medicine (NCSU) approved this study.
13 The NCSU Animal Care and Use policy applies standards and guidelines set forth in the
14 Animal Welfare Act and Health Research Extension Act of 1985. Laboratory animal
15 facilities at NCSU adhere to guidelines set forth in the Guide for the Care and Use of
16 Laboratory Animals. The animals' health statuses were assessed daily, and moribund
17 animals were humanely euthanized by CO_2 asphyxiation followed by secondary
18 measures (cervical dislocation). Trained animal technicians or a veterinarian performed
19 animal husbandry in an AAALAC-accredited facility during this study.

20 **Animals and housing.** C57BL/6J mice (females and males) were purchased from
21 Jackson Laboratories (Bar Harbor, ME) and quarantined for 1 week prior to starting the
22 antibiotic water administration to adapt to the new facilities and avoid stress-associated
23 responses. Following quarantine, the mice were housed with autoclaved food, bedding,

1 and water. Cage changes were performed weekly by laboratory staff in a laminar flow
2 hood. Mice had a 12 hr cycle of light and darkness.

3 **Ursodiol dosing experiment and sample collection.** Groups of 5 week old C57BL/6J
4 mice (male and female) were treated with ursodiol at three doses (50, 150, and 450
5 mg/kg dissolved in corn oil; Ursodiol U.S.P., Spectrum Chemical, CAS 128-13-2) given
6 daily via oral gavage for 14 days. Concurrently mice were administered cefoperazone
7 (0.5 mg/ml), a broad spectrum antibiotic, in their drinking *ad libitum* for 5 days. A control
8 group of mice were only administered cefoperazone in their drinking water *ad libitum* for
9 5 days. Two independent experiments were performed, with a total of n = 8
10 mice/treatment group. Fecal pellets were collected twice daily, flash-frozen and stored
11 at -80°C until further analysis.

12 **Murine model of *C. difficile* infection and sample collection.** Groups of 5 week old
13 C57BL/6J mice (male and female, n = 12 mice/treatment group) were given
14 cefoperazone (0.5 mg/ml), in their drinking *ad libitum* for 5 days followed by a 2-day
15 wash out with regular drinking water *ad libitum* (Fig. 3A). A group of cefoperazone
16 treated mice were pretreated with ursodiol (450 mg/kg dissolved in corn oil; Ursodiol
17 U.S.P., Spectrum Chemical, CAS 128-13-2) daily via oral gavage. Mice were then
18 challenged with 10⁵ spores of *C. difficile* R20291 via oral gavage at Day 0 (*C. diff* +
19 U450). Mice were monitored for weight loss and clinical signs of CDI (lethargy,
20 inappetence, diarrhea, and hunched posture) for 7 days post challenge. Fecal pellets
21 were collected daily for *C. difficile* enumeration. Controls for this experiment included
22 cefoperazone treated mice, no ursodiol, challenged with *C. diff* (*C. diff* only), and
23 cefoperazone treated mice pretreated with the corn oil vehicle (equivalent volume, 75

1 μ l) daily via oral gavage and then challenged with 10^5 spores of *C. difficile* R20291 via
2 oral gavage at Day 0 (*C. diff* + corn oil). Uninfected controls included cefoperazone
3 treated mice with (Cef only), and with pretreatment of ursodiol (450 mg/kg dissolved in
4 corn oil; Cef + U450). Animals were euthanized after losing 20% of initial baseline
5 weight or after developing any serve clinical signs noted above.

6 Necropsy was performed on days 2, 4, and 7 post challenge. Contents and
7 tissue from the cecum and colon were collected immediately at necropsy, flash frozen in
8 liquid nitrogen, and stored at -80°C until further analysis. To prevent RNA degradation,
9 snips of cecal and colonic tissue were also stored in RNA-Later at 80°C until further
10 analysis. Cecum and colon were prepared for histology by placing intact tissue into
11 histology cassettes and stored in 10% neutral buffered formalin at a ratio of 1:10
12 tissue:fixative at room temperature for 72 hr then transferred to room temperature 70%
13 ethyl alcohol. Tissues were processed, paraffin embedded, sectioned at $4\ \mu\text{m}$ thick, and
14 stained by routine hematoxylin and eosin for histopathological examination (LCCC
15 Animal Histopathology Core Facility at the University of North Carolina at Chapel Hill).
16 Histopathologic evaluation was conducted by a board-certified veterinary pathologist
17 (S. Montgomery) in an *a priori* blinded manner in which individuals within groups were
18 masked at time of scoring. For tissue scoring, a 0-4 numerical scoring system was
19 employed to separately assess edema, inflammatory cell infiltration, and epithelial cell
20 damage in the cecum and colon based upon a previously published scoring scheme.⁴⁸

21 **Bile acid quantitation by UPLC-MS/MS of murine cecal and fecal content.** Targeted
22 analysis of bile acids on fecal pellets ($n = 3$ mice/treatment group were selected) were
23 performed with an ACQUITY ultraperformance liquid-chromatography system using a

1 C8 BEH column (2.1 × 100 mm, 1.7 μm) coupled with a Xevo TQ-S triplequadrupole
2 mass spectrometer equipped with an electrospray ionization (ESI) source operating in
3 negative ionization mode (All Waters, Milford, MA) as previously described.⁴⁹ The
4 sample was thawed on ice and 25 mg was added to 1 mL of pre-cooled methanol
5 containing 0.5 μM stable-isotope-labeled bile acids as internal standards (IS), followed
6 by homogenization (Precellys, Bertin Technologies, Rockville, MD) with 1.0 mm
7 diameter zirconia/silica beads (BioSpec, Bartlesville, OK) and centrifugation (Eppendorf,
8 Hamburg, Germany) at 13 200g, 4 °C, and 20 min. 200 μl of the supernatant was
9 transferred to an autosampler vial. Following centrifugation, the supernatant was
10 transferred to an autosampler vial for quantitation. Bile acids were detected by either
11 multiple reaction monitoring (MRM) (for conjugated bile acid) or selected ion monitoring
12 (SIM) (for non-conjugated bile acid). MS methods were developed by infusing individual
13 bile acid standards. Calibration curves were used to quantify the biological
14 concentration of bile acids. Bile acid quantitation was performed at Penn State
15 University.

16 Heatmaps and box and whisker plots of bile acid concentrations, and nonmetric
17 multidimensional scaling (NMDS) depicting the dissimilarity indices via Horn distances
18 between bile acid profiles were generated using R packages (<http://www.R-project.org>).

19 **Illumina MiSeq sequencing of bacterial communities.** Microbial DNA was extracted
20 from murine feces (n = 3 mice/treatment group were selected) using the PowerSoil-htp
21 96-well soil DNA isolation kit (Mo Bio Laboratories, Inc.). The V4 region of the 16S
22 rRNA gene was amplified from each sample using a dual-indexing sequencing
23 strategy.⁵⁰ Each 20 μl PCR mixture contained 2 μl of 10× Accuprime PCR buffer II (Life

1 Technologies), 0.15 μ l of Accuprime high-fidelity Taq (catalog no. 12346094) high-
2 fidelity DNA polymerase (Life Technologies), 2 μ l of a 4.0 μ M primer set, 1 μ l DNA, and
3 11.85 μ l sterile double-distilled water (ddH₂O) (free of DNA, RNase, and DNase
4 contamination). The template DNA concentration was 1 to 10 ng/ μ l for a high bacterial
5 DNA/host DNA ratio. PCR was performed under the following conditions: 2 min at 95°C,
6 followed by 30 cycles of 95°C for 20 sec, 55°C for 15 sec, and 72°C for 5 min, followed
7 by 72°C for 10 min. Each 20 μ l PCR mixture contained 2 μ l of 10 \times Accuprime PCR
8 buffer II (Life Technologies), 0.15 μ l of Accuprime high-fidelity Taq (catalog no.
9 12346094) high-fidelity DNA polymerase (Life Technologies), 2 μ l of 4.0 μ M primer set,
10 1 μ l DNA, and 11.85 μ l sterile ddH₂O (free of DNA, RNase, and DNase contamination).
11 The template DNA concentration was 1 to 10 ng/ μ l for a high bacterial DNA/host DNA
12 ratio. PCR was performed under the following conditions: 2 min at 95°C, followed by 20
13 cycles of 95°C for 20 sec, 60°C for 15 sec, and 72°C for 5 min (with a 0.3°C increase of
14 the 60°C annealing temperature each cycle), followed by 20 cycles of 95°C for 20 sec,
15 55°C for 15 sec, and 72°C for 5 min, followed by 72°C for 10 min. Libraries were
16 normalized using a Life Technologies SequelPrep normalization plate kit (catalog no.
17 A10510-01) following the manufacturer's protocol. The concentration of the pooled
18 samples was determined using the Kapa Biosystems library quantification kit for
19 Illumina platforms (KapaBiosystems KK4854). The sizes of the amplicons in the library
20 were determined using the Agilent Bioanalyzer high-sensitivity DNA analysis kit (catalog
21 no. 5067-4626). The final library consisted of equal molar amounts from each of the
22 plates, normalized to the pooled plate at the lowest concentration.

1 Sequencing was done on the Illumina MiSeq platform, using a MiSeq reagent kit
2 V2 with 500 cycles (catalog no. MS-102-2003) according to the manufacturer's
3 instructions, with modifications.⁵⁰ Libraries were prepared according to Illumina's
4 protocol for preparing libraries for sequencing on the MiSeq (part 15039740 Rev. D) for
5 2 or 4 nM libraries. The final load concentration was 4 pM (but it can be up to 8 pM) with
6 a 10% PhiX spike to add diversity. Sequencing reagents were prepared according to
7 Illumina's protocol for 16S sequencing with the Illumina MiSeq personal sequencer.⁵⁰
8 (Updated versions of this protocol can be found at
9 http://www.mothur.org/wiki/MiSeq_SOP.) Custom read 1, read 2, and index primers
10 were added to the reagent cartridge, and FASTQ files were generated for paired-end
11 reads.

12 **Microbiome analysis.** Analysis of the V4 region of the 16S rRNA gene was done using
13 mothur (version 1.40.1).^{50,51} Briefly, the standard operating procedure (SOP) at
14 http://www.mothur.org/wiki/MiSeq_SOP was followed to process the MiSeq data. The
15 paired-end reads were assembled into contigs and then aligned to the SILVA 16S rRNA
16 sequence database (release 132)^{52,53} and were classified to the mothur-adapted RDP
17 training set v16⁵⁴ using the Wang method and an 80% bootstrap minimum to the family
18 taxonomic level. All samples with <500 sequences were removed. Chimeric sequences
19 were removed using UCHIME.⁵⁵ Sequences were clustered into operational taxonomic
20 units (OTU) using a 3% species-level definition. The OTU data were then filtered to
21 include only those OTU that made up 1% or more of the total sequences. The
22 percentage of relative abundance of bacterial phyla and family members in each sample
23 was calculated. A cutoff of 0.03 (97%) was used to define operational taxonomic units

1 (OTU) and Yue and Clayton dissimilarity metric (θ_{YC}) was utilized to assess beta
2 diversity. Standard packages in R were used to create NMDS ordination on serial fecal
3 samples.

4 **mRNA isolation and gene expression analysis.** RNA extraction was performed on
5 cecal and colonic tissue using Zymo Research RNA extraction kit (Irvine, CA). After
6 RNA extraction, samples were quantified using an Invitrogen Qubit (Thermo Fisher
7 Scientific) and run on an Agilent Bioanalyzer NanoChip (Santa Clara, CA) to assess
8 quality of RNA. RNA extraction, quantification, and purity assessment were performed
9 at the Microbiome Shared Resource Lab, Duke University, Durham, NC.

10 The nCounter Mouse Inflammation v2 gene expression panel and custom code
11 set was purchased from NanoString Technologies (Seattle, WA) and consists of 254
12 inflammation-related mouse genes including six internal reference genes (see Table S1)
13 and 10 custom genes of interest (see Table S2). The nCounter assay was performed
14 using 100 ng of total RNA. Hybridization reactions were performed according to the
15 manufacturer's instructions with 5 μ l diluted sample preparation reaction and samples
16 were hybridized overnight. Hybridized reactions were purified using the nCounter Prep
17 Station (NanoString Technologies) and data collection was performed on the nCounter
18 Digital Analyzer (NanoString Technologies) following the manufacturer's instructions to
19 count targets. Nanostring nCounter Max platform was utilized at UNC Translational
20 Genomics Laboratory to perform these assays.

21 The raw data was normalized to six internal reference genes within each tissue
22 type with the lowest coefficient of variation using nSolver software following the
23 manufacturer's instructions (Nanostring Technologies). Using the nSolver Advanced

1 analysis software, after pre-processing and normalization counts were \log_2 transformed
2 and z-scores were calculated (Nanostring Technologies).

3 To identify highly significant genes and molecular pathways, the gene set was
4 subsequently uploaded to Ingenuity Pathway Analysis (IPA) database (Qiagen,
5 Redwood City, CA) and analyzed in the context of known biological response and
6 regulatory networks. IPA was also used to assess gene expression fold changes
7 comparing ursodiol pretreated CDI mice to untreated CDI mice.

8 **Statistical analysis.** Statistical tests were performed using Prism version 7.0b for Mac
9 OS X (GraphPad Software, La Jolla California USA) or using R packages ([http://www.R-](http://www.R-project.org)
10 [project.org](http://www.R-project.org)). Statistical significance was set at a p value of < 0.05 for all analyses (*, p
11 < 0.05 ; **, p < 0.01 ; ***, p < 0.001 ; ****, p < 0.0001). For *in vitro* experiments,
12 significance between treatments in spore germination was calculated by Student's
13 parametric t-test with Welch's correction. Two-way analysis of variance (ANOVA) test
14 followed by Dunnett's multiple comparisons *post hoc* test was used to calculate
15 significance between treatments for the anaerobic growth assay and toxin activity
16 assay.

17 For the *in vivo* experiments, a two-way ANOVA followed by Tukey's multiple
18 comparisons *post hoc* test was used to calculate significance between treatment groups
19 in bile acid profiles, individual bile acids, *C. difficile* load (CFU/g) cecal content, and total
20 histologic score. A two-way ANOVA followed by Sidak's multiple comparisons *post hoc*
21 test was used to calculate significance between treatment groups in baseline weight
22 loss. A Student's t-test corrected for multiple comparisons using the Holm-Sidak method
23 was used to calculate significance between treatment groups in cecal content toxin

1 activity and edema score. Analysis of molecular variance (AMOVA) was used to detect
2 significant microbial community clustering of treatment groups in NMDS plots.⁵⁶ nSolver
3 software and IPA were used to calculate significance in gene expression fold change
4 comparing mice pretreated with ursodiol to untreated mice.

5 **Availability of data and material.** Raw 16S sequences have been deposited in the
6 Sequence Read Archive (SRA) with SRA accession number: SUB6809988. All other
7 data including bile acid metabolomics and Nanostrings expression data is provided in
8 Tables S3-S7 in the supplemental material.

9 **Results**

10 **Ursodiol directly inhibits different stages of the *C. difficile* life cycle *in vitro*.**

11 To determine the direct effects of ursodiol on the life cycle of clinically relevant *C.*
12 *difficile* strain R20291, *in vitro* assays to assess spore germination and outgrowth,
13 growth, and toxin activity were performed (Fig. 1). Taurocholic acid (TCA) mediated
14 spore germination and outgrowth of *C. difficile* was significantly inhibited by ursodiol in a
15 dose dependent manner (Fig. 1A, blue bars). No significant difference was observed
16 between positive controls, sterile ethanol, and water supplemented with 0.1% TCA (Fig.
17 1A, ethanol represented as solid grey bar). As expected, the negative control, 0.04%
18 chenodeoxycholic acid (CDCA) supplemented with 0.1% TCA resulted in significant
19 inhibition of spore germination and outgrowth (Fig. 1A, red bar). CDCA is a known
20 inhibitor of spore germination.⁵⁷

21 *C. difficile* growth decreased when exposed to ursodiol at 8, 12 and 24 hr in a
22 dose dependent manner compared to the ethanol control (Fig. 1B, blue lines). For the
23 ursodiol 3.82 mM concentration, a significantly lower optical density (OD) compared to

1 the ethanol control was observed at time points 8, 12, and 24 hr ($p = 0.0316$, $p =$
2 0.0001 , $p = 0.0001$, respectively). For the ursodiol 0.76 mM treatment, a significantly
3 lower OD compared to ethanol control was observed at time points 12 and 24 hr ($p =$
4 0.0001 for both time points), and for the ursodiol 0.076 mM treatment at time point 24 hr
5 ($p = 0.0208$). No significant difference in OD between the ethanol control and treatments
6 was noted at time point 0 or 4 hr. The highest concentration of ursodiol (7.64 mM)
7 interfered with OD measurement and was not included in the analysis.

8 Since OD measurements do not capture viability and spore formation of *C.*
9 *difficile*, we also did differential plating to evaluate the colony forming units (CFU)/ml at
10 each time point (Fig. S1). At 24 hr ursodiol inhibited *C. difficile* total vegetative cells and
11 spores compared to the ethanol control. The highest concentration of ursodiol (7.64
12 mM) had a significant and immediate inhibitory effect on *C. difficile* viability compared to
13 ethanol at time 0 hr. Ursodiol treatment also significantly decreased *C. difficile* spores.

14 Toxin activity was not detected until eight hours of *C. difficile* growth (Fig. 1C).
15 Higher concentrations of ursodiol resulted in significant decreases in *C. difficile* toxin
16 activity compared to the ethanol control at 8, 12, and 24 hr (Fig. 1C, blue bars). Using a
17 Spearman's correlation (r) the CFU/ml and toxin activity at 8 and 12 hr were significantly
18 correlated (8 hr, $r = 0.6399$, $p = 0.0042$; 12 hr, $r = 0.6981$, $p = 0.0013$), suggesting low
19 toxin activity is due to decreased growth.

20 **Ursodiol alters the fecal bile acid metabolome with minimal change to the** 21 **microbiota.**

22 Since the gut microbiota and bile acids mediate colonization resistance against *C.*
23 *difficile*, we aimed to determine how different doses of ursodiol administration alone

1 impacts them in our antibiotic treated mouse model. C57BL/6J mice were administered
2 three doses of ursodiol (50, 150, 450 mg/kg/day) via oral gavage for 14 days (Fig. 2A).
3 Mice were also concurrently administered cefoperazone (in drinking water *ad libitum*) for
4 5 days, in order to mimic the CDI mouse model. Paired fecal samples were collected
5 from the same mice serially over the 14 day period to evaluate how ursodiol affected the
6 gut microbial community structure and bile acid metabolome.

7 The fecal microbial community structures of all treatment groups were
8 significantly different from pretreatment at all time points, based on an analysis of
9 molecular variance (AMOVA) performed for each day (Fig. 2B). There were no
10 significant differences between the fecal microbial community structures based on
11 ursodiol pretreatment. The shifts in the fecal microbiota seen in the NMDS were
12 predominantly due to the cefoperazone treatment and experiment day, and not as a
13 result of the ursodiol pretreatment.

14 Targeted bile acid analysis via UPLC-MS/MS detected 38 bile acids in the feces
15 of mice. During the first 9 days of the experiment (Day -2 to Day 7), the fecal bile acid
16 profiles of mice cluster based on the dose of ursodiol administered (Fig. 2C). Ursodiol
17 dramatically alters the fecal bile acid metabolome compared to pretreatment as seen in
18 Fig. 2D and Table S3. Bile acids that significantly changed over the course of the
19 experiment in mice that received ursodiol compared to mice that only received
20 cefoperazone included T β MCA, TCA, TUDCA, β MCA, and UDCA (Fig. S2, two-way
21 ANOVA with Tukey's multiple comparisons *post hoc* performed on each experiment
22 day, p values ranged from p <0.0001 to p = 0.0012).

23 **Ursodiol attenuates disease in mice early during *C. difficile* infection.**

1 Due to potent inhibitory effect of ursodiol on *C. difficile in vitro*, and increased
2 gastrointestinal bile acids *in vivo*, we sought to determine the extent to which
3 pretreatment with ursodiol influences the natural course of CDI in a reproducible mouse
4 model. Since supra-physiologic UDCA intestinal concentrations were achieved in Fig.
5 2D when mice were dosed with 450 mg/kg/day of ursodiol, we selected this dose.⁵⁸
6 All C57BL/6J mice in each group received cefoperazone in their drinking water from
7 Day -7 to Day -2 with a two day washout on normal drinking water. Control groups
8 included two uninfected groups that received cefoperazone no ursodiol (Cef only), and
9 cefoperazone with ursodiol (Cef + U450) (Fig. 3A). An additional control group received
10 cefoperazone no ursodiol, with *C. difficile* challenge (*C. diff* only). Mice were orally
11 gavaged with ursodiol (450 mg/kg in corn oil vehicle) daily for 7 days prior to challenge
12 with *C. difficile* on Day 0, and throughout the infection (*C. diff* + U450) (Fig. 3A).

13 Two groups of mice (*C. diff* only and *C. diff* + U450) were challenged with 10^5
14 spores of *C. difficile* R20291 on Day 0 and within 24 hr were colonized with greater than
15 10^7 CFUs per gram of feces (data not shown). Mice challenged with *C. difficile* with and
16 without ursodiol pretreatment lost a significant amount of their baseline weight by Day 2
17 post challenge (*C. diff* only, red circles and *C. diff* + U450, blue circles Fig. 3B).
18 Additionally, there was no significant difference in weight loss between these groups
19 over the course of the experiment (based on two-way ANOVA). However, mice
20 pretreated with ursodiol (*C. diff* + U450) had a 24-36 hr delay in clinical signs (lethargy,
21 inappetence, diarrhea, and hunched posture) related to CDI compared to untreated
22 mice (*C. diff* only). Uninfected control mice (Cef only and Cef + U450) revealed no
23 clinical signs of disease during the experiment and remained *C. difficile* free. The Cef

1 only group of mice displayed weight gain over the course of the experiment as expected
2 (Fig. 3B, gray circles). The Cef + U450 group did have significant weight loss compared
3 to Cef only mice at Day 5 and Day 6 of the experiment (two-way ANOVA with Sidak's
4 multiple comparisons *post hoc* test; $p = 0.0013$, $p = 0.0001$ respectively; Fig. 3B, orange
5 circles). However, no significant difference was noted between these groups by Day 7
6 (Fig. 3B). An additional control group of mice challenged with *C. difficile* and pretreated
7 with the vehicle corn oil alone (*C. diff* + corn oil) displayed no significant difference in
8 disease outcome compared to the *C. diff* only group (Fig. S3).

9 Mice pretreated with ursodiol (*C. diff* + U450) had equivalent total cecal bacterial
10 loads of *C. difficile* (CFU/g cecal content) compared to untreated mice (*C. diff* only) (Fig.
11 3C). However, mice pretreated with ursodiol had a significant reduction in *C. difficile*
12 spores within cecal content at Day 2 compared to untreated mice (log transferred CFU/g
13 content, two-way ANOVA with Tukey's multiple comparisons *post hoc* test, $p < 0.0001$;
14 Fig. 3C). Mice ceca remained persistently colonized with *C. difficile* throughout the 7
15 day experiment. At Day 4, *C. difficile* toxin activity within cecal content was significantly
16 reduced in ursodiol pretreated compared to untreated mice (Student's t- test corrected
17 with the Holm-Sidak method for multiple comparisons, $p = 0.014$; Fig. 3D).

18 Histopathologic evaluation of cecal and colonic tissue revealed no significant
19 difference in total histologic score between ursodiol pretreated and untreated mice (Fig.
20 S4). When assessing individual parameters, there was no significant difference between
21 epithelial damage or inflammatory infiltrate between the treatment groups. At Day 2,
22 mice pretreated with ursodiol had a significant reduction in edema score (scored 0-4) in
23 both cecal and colonic tissue compared to untreated mice (Student's t- test corrected

1 with the Holm-Sidak method for multiple comparisons, $p = 0.00025$, $p = 0.0049$
2 respectively; Fig. 3E and F).

3 **Ursodiol alters the host inflammatory transcriptome early during CDI.**

4 Since there were minimal changes in the *C. difficile* life cycle early during infection to
5 explain the decrease in edema, we wanted to examine the impact of ursodiol
6 pretreatment on the host inflammatory response during CDI. We interrogated
7 transcriptional changes related to the cecal and colonic inflammatory response at Day 2
8 post challenge using a murine specific panel of inflammatory markers (Nanostring
9 nCounter) that included 264 transcripts. After processing the raw data, mRNA log₂
10 normalized z-scores for 261 genes were obtained and genes significantly altered by
11 ursodiol pretreatment compared to untreated mice with CDI were evaluated. Ursodiol
12 mediated alterations in gene expression fold change were different between cecal and
13 colonic tissue (Fig. 4 and Fig. S5). In cecal tissue, 173 genes had a significant
14 expression fold change when comparing ursodiol pretreated mice to untreated mice,
15 with increased expression in 74 genes and decreased expression in 99 genes (Fig. 4A
16 and B, Table S4 and S5). In colonic tissue, 139 genes had a significant expression fold
17 change when comparing ursodiol pretreated to untreated mice, with increased
18 expression in 58 genes and decreased expression in 81 genes (Fig. S5A and B, Table
19 S4 and S5).

20 Ingenuity Pathway Analysis (IPA) identified NF- κ B signaling as an inflammatory
21 canonical pathway in both cecum and colon significantly altered in ursodiol pretreated
22 mice ($p < 0.0001$ for both tissues). Based on unsupervised clustering, genes involved
23 with NF- κ B signaling in ursodiol pretreated mice clustered distinctly and separately from

1 untreated mice in both cecal and colonic tissue (Fig. 4C and Fig. S5C). Differences in
2 individual gene expression fold changes were noted in NF- κ B signaling between the
3 cecal and colonic tissue (Fig. 4D and Fig. S5D). In both tissues, components important
4 for mediating the host inflammatory response to CDI, such as IL-1R1 and various TLRs,
5 were significantly decreased in expression in the ursodiol pretreated mice compared to
6 untreated mice at Day 2 (Fig. 4D and Fig. S5D, see Table S6 for p-values). Collectively,
7 these results suggest that ursodiol pretreatment in CDI mice alters the inflammatory
8 transcriptome in cecal and colonic tissue by impacting essential host inflammatory
9 signaling pathways, such as NF- κ B, early in the course of CDI.

10 **Ursodiol induces bile acid transport and signaling via FXR/FGF15 pathway.**

11 Since ursodiol treatment alters the bile acid metabolome in mice (Fig. 2D) and bile acids
12 are known to modulate signaling via the nuclear bile acid receptor FXR and the plasma
13 membrane receptor TGR5⁵⁹, we sought to define transcriptional changes in bile acid
14 transport and signaling via the FXR/FGF15 pathway. We selected seven genes in the
15 FXR/FGF15 pathway to interrogate within cecal and colonic tissue using a customized
16 murine panel (Nanostring nCounter). Bile acids within the gastrointestinal tract are
17 transported back to the liver via enterohepatic recirculation by several active
18 transporters, ASBT on the apical surface, and OST α/β on the basolateral surface of
19 enterocytes (Fig. 5C)⁵⁹. Cytoplasmic Ibabp helps to facilitate bile acid transport (Fig.
20 5C)⁵⁹. Bile acids in ileal enterocytes and colonic L cells bind to FXR and activate the
21 FXR-RXR heterodimer complex, resulting in transcription of target genes, such as
22 FGF15/19. FGF15/19 is secreted into the portal vein and transported back to the liver,

1 where it inhibits expression of *CYP7A1* in hepatocytes to directly regulate bile acid
2 synthesis.⁵⁹

3 In cecal tissue, mRNA expression of the receptors FXR and TGR5, and the bile
4 acid transporters ASBT and OST α/β were significantly increased in ursodiol pretreated
5 mice compared to untreated mice at Day 2 post challenge (Fig. 5A, Student's
6 parametric t-test with Welch's correction, see Table S7 for p values). In colonic tissue,
7 mRNA expression of the receptors FXR and TGR5, the signaling molecule FGF15, and
8 the bile acid transporters ASBT and OST α/β were significantly increased in ursodiol
9 pretreated mice compared to untreated mice (Fig. 5B, Student's parametric t-test with
10 Welch's correction, see Table S7 for p values). Collectively, these results demonstrate
11 that pretreatment of ursodiol in mice with CDI increases the expression of the
12 FXR/FGF15 pathway transcriptome in both cecal and colonic tissue.

13 **Discussion**

14 Ursodiol, the FDA approved commercial formulation of UDCA, is used to treat a variety
15 of hepatic and biliary diseases including: cholesterol gallstones, primary biliary cirrhosis,
16 primary sclerosing cirrhosis, cholangitis, non-alcoholic fatty liver disease, chronic viral
17 hepatitis C, and cholestasis of pregnancy.³¹⁻⁴⁰ However, limited evidence is available for
18 use of ursodiol in CDI patients. To date, only a single case report demonstrating
19 successful prevention of recurrent *C. difficile* ileal pouchitis with oral UDCA
20 administration is available,²⁹ suggesting that exogenous administration of the secondary
21 bile acid UDCA may be able to restore colonization resistance against *C. difficile in*
22 *vivo*.²⁹ Although the mechanisms by which ursodiol exerts its impact on *C. difficile* are
23 unknown, off-label use of ursodiol is currently in Phase 4 clinical trials for the prevention

1 of recurrent CDI.⁴¹ In the present study, ursodiol pretreatment impacted the life cycle of
2 *C. difficile* *in vivo*. Although, *C. difficile* bacterial load and toxin activity was not affected
3 at day 2, pretreatment with ursodiol resulted in a 24-36 hr delay in the clinical course of
4 CDI, suggesting attenuation of CDI pathogenesis early during infection. Although
5 ursodiol pretreatment did not alter the gut microbiota composition, significant
6 modifications in the bile acid metabolome were observed. Several key bile acid species
7 were significantly increased following ursodiol administration, namely UDCA, TUDCA,
8 and T β MCA. The tissue inflammatory transcriptome was altered, including gene
9 expression of bile acid activated receptors nuclear farnesoid X receptor (FXR) and
10 transmembrane G protein-coupled membrane receptor 5 (TGR5), which are able to
11 modulate the innate immune response through signaling pathways such as nuclear
12 factor κ -B (NF- κ B). This is the first study to investigate the impact of innate immune
13 response regulation by bile acid activated receptors, FXR and/or TGR5, during CDI.

14 Modulation of bile acid pools to restore colonization resistance against *C. difficile*
15 is a rational therapeutic option, based on the importance bile acids play in the life cycle
16 of *C. difficile*.^{25,27} Traditional therapeutic approaches to CDI have focused on restoring
17 bile acid pools by reestablishing the entire microbial community, such as with fecal
18 microbiota transplantation.^{13,28} Or supplementation of key commensals, such as
19 *Clostridium scindens*, a bacterium capable of synthesizing microbial derived secondary
20 bile acids, which when supplemented leads to partial restoration of colonization
21 resistance against *C. difficile*.¹¹ In the present study, we deduced that direct
22 supplementation of bile acids, such as UDCA or ursodiol which are known to inhibit the
23 life cycle of *C. difficile*,²⁶ may be sufficient to restore colonization resistance against this

1 enteric pathogen by directly impacting *C. difficile*, but also by modulating the host
2 inflammatory response.

3 The host innate immune response to CDI is an essential component of *C. difficile*
4 pathogenesis.¹ The acute host innate immune response is initiated by *C. difficile* toxin-
5 mediated damage to the intestinal epithelial cells, loss of the epithelial barrier, and
6 subsequent translocation of luminal gut microbes.^{1,60} *C. difficile* toxin A (TcdA) and toxin
7 B (TcdB), directly disrupt the cytoskeleton of intestinal epithelial cells, resulting in
8 disassociation of tight junctions and loss of epithelial integrity.^{1,61} In response to toxin-
9 mediated destruction, resident immune cells and intoxicated epithelial cells release pro-
10 inflammatory cytokines and chemokines to recruited circulating innate immune cells and
11 adaptive immune cells (reviewed in Abt et al.).¹ A prominent inflammatory pathway that
12 is activated in intestinal epithelial cells by TcdA and TcdB is the NF κ -B signaling
13 pathway through the phosphorylation of mitogen-activated protein kinase (MAPK),
14 which leads to the transcription of pro-inflammatory cytokines, including IFN- γ , TNF- α ,
15 IL-6, and IL-1 β .⁶²⁻⁶⁵ In the present study, pretreatment with ursodiol resulted in
16 alterations in the host intestinal inflammatory transcriptome. Genes involved with NF- κ B
17 signaling pathway in both cecum and colon, segregated based on ursodiol
18 pretreatment. This is not surprising given the broad ursodiol mediated alterations in bile
19 acid metabolome observed and the building evidence that bile acid activated receptors
20 regulate the host innate immune response (recently reviewed in Fiorucci et al. 2018).⁴⁴
21 In particular, interactions between the gut microbiota and bile acids result in alterations
22 in bile acid pools, which in turn modulate signaling via the FXR and TGR5, both of
23 which can regulate the innate immune response.^{44,59,66} Bile acid activation of TGR5

1 involves cAMP-protein kinase A (PKA)-mediated inhibition of NF- κ B, and bile acid
2 activation of FXR leads to FXR-nuclear receptor corepressor 1 (NCoR1)-mediated
3 repression of NF- κ B responsive elements (NRE). Bile acid activation of both pathways
4 blunt NF- κ B gene transcription including pro-inflammatory cytokines IFN- γ , TNF- α , IL-6,
5 and IL-1 β .^{67,68} Additionally, TGR5 directly regulates IL-10 expression by a cAMP/PKA/p
6 CAMP-responsive element binding protein pathway, thus further attenuating the
7 inflammatory response.⁶⁹

8 Bile acids differ in their affinity and agonistic/antagonistic effects for FXR and
9 TGR5.⁴⁴ We observed global alterations in bile acids that are both FXR and TGR5
10 agonists and antagonists, lending to the complexity of unravelling the cumulative effects
11 on bile acid activated receptors and how this impacts the overall innate immune
12 response during CDI. Based on FXR and TGR5 signaling transcriptomics, ursodiol
13 pretreatment in the present study resulted in increased cecal and colonic expression of
14 genes in these pathways during CDI. Collectively, this suggests that ursodiol induced
15 alterations in the bile acid metabolome results in alterations of host inflammatory
16 transcriptome, including alterations in NF- κ B signaling pathway, likely via the bile acid
17 activated receptors FXR and TGR5. Furthermore, this principle has also recently been
18 demonstrated in recurrent CDI patients receiving FMTs, where effectiveness of FMT
19 was associated with an upregulation of the FXR-FGF pathway.⁷⁰

20 Whilst the present study demonstrated ursodiol-mediated attenuation of CDI
21 early in the disease course, complete restoration of colonization resistance against this
22 enteric pathogen was not achieved. Several limitations of the use of ursodiol
23 pretreatment prior to CDI need to be considered. First, once daily dosing may be

1 sufficient to obtain supraphysiologic pulses of UDCA, but these concentrations may not
2 be sustained in the gastrointestinal tract throughout the day. Consequently, *C. difficile* *in*
3 *vivo* is exposed to a daily pulse of ursodiol that inhibits its life cycle, similar to our *in vitro*
4 results, only during a limited window. Alterations in the dosing scheme such as dividing
5 the dose over two or four administration times may ameliorate this potential limitation.
6 Secondly, alterations in the inflammatory microenvironment during the initial phases of
7 CDI were observed in this study, however these effects were not sustained throughout
8 CDI. Potentially, longer pretreatment with ursodiol is needed to result in sustained
9 alterations to the host innate immune response. Lastly, ursodiol may be more effective
10 as a preventative for relapsing CDI, meaning that following successful treatment of CDI
11 with antibiotic treatment ursodiol could be administered to prevent relapse of infection.

12 Regardless, our results highlight that ursodiol pretreatment was able to
13 significantly alter the bile acid metabolome and host inflammatory transcriptome during
14 CDI. Although ursodiol pretreatment did not result in colonization resistance against *C.*
15 *difficile*, attenuated pathogenesis of *C. difficile* was noted early in the disease course.
16 Collectively, we theorize that ursodiol induced alterations within the intestinal bile acid
17 metabolome result in activation of bile acid receptors, such as FXR and TGR5, which
18 are able to modulate the innate immune response through signaling pathways such as
19 NF- κ B, thus mitigating an overly robust host inflammatory response that can be
20 detrimental to the host. Given these results and the effectiveness of ursodiol to inhibit
21 the *C. difficile* life cycle, ursodiol remains a viable non-antibiotic treatment and/or
22 prevention strategy against CDI. Moreover, targeting bile acid activated receptors, FXR

1 and TGR5, to attenuate an overly robust detrimental host immune response during CDI
2 merits further consideration as a potential therapeutic intervention for CDI patients.

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13 Medicine.

14 **Disclosure statement.**

15 CMT is a scientific advisor to Locus Biosciences, a company engaged in the
16 development of antimicrobial technologies. CMT is a consultant for Vedanta
17 Biosciences and Summit Therapeutics.

18 **Figure legends**

19 **Figure 1: Ursodiol inhibits different stages of the *C. difficile* life cycle.** *In vitro*
20 assay to assess if the addition of ursodiol (0.0076, 0.076, 0.76, and 7.64 mM) **(A)**
21 inhibits *C. difficile* R20291 TCA-mediated spore germination and outgrowth compared to
22 positive control, ethanol (gray bar) and negative control, 0.04% CDCA (red bar). The
23 data presented represents triplicate experiments. Statistical significance between

1 treatment groups and the positive controls was determined by Student's parametric t-
2 test with Welch's correction. **(B)** alters the growth kinetics of *C. difficile* over a 24 hr
3 period. Growth curves were done in BHI media with ethanol (gray line) and addition of
4 varying concentrations of ursodiol (blue lines). The data presented represents $OD_{600} \pm$
5 SEM from triplicate experiments. **(C)** Culture supernatants, taken throughout a 24 hr
6 growth curve in 1B, were used for a Vero cell cytotoxicity assay and the data is
7 expressed as \log_{10} reciprocal dilution toxin per 100 μ L of *C. difficile* culture supernatant.
8 The data presented represents triplicate experiments. Statistical significance between
9 treatment groups and the positive controls was determined by a two-way ANOVA with
10 Dunnett's multiple comparisons (*, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$).

11 **Figure 2: Ursodiol does not alter the gut microbiota but does alter the bile acid**
12 **metabolome.** Groups of C57BL/6J mice (n=8 mice/treatment group) were **(A)** given
13 cefoperazone in their drinking water ad libitum for 5 days alone and or with ursodiol at
14 three doses (50, 150, and 450 mg/kg) given daily via oral gavage for 14 days. An n = 3
15 mice/treatment group were selected for further microbiome and bile acid metabolomic
16 analysis. **(B)** NMDS ordination of the fecal microbial communities from mice treated and
17 untreated with ursodiol was calculated from Yue and Clayton dissimilarity metric (θ_{YC})
18 on OTU at a 97% cutoff. **(C)** NMDS ordination illustrates dissimilarity indices via Horn
19 distances between the bile acid profiles of fecal samples from treated and untreated
20 mice. **(D)** A heatmap of bile acid concentrations present in nanomoles (nmol) per gram
21 of feces, ranging from 0 to 38221.91 (nmol/g feces). Asterisks next to bile acids
22 represent ones that were significantly altered during ursodiol treatment.

23

1 **Figure 3: Ursodiol attenuates disease early during *C. difficile* infection. (A)**
2 Groups of C57BL/6J mice (n=12 mice/treatment group) were given cefoperazone in
3 their drinking water *ad libitum* for 5 days alone and or with ursodiol at 450 mg/kg given
4 daily via oral gavage for 14 days. Two groups of mice were then challenged with 10^5
5 spores of *C. difficile* via oral gavage at day 0. Mice were monitored for weight loss and
6 clinical signs of CDI from day 0 to day 7. Necropsy was performed on days 2, 4, 7 (open
7 circles). **(B)** Baseline weight loss of mice challenged with and without *C. difficile*, and
8 pretreated with and without ursodiol. Significance was determined by a two-way ANOVA
9 with Sidak's multiple comparisons *post hoc* test. **(C)** *C. difficile* bacterial load in the
10 cecal content throughout CDI. Solid boxes represent total vegetative cells and spores
11 and hashed boxes represent spores only. Significance was determined by a two-way
12 ANOVA with Tukey's multiple comparisons *post hoc* test. **(D)** Vero cell cytotoxicity
13 assay from cecal content throughout CDI. Significance was determined by a Student's t-
14 test corrected with the Holm-Sidak method for multiple comparisons. **(E)** Histopathologic
15 scoring of edema in the murine cecum and colon at day 2 post challenge. Significance
16 was determined by a Student's t-test corrected with the Holm-Sidak method for multiple
17 comparisons. Error bars represent the standard deviations from the mean. **(F)**
18 Representative histomicrograph images of H&E stained cecum (upper) and colon
19 (lower) for groups indicated, shown at 100X total magnification with 500 μ m scalebar.
20 For all graphs (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$).

21 **Figure 4: Ursodiol alters the host inflammatory transcriptome of the cecum early**
22 **during CDI. (A)** Venn diagram depicting the mRNA expression fold change comparing
23 the ursodiol pretreated compared to untreated mice in cecal tissue at day 2 post

1 challenge with *C. difficile*, from two independent experiments performed with a total of
2 n=12 mice/treatment group. Of the 261 genes evaluated, a total of 173 cecal genes had
3 significant gene expression fold changes (increased expression, red; decreased
4 expression, green). **(B)** Volcano plots highlighting genes whose transcript levels
5 changed by greater than 1-fold and met the significant threshold $p \leq 0.05$. Genes
6 highlighted in red had increased transcript levels, while those highlighted in green had
7 decreased levels. Blue points represent genes whose results were significant but did
8 not meet the specify fold change. Black points represent genes whose results failed to
9 meet the significance threshold. **(C)** Cecal inflammatory transcriptome heatmap of
10 calculated z-scores for log₂ transformed count data of mRNA expression levels (y-axis)
11 comparing ursodiol pretreated (blue) to untreated mice (red). Dendrograms represent
12 unsupervised clustering. **(D)** mRNA expression fold differences in increased expression
13 (red) and decreased expression (green) of genes involved with NF-κB signaling in cecal
14 tissue comparing ursodiol pretreated to untreated mice.

15 **Figure 5: Ursodiol alters the FXF-FGF15 transcriptome during CDI.** Violin plots of
16 **(A)** cecal and **(B)** colonic mRNA expression levels of normalized log₂ counts of seven
17 genes involved with the FXF-FGF15 pathway. Significance determined by a Student's
18 parametric t-test with Welch's corrected (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq$
19 0.0001). **(C)** Schematic of seven genes within the FXF-FGF15 pathway. Bile acids
20 modulate signaling via the nuclear bile acid receptor FXR (farnesoid X receptor) and
21 plasma membrane receptor TGR5 (or G-protein coupled bile acid receptor Gpbar1). Bile
22 acid within the gastrointestinal tract are transported back to the liver via enterohepatic
23 recirculation by several active transporters, ASBT (apical sodium dependent bile acid

1 transporter) on the apical surface, and OST α/β (organic solute transporters) on the
2 basolateral surface of enterocytes. Cytoplasmic Ibabp (ileal bile acid-binding protein)
3 helps to facilitate bile acid transport. Bile acids in ileal enterocytes and colonic L cells
4 bind to FXR and activate the FXR-RXR heterodimer complex, resulting in transcription
5 of target genes, such as FGF15/19. FGF 15/19 is secreted into the portal vein and
6 transported back to the liver, where it inhibits expression of *CYP7A1* in hepatocytes to
7 directly regulate bile acid synthesis.

8 **Supplemental Figure 1: Effect of ursodiol on viability and spore formation of *C.***
9 ***difficile*.** Culture aliquots (100 μ L) of *C. difficile* R20291, grown in BHI media, were
10 taken over a 24 hr period and enumerated on TBHI plates to obtain total CFU/ml of total
11 vegetative cells and spores (solid bars) and spores only (hashed bars). The positive
12 controls (EthOH, gray bars) represent *C. difficile* grown in BHI media with ethanol and
13 the treatments groups (blue bars) represents *C. difficile* grown in BHI media with varying
14 concentrations of ursodiol from lowest to highest concentration (left to right). The limit of
15 detection for this assay is 10^2 CFU/ml. The data presented represents triplicate
16 experiments. Significance was determined by Welch's t-test using log transformed
17 CFU/ml.

18 **Supplemental Figure 2: Ursodiol treatment alters the bile acid metabolome.** Box
19 and whisker plots of bile acids that were significantly altered over the course of ursodiol
20 treatment compared to mice that only received cefoperazone (based on a Two-way
21 ANOVA with Tukey's multiple comparisons post hoc test). An n=3 mice/treatment group
22 were then selected for bile acid metabolomic analysis.

1 **Supplemental Figure 3: No effect with corn oil vehicle alone on CDI in mice. (A)**

2 Baseline weight loss of cefoperazone treated mice challenged with *C. difficile* pretreated
3 with (white, *C. diff* + corn oil) and without corn oil vehicle (red, *C. diff* only) (n=8
4 mice/treatment group). **(B)** *C. difficile* bacterial load in feces and **(C)** cecal content over
5 the first 2 days post challenge. Solid boxes represent total vegetative cells and spores
6 and hashed boxes represent spores only. Significance was determined by a two-way
7 ANOVA test.

8 **Supplemental Figure 4: Total histologic scores were not significantly different**

9 **with ursodiol pretreatment.** Histopathologic scoring of murine cecum and colon
10 throughout CDI. Total histologic scores were calculated by adding all three scores from
11 parameters assessed: epithelial damage, inflammation, and edema. Significance was
12 determined by a two-way ANOVA with Sidak's multiple comparisons *post hoc* test.
13 Error bars represent the standard deviations from the mean.

14 **Supplemental Figure 5: Ursodiol alters the host inflammatory transcriptome of**

15 **the colon early during CDI. (A)** Venn diagram depicting the mRNA expression fold
16 change comparing the ursodiol pretreated to untreated mice in colonic tissue at day 2
17 post challenge with *C. difficile*, from two independent experiments performed with a total
18 of n=12 mice/treatment group. Of the 261 genes evaluated, a total of 139 colonic genes
19 had significant gene expression fold changes (increased expression, red; decreased
20 expression, green). **(B)** Volcano plots highlighting genes whose transcript levels
21 changed by greater than 1-fold and met the significant threshold $p \leq 0.05$. Genes
22 highlighted in red had increased transcript levels, while those highlighted in green had
23 decreased levels. Blue points represent genes whose results were significant but did

1 not met the specify fold change. Black points represent genes whose results failed to
2 meet the significance threshold. **(C)** Colonic inflammatory transcriptome heatmap of
3 calculated z-scores for log₂ transformed count data of mRNA expression levels (y-axis)
4 comparing ursodiol pretreated (blue) to untreated mice (red). Dendrograms represent
5 unsupervised clustering. **(D)** mRNA expression fold differences in increased expression
6 (red) and decreased expression (green) of genes involved with NF-κB signaling in
7 colonic tissue comparing ursodiol pretreated to untreated mice.

8 **Supplemental Table 1:** Nanostrings nCounter Mouse Inflammation V2 Panel Gene List
9 (in word document)

10 **Supplemental Table 2:** Nanostrings nCounter Mouse Custom Code Set Gene List (in
11 word document)

12 **Supplemental Table 3:** Targeted bile acid metabolomics data from mice treated with
13 cefoperazone with and without different doses of ursodiol.

14 **Supplemental Table 4:** Nanostrings differential gene expression data from cecal and
15 colonic tissue comparing ursodiol pretreated to untreated mice.

16 **Supplemental Table 5:** Nanostrings z-score data from the cecal and colonic tissue
17 comparing ursodiol pretreated to untreated mice.

18 **Supplemental Table 6:** Cecal and Colonic Tissue mRNA Expression Fold Change for
19 NF-κB Signaling

20 **Supplemental Table 7:** Cecal and Colonic Tissue mRNA Expression Fold Change for
21 FXR-FGF15 Pathway

Continue Table S1: Nanostring nCounter SC Mouse Inflammation V2 Panel Gene List

Table S1	NM_011455.4	A4468537, A4596182, B13002C1, 48k, Mboaa15	Cytoplasm, Cytoskeleton, Microtubule, Protein Complex, Microtubule Based Process, Protein Polymerization, Microtubule Based Movement, GTP Binding, Nucleotide Binding, Protein Complex Binding, Structural Molecular Activity, Structural Constituent Of Microtubules	ATTGGGAGGTGCTTCCCTGCTATTTGGTCTTCCTTCCTCCGGAGAGATGGGGGCTTGGGGGTCGGGGGAGGCTTTGGTCTT GGTCTCTGGAGCTGGGAAATTCG	tubulin, beta 5 class I
© 2013 NanoString Technology Inc.					

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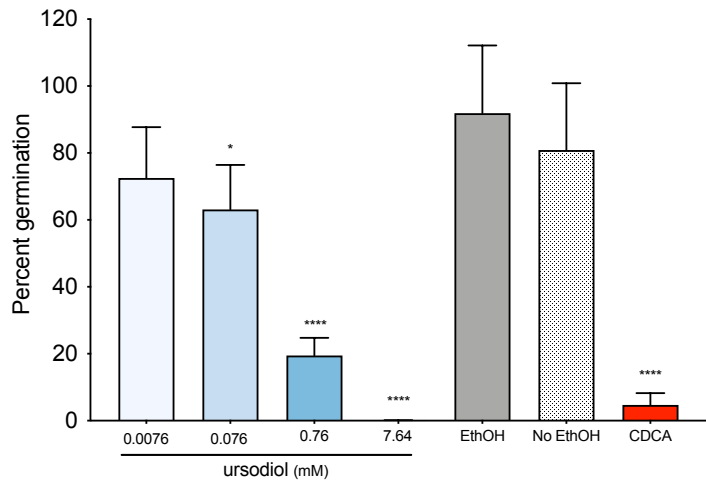
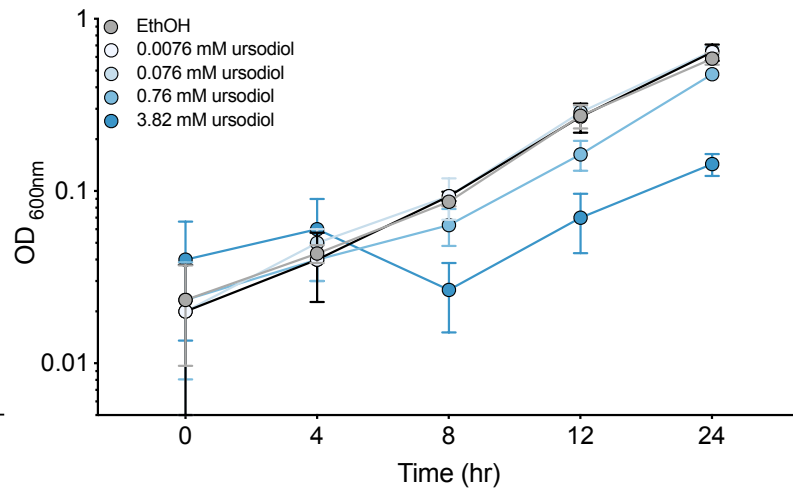
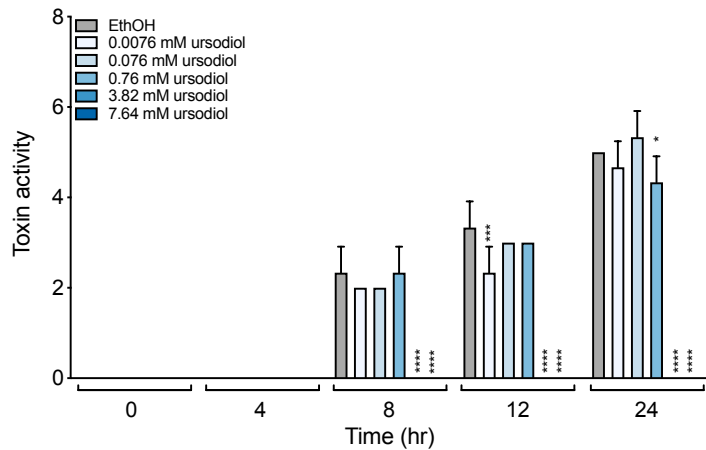
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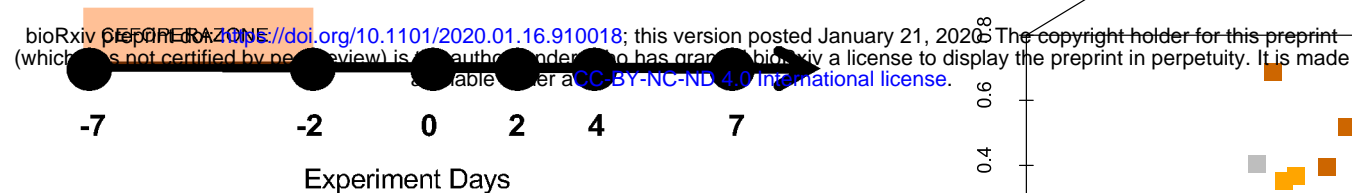
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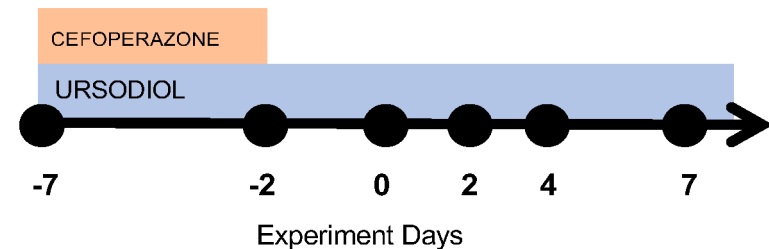
A**B****C**

A

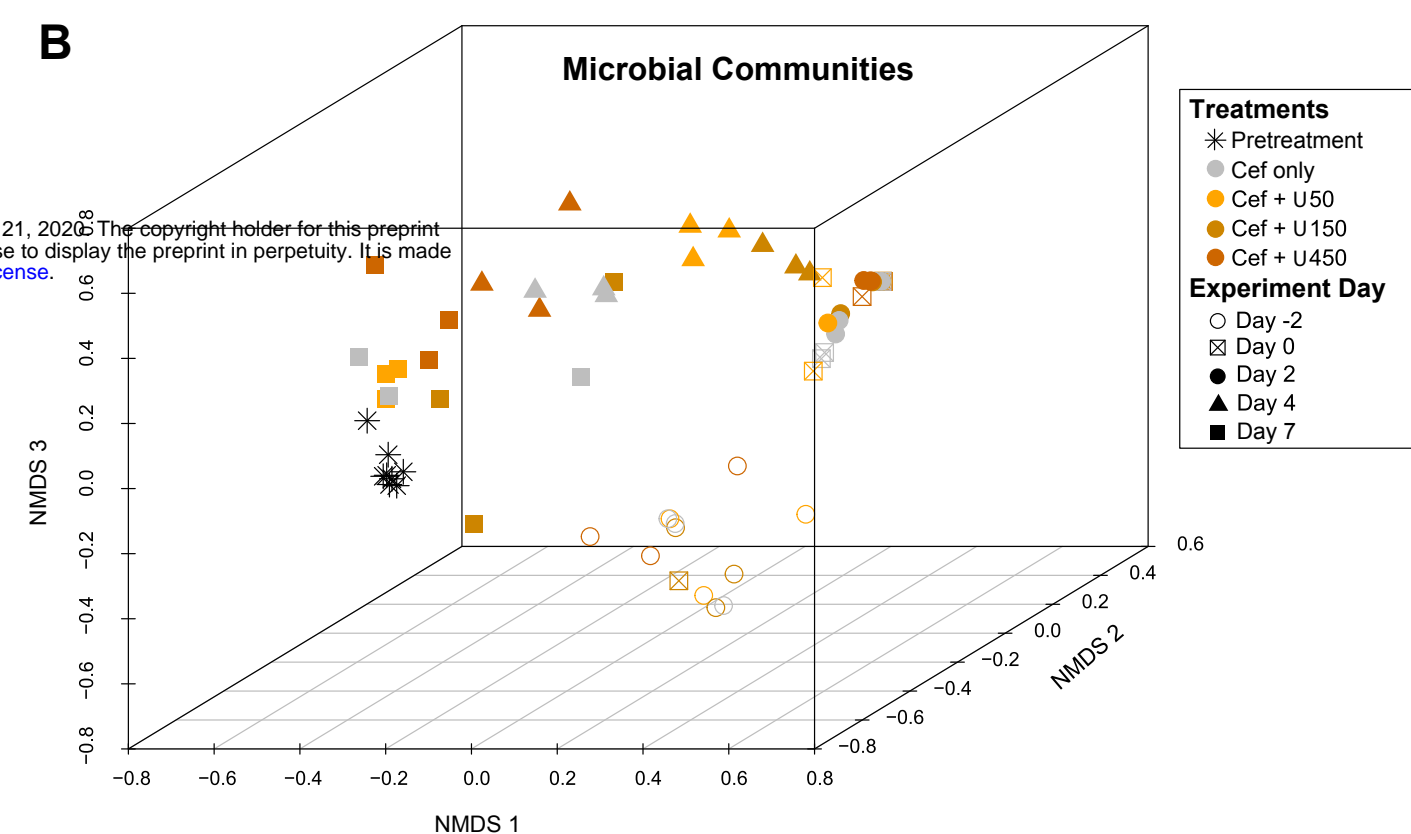
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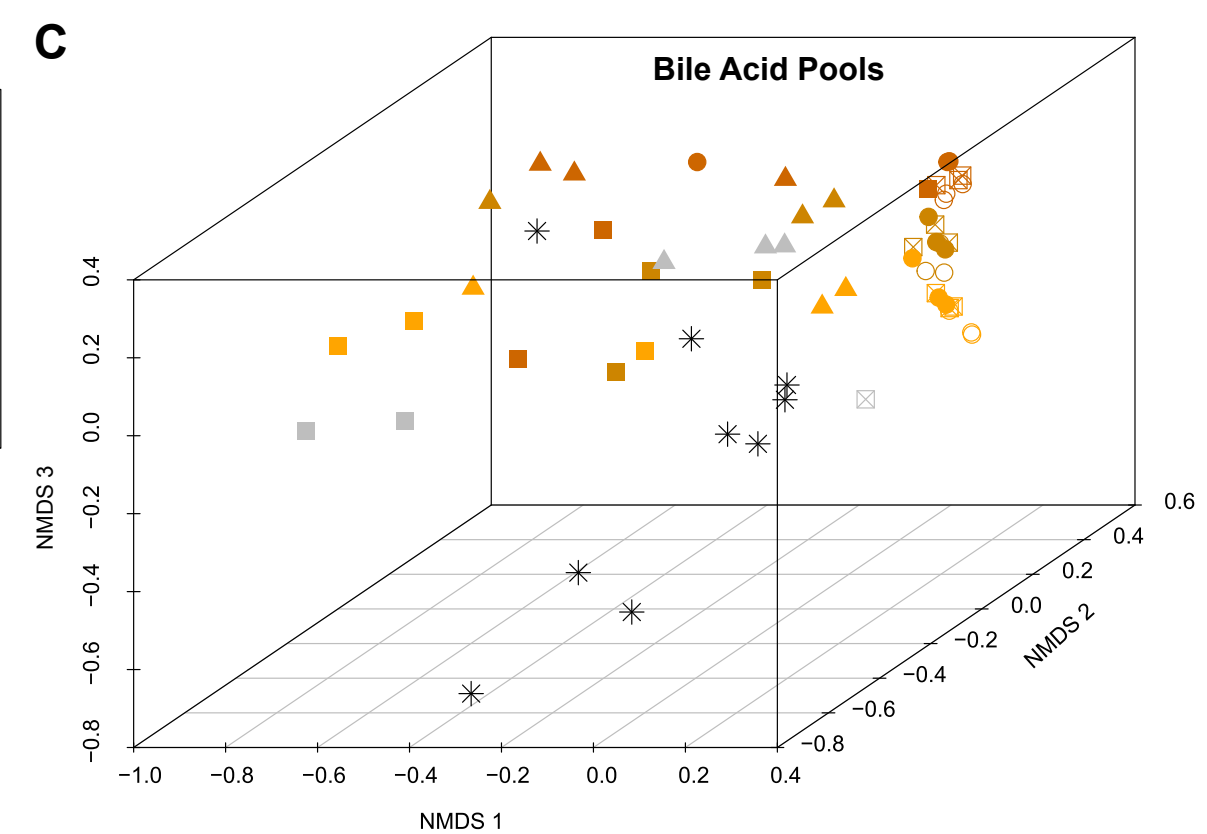
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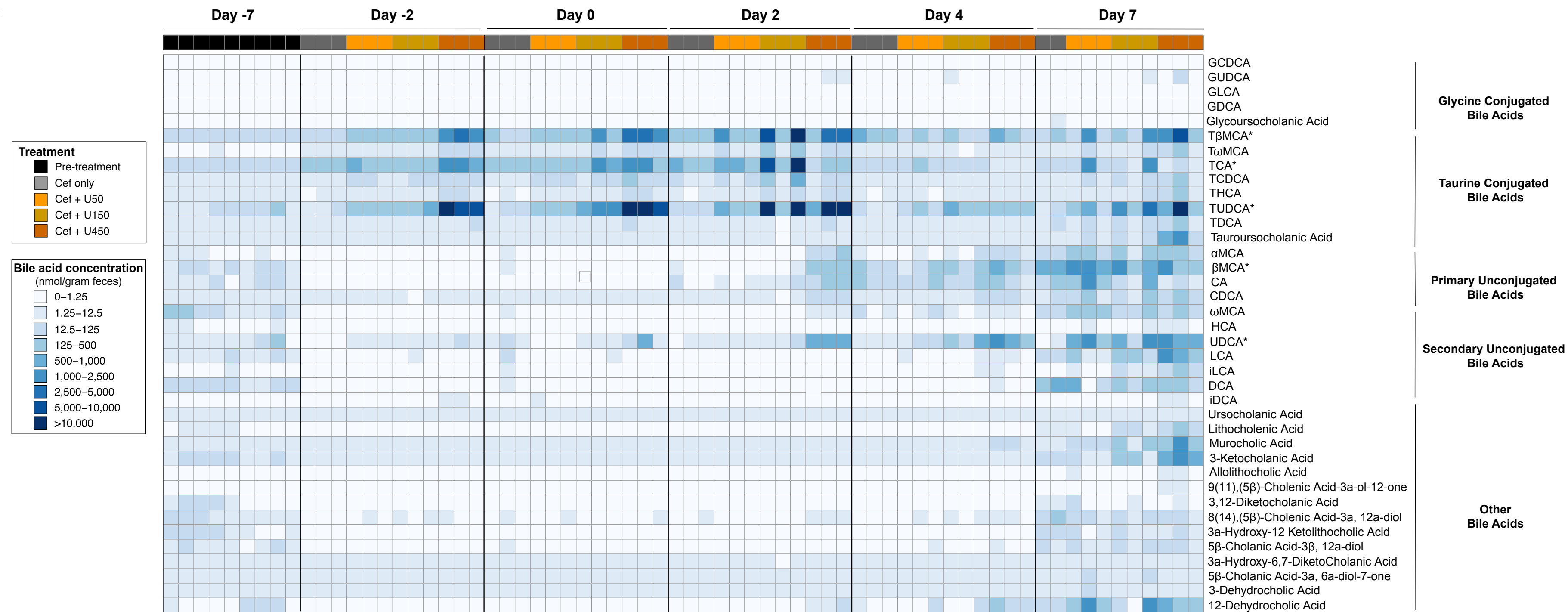
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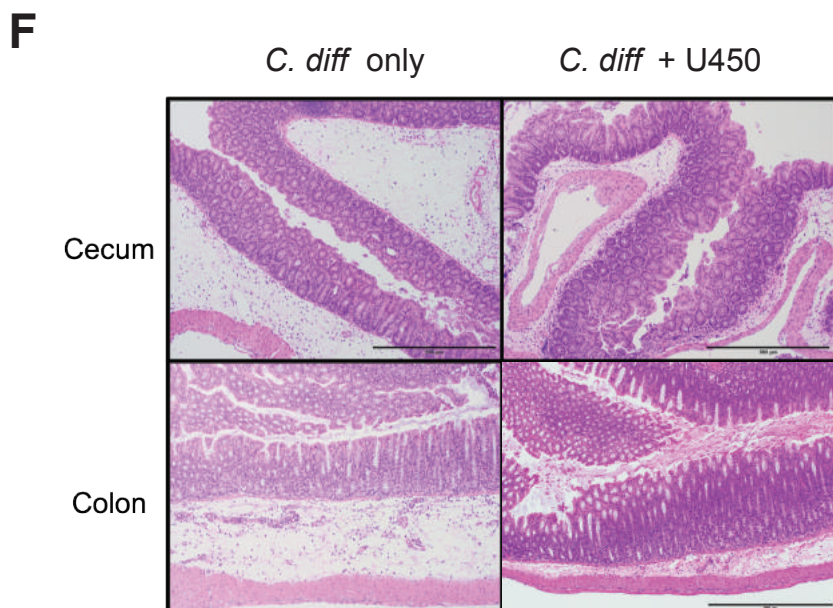
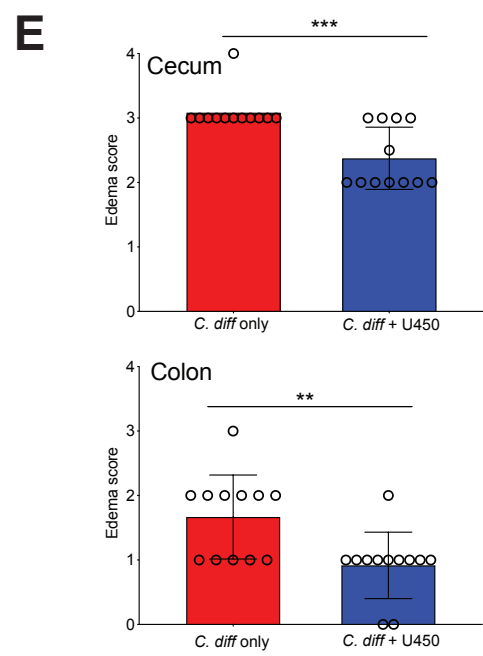
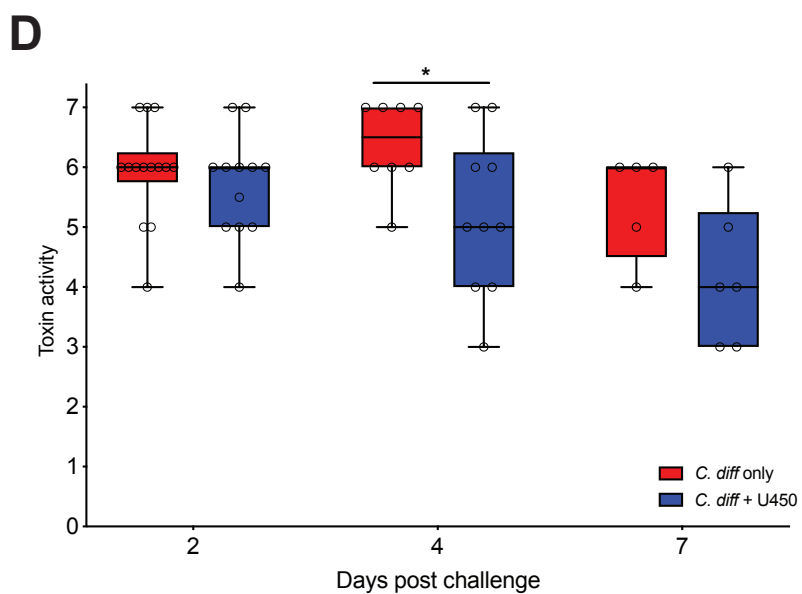
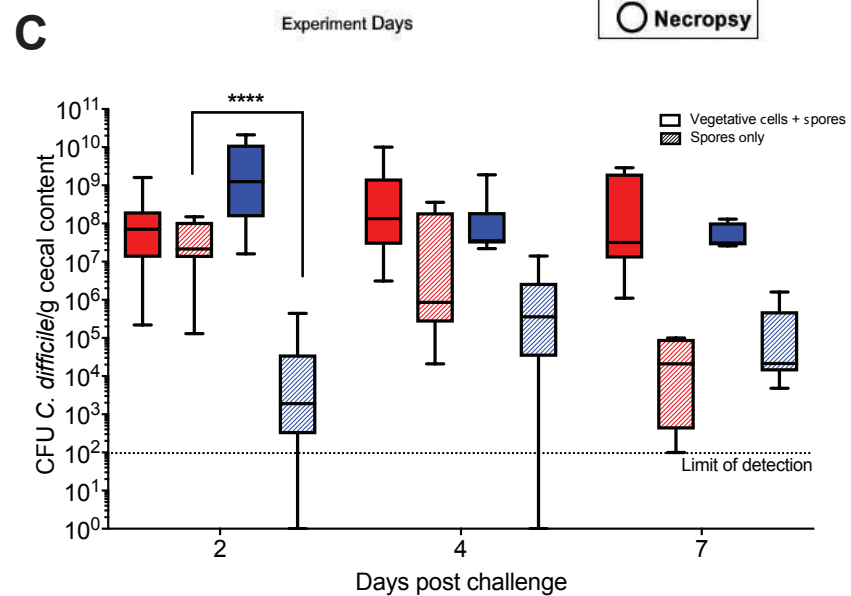
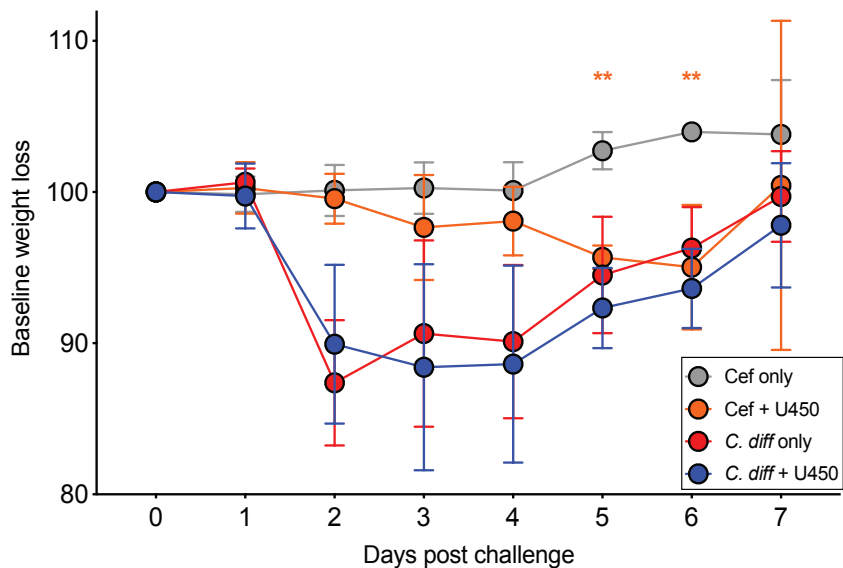
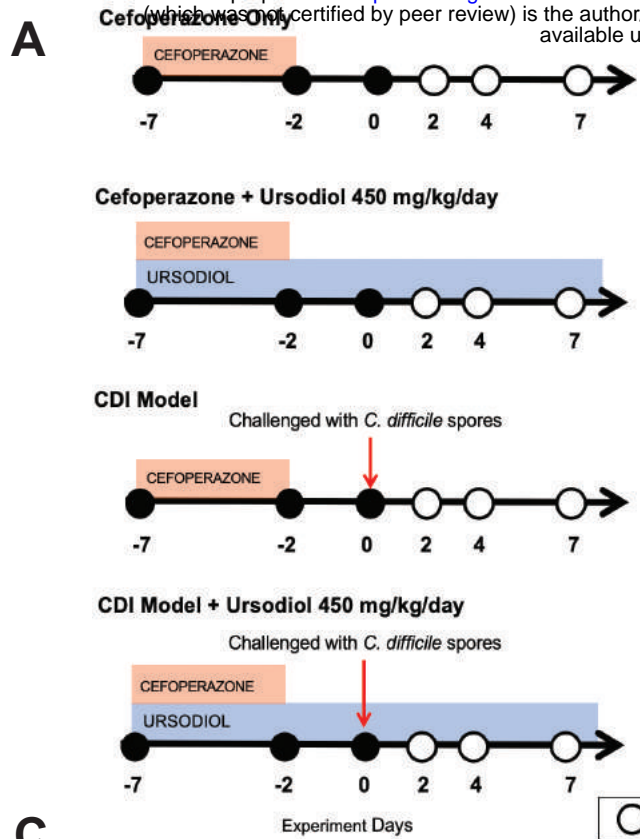


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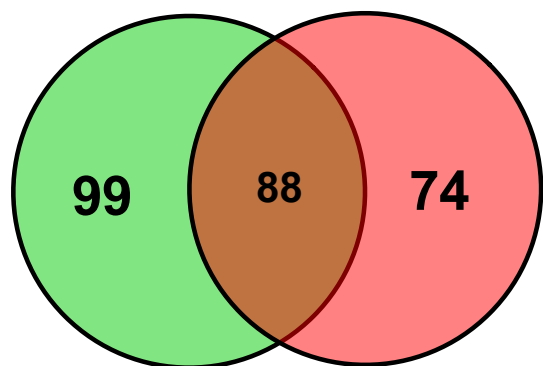
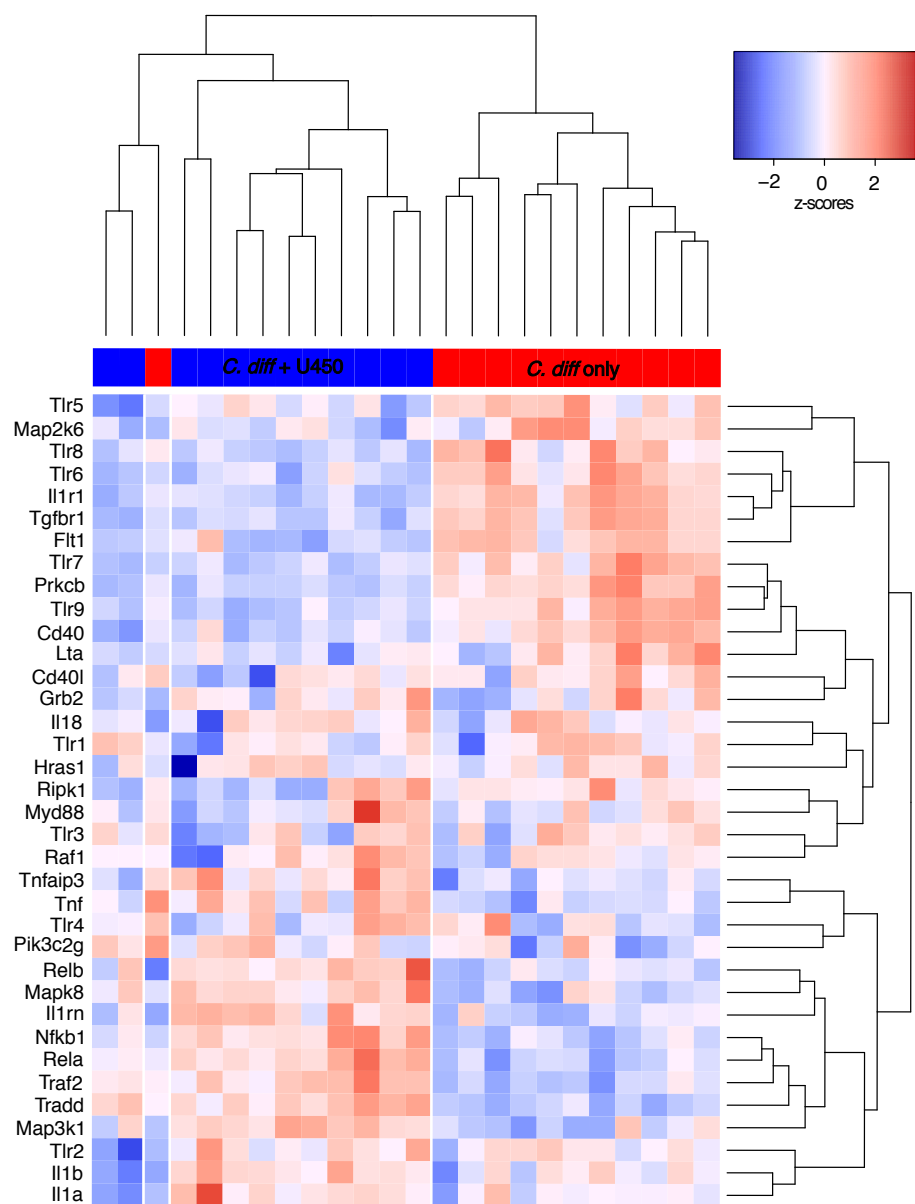
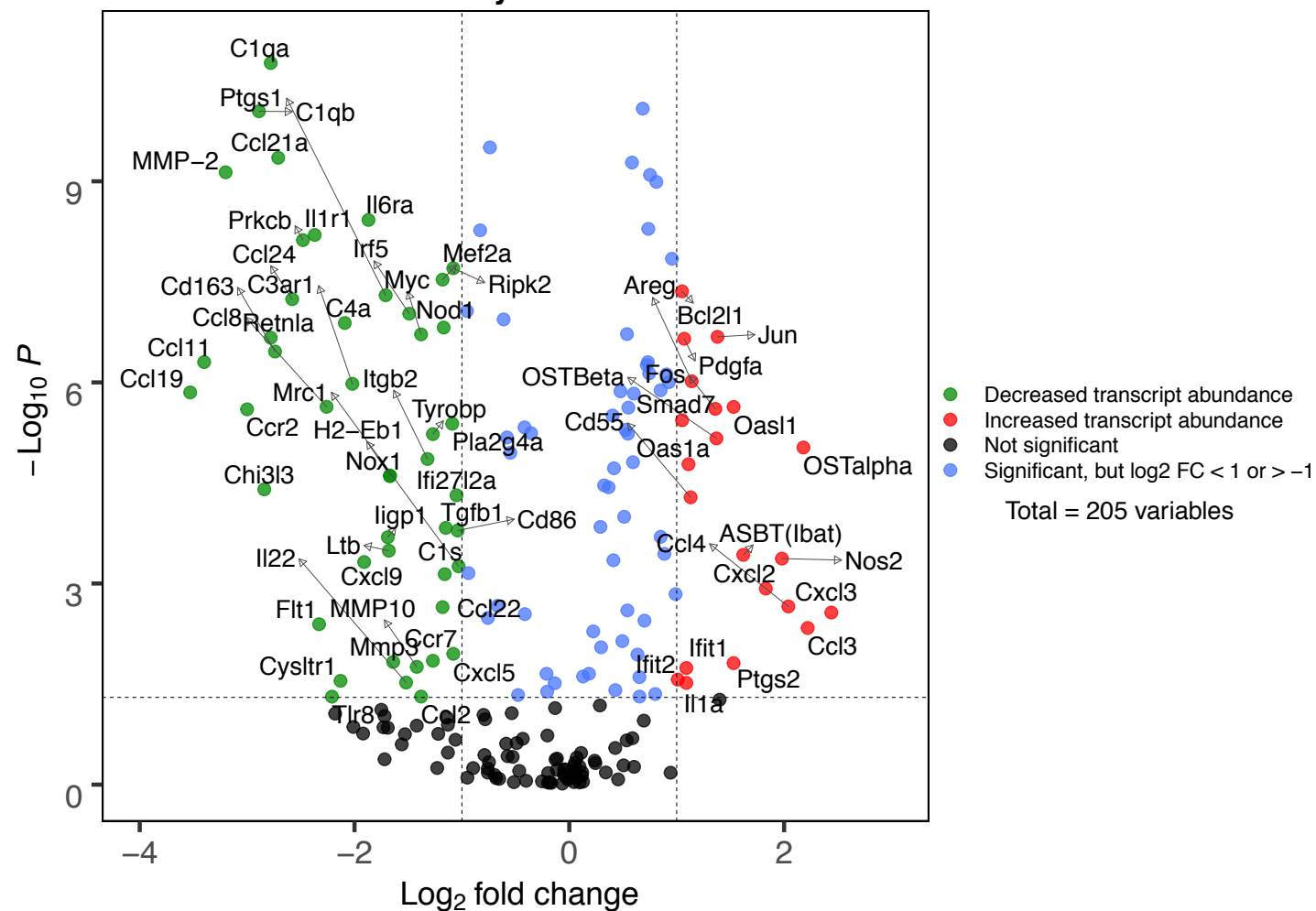
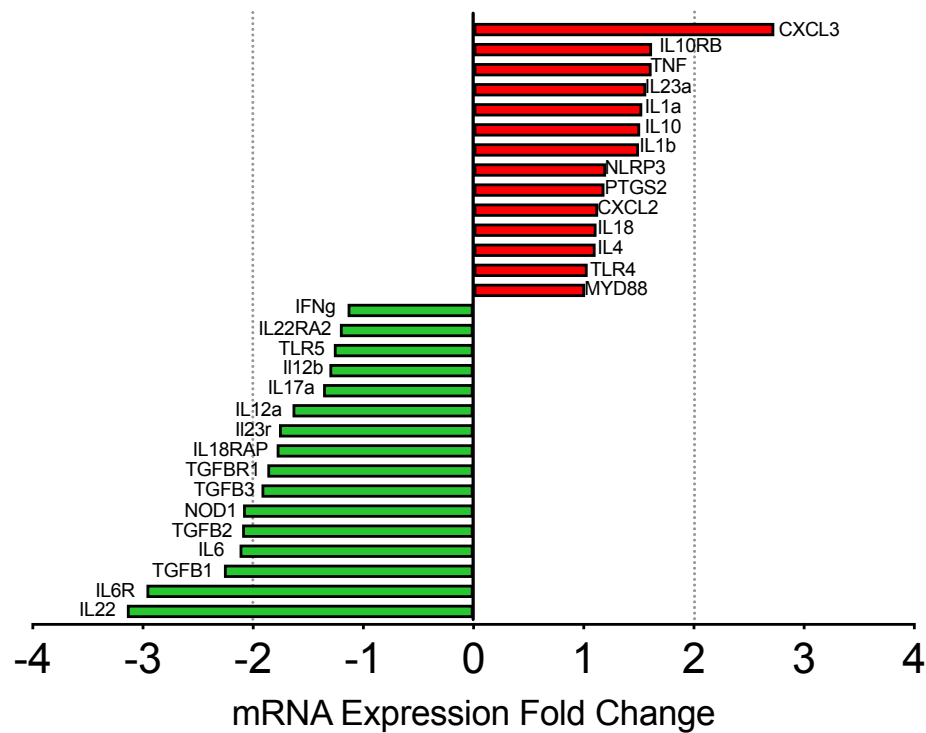


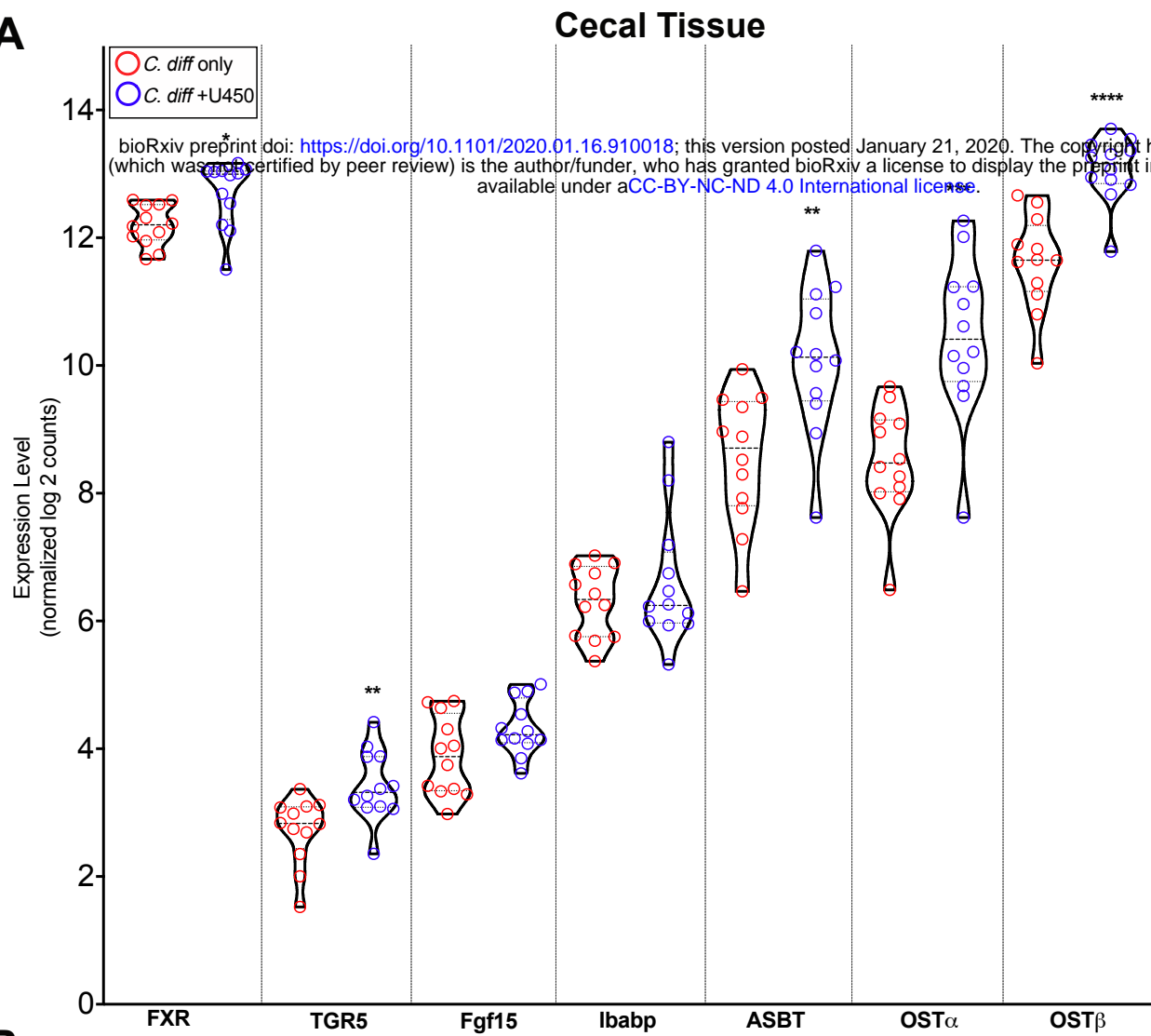
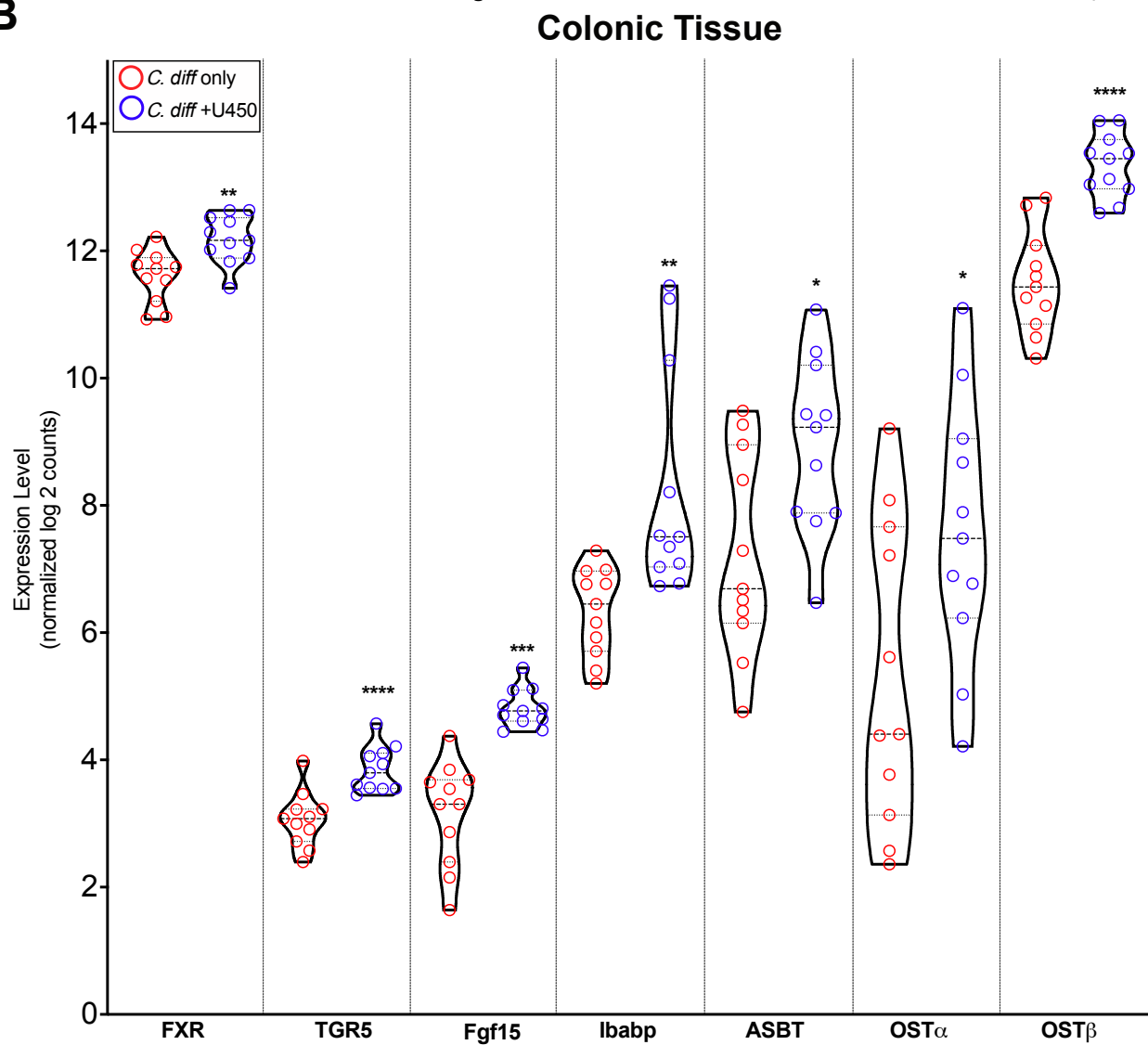


A**Cecal Tissue**

Decreased expression

Increased expression

**C****B*****C. diff* + U450 vs *C. diff* only****D**

A**B****C**