Methods

Epidemiology study design and analysis

Epidemiological design and data source. To investigate therapeutic drug classes and individual drugs that may impact colonization resistance, we conducted a case cross-over study in a population-based dynamic cohort. The case cross-over design was selected as it allowed us to use each case as their own control, thereby adjusting for known and unknown individual characteristics that may influence the outcome of gastrointestinal infection. The population-based dynamic cohort was comprised of a population-based 25% random sample of the 4.1 million residents of Montreal, Quebec, followed over a period of 15 years from 1999 to 2014. The cohort is dynamically updated each year to account for in- and out-migration. Each member of the cohort is provided with universal health insurance for all medical and hospital care through the provincial health insurer (RAMQ). In addition, all essential drugs are covered for residents 65 years of age or older, those receiving income security and those without drug insurance through their employer (approximately 50% of Quebec residents). For each member of the cohort, the RAMQ provided the research group with all medical services received including the date and location of the service, patient and physician identification, primary diagnosis, type of visit, and reimbursement provided, as well as records of all RAMQ-covered drugs dispensed through community pharmacies (date, drug, patient and pharmacy identification). These data were linked by patient identification to the provincial birth and death registry, which records the date, time and cause of death, as well as to the Ministry of Health database on all hospitalizations in the province (patient identification, dates of admission and discharge, primary and secondary discharge diagnoses, and procedures).

Case and control period definition and sampling relative to drug exposure. Within this cohort of 1,434,375 individuals, we identified all individuals who had a physician claim or hospital admission with the primary diagnosis of a gastrointestinal (GI) problem suspected to be of infectious origin (events). These included ICD9 (001x, 002x, 003x, 004x, 005x, 0060, 0061,

0062, 0069, 007x, 008x, 009x) and ICD10 codes (A00x, A01x, A02x, A03x, A04x, A05x, A060, A061, A062, A069, A07x, A08x, A09). If an individual had multiple visits for an infectious GI problem, we selected the first visit. Prescription drug use in the 60 days (~2 months) prior to the visit for an infectious GI problem was assessed (case period). To avoid misclassification of the timing of drug exposure (i.e., the drug was prescribed to treat the GI problem), we excluded drugs prescribed in the 3-day period directly prior to the GI visit. For each patient, we selected a control date that was ~2 months before the first GI visit. Drug use was assessed in the 2 months prior to the control date, excluding the same 3-day gap period prior to the control date to assess drug use (Extended Data Fig. 1a).

Measurement of Prescription Drug Use. In each 2-month case and control period, we created a drug-by-day matrix using the date of dispensing and the duration of supply for each prescription filled by a patient. Drugs were classified by ingredient and therapeutic class using the American Hospital Formulary System[1].

Analysis and visualization of epidemiological data. Descriptive statistics were used to characterize the study population. Conditional logistic regression was used to evaluate the association between GI infection risk with each therapeutic class and drugs frequently prescribed within each therapeutic class. Several drug classes were considered expected associations (including anti-microbial agents, immunosuppressive agents, antidiarrhea agents, analgesics and antipyretics, antiemetics, and cathartics and laxatives) and were ruled out from further investigation. Based on the selected inclusion criteria (>100 prescriptions, odds ratio >1.5, *P* value <0.05), a subset of drugs were identified for further investigation in mouse models (Fig. 1b, Extended Data Table 2). Drugs that were not well-tolerated in mice (*e.g.*, risperidone) were excluded, constituting a final list of 21 drugs. Epidemiological data were visualized using a complex heatmap[2] to illustrate the criteria used for drug selection (Fig. 1b).

For the association between digoxin and GI infection, we assessed whether the concurrent use of antibiotics, immunosuppressants, and/or antidiarrheals modified the association by including binary indicators for use (yes/no) in multivariate models (Extended Data Table 4).

Chemicals and primers

Drugs for animal studies were purchased individually, and drug dosages were determined based on previous studies (Extended Data Table 3). All primers used in the study are listed in Extended Data Table 5.

Microbiological culture and growth measurements

Bacterial strains. All bacterial strains and isolates used in this study are listed in Extended Data Table 6.

Aerobic growth conditions. Routine aerobic culturing was conducted in Luria Bertani (LB) broth using shaking conditions (220 RPM) at 37°C for 16 hours. Stocks were prepared from 16-hour cultures using autoclaved glycerol (20% final concentration) and stored at -80°C.

Anaerobic growth conditions. All anaerobic microbiology steps were performed in a flexible anaerobic chamber (Coy Laboratory Products) containing 20% CO_2, 10% H_2, and 70% N_2 using pre-reduced media and materials. Routine anaerobic culturing was conducted using liquid mega medium[3] or Brucella blood agar plates. Stocks were prepared under anaerobic conditions from 16-hour cultures using autoclaved glycerol (20% final concentration) and stored in single-use aliquot vials at -80°C.

In vitro growth assays. To measure the impact of medical drugs on the growth of representative gut microbial species, growth measurements were conducted at three relevant drug concentrations (20, 40, 80 μM) as described in previous publications[4]. To this end, 384-well

plates were freshly prepared with each of the 21 identified drugs (Fig. 1b) at each concentration in modified Gifu Anaerobic Medium (mGAM; HyServe) and stored in the anaerobic chamber for 24 hours before bacterial inoculation. Twelve representative human gut isolates, *S*. Tm, and *E. coli* (Extended Data Table 6) were cultured for 16 hours and inoculated in quadruplicate into the 384-well plate at an initial optical density (OD_{600}) of 0.025. Plates were incubated anaerobically at 37°C for 24 hours, and OD_{600} was recorded every 15 minutes after mixing for 1 minute prior to measurement. Media controls were used as blanks. The area under the curve (AUC) was calculated to evaluate bacteria growth under different drug concentrations, which were compared with the AUC for the corresponding vehicle concentrations (DMSO controls). Double-sided Mann–Whitney U tests were conducted to test the significance of AUC differences, and *P* values were corrected by the Benjamini-Hochberg method with $P < 0.05$ after multiple hypothesis testing considered significant. AUC differences were converted into a heatmap using the R package "pheatmap" [5], and clustering was performed based on bacterial growth AUC.

Animal Experiments

Conventional and gnotobiotic husbandry. All mice experiments were performed using protocols approved by the Yale University Institutional Animal Care and Use Committee (IACUC), and all mice were kept in a 12-hour light/dark cycle. 6-8-week-old mice were used in these studies unless stated otherwise. No statistical methods were used to predetermine sample sizes, but sample sizes are similar to those reported in previous publications studying colonization resistance[6,7]. Female mice were used for colonization resistance screening and co-housing experiments. Both sexes were used for all other experiments. Conventional mice were housed under specific pathogen-free conditions with unlimited access to water and food (Telkad Global 16% Protein Rodent Diet, Envigo). Germ-free (GF) animals were housed in gnotobiotic isocages (Sentry Sealed Positive Pressure (SPP) isolation cage system (Allentown, Inc., Allentown, NJ, USA)). In experiments using gnotobiotic mice carrying fecal microbiome transplants from

individual donor animals or pooled human fecal samples, gnotobiotic recipient mice were individually housed in flexible plastic gnotobiotic isolators. Gnotobiotic mice were fed a standard, autoclaved mouse chow (5K67 LabDiet, Purina) *ad libitum* and autoclaved water for the duration of the experiment. GF status was routinely monitored by 16S rRNA-targeted PCR of fecal DNA and culturing of fecal samples under aerobic and anaerobic conditions.

Investigation of candidate drugs in mice. Groups of 6-8 week old female C57BL/6N mice (Taconic Biosciences) were treated with each of the 21 candidate drugs identified in the epidemiological analysis (Fig. 1b; n=5/group) in cohorts of 4-5 drugs per cohort. All mice within a cohort were obtained concurrently and a vehicle control group was included in each cohort. All drugs were dissolved in 90% Phosphate Buffered Saline (PBS), 5% DMSO, and 5% Tween 80 to create a consistent treatment condition between the groups. Drug dosage was determined based on the literature (Extended Data Table 3), and drugs that were not completely dissolved were administered as a slurry; each drug was prepared fresh prior to gavage. Mice were orally gavaged with individual drugs twice daily for two days (four doses total). The first drug dose was administered in the evening (Zeitgeber time ZT12), with the three subsequent doses at 12-hour intervals. Fecal samples were collected for microbiome assessment immediately before the 1st drug treatment (D(-2)) and 12 hours after the final dose (D0; also ZT12). For infections, 10^8 colony-forming units (CFUs) of *S.* Tm Δ*invA*, prepared as described below, were used for infection by oral gavage at the D0 time-point (ZT12, 12 hours after the final drug dose). *S.* Tm Δ*invA* CFUs were enumerated from feces 12h, D1, D2, and D4 after infection.

Pathogen infection. WT[8] and Δ*invA* (SB1901)[9] genotypes of *S*. Tm strain SL1344 were grown in LB broth overnight at 37 ºC with shaking at 220 rpm. After 16 hours of growth, bacteria were washed twice and resuspended in autoclaved PBS. Mice were infected with 10^8 CFUs by oral gavage and were weighed at the same time daily before and after infection. Animals were

euthanized 4 days post-infection or monitored for survival as described below. For fecal *S.* Tm CFU quantification, feces were weighed, homogenized by vortexing in 1 mL of sterile PBS, serially diluted in PBS, and plated on agar plates supplemented with 200µg/ml streptomycin. Plates were incubated for ≥ 16 hours at 37 ºC prior to CFU enumeration, and CFU was reported per gram of feces for each mouse. *S.* Tm-selective CFU plating was also conducted prior to infection to confirm the absence of streptomycin-resistant aerobic taxa in each microbiome.

Survival analysis. Mice were observed twice daily during the experimental period, and those with ≥ 20% weight loss and/or clear morbidity (lethargy, huddling, shivering, hunched posture) were euthanized immediately.

Digoxin administration. Digoxin was freshly prepared in PBS with 5% DMSO before each gavage and administered (5 mg/kg or 0.5 mg/kg) as an oral suspension to mice. PBS 5% DMSO (referred to as PBS controls) was used as vehicle controls for all digoxin experiments in mice. The dose of 5mg/kg of mouse body weight was selected based on previous literature[10–12]. This dose led to a slight reduction in weight in C57BL/6N, C57BL/6J, and *Rorγ*$^{-/-}$ mouse strains as previously observed[13] (Extended Data Fig. 5g) , but did not cause other observed distress, morbidity, or mortality to mice. While both 5 mg/kg and 0.5 mg/kg doses significantly reduced SFB levels and increased *S.* Tm infection risk in mice, we used the higher dose unless otherwise indicated in order to reduce the number of animals required in light of the intrinsic variability of *S.* Tm infection in mice that are not receiving streptomycin pretreatment prior to infection. In some experiments, mice were sacrificed at the D0 time point (12 hours after the final drug dose, prior to pathogen infection) for measurement of host gene expression.

Vancomycin administration. 6-8 week-old mice were treated either with vancomycin orally (25 mg/kg) or intraperitoneally (2.5 mg/kg to account for the reported 10% bioavailability of oral

vancomycin[14]) for two days (2 doses/day), and intraperitoneally administered PBS was used as a control. Mice were sacrificed 12 hours after the final drug dose, and ileum tissues were collected for gene expression analysis. Feces were collected pre- and post-drug treatment for SFB enumeration.

Co-housing experiments. 3-4 week old female C57BL/6J and C57BL/6N mice were purchased from the Jackson Laboratory and Taconic Biosciences, respectively. Mice were tagged and co-housed (two randomly selected C57BL/6J and two C57BL/6N mice/cage) for two weeks and then segregated into new cages based on vendor origin. Segregated C57BL/6J mice were housed together, and digoxin administration and pathogen infection were conducted as described above. Experiments with non-cohoused control animals grouped in cages were concurrently performed.

Studies using CV and GF C57BL/6N Nramp1$^{+/+}$ mice. CV C57BL/6N *Nramp1*$^{+/+}$ mice[15] were generously provided by Dr. Jorge Galán (Yale University) and were bred in-house at the Yale Animal Resources Center (YARC). 6-10 week old male and female animals were used for drug administration and infection studies as described above. GF C57BL/6N *Nramp1*$^{+/+}$ mice were re-derived to the germ-free state by sterile hysterectomy of the CV *Nramp1*$^{+/+}$ C57BL/6N animals. Briefly, donor CV *Nramp1*$^{+/+}$ C57BL/6N mice were time-mated along with GF Swiss Webster dams used for fostering. 40μl of progesterone (NDC #00143972501) was subcutaneously injected on the back near the rump into the pregnant donor mice for three consecutive days before the C-section. On the day of the C-section, the pregnant donor mouse is sacrificed via cervical dislocation and submerged in hot (~40°C) Virkon S (Lanxess #57811373) to disinfect. The pup-loaded uterus was removed and submerged in a 50-mL tube filled with Virkon S. The tube with pups was inserted into the germ-free isolator containing the GF Swiss Webster foster

female. The pups are removed from their amniotic sacs, cleaned with Q-tips, placed with the foster female, and monitored until they are accepted. Sterilizing levels of chlorine dioxide (CLIDOX-S: 1:3:1 (Pharmacal, #95120F)) were used to maintain sterility during the procedure. Germ-free status was monitored as described above.

Rorγ$^{-/-}$ *mice*. Heterozygous *B6.129P2-Rorctm1Litt/J* were obtained from the Jackson Laboratory (Strain #007571). Mice were separated according to genotype at weaning, and heterozygous animals were used to maintain the colony. Because this strain is SFB-negative, homozygous (*Rorγ*$^{-/-}$) and their WT littermate controls were colonized with SFB prior to drug and infection experiments. To this end, cecal contents from SFB-monocolonized mice (provided by Dr. Ivaylo I. Ivanov, Columbia University) were used to colonize GF C57BL/6N mice; after 4 weeks, gastrointestinal contents from 5 animals were collected under sterile anaerobic conditions. All subsequent steps prior to -80°C storage were also conducted under sterile anaerobic conditions. Contents were weighed, resuspended in 15 mL of sterile, pre-reduced PBS per gram gut contents, vortexed, and passed through a 70μm cell strainer (Fisher #08-771-2). An equal volume of pre-reduced 40% glycerol (in PBS + cysteine) was added to the flow-through and mixed by inverting. These "biobanked" samples were stored in single-use aliquots in sealed Wheaton vials (Fisher #03-140-390) at -80ºC. These aliquots were used to colonize *Rorγ*$^{-/-}$ mice and their WT littermates by daily oral gavage beginning at 4-6 weeks of age and continuing for 3 days, followed by an 11-day rest period to allow for SFB expansion and stabilization. SFB engraftment was confirmed using qPCR with SFB-specific primers (Extended Data Table 5). Digoxin administration and pathogen infection was conducted as described above.

ΔILC3 mice. *Rorγ*^{STOP}, *Cd4-cre*$^{+}$ mice (which retain *Rorγ* expression in all T cells but lack *Rorγ* expression in ILC3 cells)[16] were bred with *Rorγ*^{STOP}, *Cd4-cre*$^{-}$ mice (which lack *Rorγ* expression

in both ILC3 cells and T cells)[16] to obtain littermate animals that either specifically lack ILC3 cells (designated ΔILC3) or lack both ILC3 and Th17 cells (designated *Rorγ*^{STOP}). SFB colonization and digoxin administration were conducted as described above.

Transgenic C57BL/6N vil-defb39 mice. Transgenic mouse generation was conducted at the Yale Genome Editing Center. The *defb39* gene insert was obtained as a gBlock (Integrated DNA Technologies). The plasmid 12.4kbVillin-ΔATG[16] was obtained as a gift from Deborah Gumucio through Addgene (Addgene plasmid 19358). The *defb39* insert was cloned into the 12.4kbVillin-ΔATG plasmid by InFusion cloning to yield p12.4kbVillin-ΔATG-defb39 (Extended Data Table 6). The villin promoter and transgene was excised from the plasmid backbone and microinjected into zygote pronuclei as described[17]. Embryos were transferred to the oviducts of pseudopregnant CD-1 foster females as described[17]. The presence of the transgene was confirmed by PCR using primers defb39_F and defb39_R (Extended Data Table 5).

Microbiome transplantation. C57BL/6N donor mice were treated with digoxin or PBS control (standard 2-day treatment regimen and extended treatment regimens, including a 12-hour drug washout period after the final dose). Gastrointestinal contents were biobanked as described above, except biobanks were established from individual animals without pooling. Male and female GF C57BL/6N recipient mice were singly housed in flexible plastic gnotobiotic isolators, with separate isolators for recipients of digoxin-treated and PBS-treated biobanked samples. Recipient mice were colonized with biobanked gut microbiomes from digoxin-treated or PBS-treated donor animals in a 1:1 ratio (individual donor to individual recipient). After 7 days, recipient mice were sacrificed to measure host gene expression or infected with *S*. Tm Δ*invA* as described above. SFB abundance was measured during the course of the transplant by qPCR. Mouse weight, *S.* Tm Δ*invA* CFU, and survival was measured as described above.

SFB monoassociation experiments. GF C57BL/6N mice were colonized by oral gavage with a single dose of biobanked material from SFB-monoassociated mice (described above). After 14 days, SFB-monoassociated mice were treated with digoxin or PBS as described above. At the D0 timepoint (12 hours after the final digoxin/PBS dose), mice were sacrificed and ileum tissues and contents collected for gene expression studies and SFB abundance quantification, respectively.

Identification of cgr2-negative human gut communities. Fecal samples from 28 healthy, unrelated human donors were previously obtained, aliquoted, and stored at -80^{o}C under anaerobic conditions under the Yale University Human Investigation Committee protocol number 1106008725[18]. Previously published and deposited metagenomic data from each donor[18] was analyzed for *cgr2* gene abundance using Shortbred[19]. To this end, Cgr2 protein sequences were downloaded from NCBI in .fasta format and metagenomic data was retrieved from the European Nucleotide Archive (accession ID: PRJEB31790). Markers were created using the shortbred-identify function and the Uniref90 reference database[20]. *Cgr2* abundance was quantified using the created reference markers with shortbred_quantify function using built-in USEARCH v.11.0.667[21]. In addition, *cgr2* gene abundance was directly measured in aliquots of these samples by qPCR using gene-specific primers (Extended Data Table 5). The resulting tables for each sample were merged into one summary table (Extended Data Table 7). We selected communities from 8 human donors with *cgr2* gene levels below the level of detection by either method (Extended Data Table 8) for gnotobiotic mouse studies. These communities were also screened for SFB using qPCR and were SFB-negative.

Colonization of gnotobiotic mice with human microbiome samples. GF C57BL/6N *Nramp1*$^{+/+}$ mice (described above) were colonized with the pooled human microbiome community by oral gavage. After 14 days, mice were treated digoxin or PBS for two days as described above. At

the D0 timepoint (12 hours after the final drug dose), mice were either sacrificed to assess host gene expression using qRT-PCR or infected with 10^8 CFUs of WT *S*. Tm. Fecal pathogen burden was enumerated at indicated time points, and mice were sacrificed at 4 days post-infection for enumeration of pathogen loads in different gut compartments, and measurements of ileal gene expression by qRT-PCR.

DNA extraction from mouse feces. DNA extraction from fecal samples was performed as previously described[22]. Briefly, fecal pellets were collected directly from mice into pre-weighed 2 mL sterile cryotubes at the designated times. The fecal pellets were frozen at -20°C or directly processed. The fecal pellets were resuspended in 500 µL CP buffer (Omega), 250 µL SDS 20%, 550 µL of 25:24:1 phenol:chloroform:isoamyl alcohol mixture (Sigma), 250 µL of 0.1mm Zirconia silica beads (Biospec), and 1 sterilized 5/32" diameter low-carbon steel ball (McMaster-Carr Supply Co). The samples were subsequently subjected to disruption using a BeadBeater for 2 cycles of 2 minutes, centrifuged at 4000 rpm at 4°C, and 200 µL of supernatant was used for DNA extraction using the EZ-96 Cycle Pure kit (Omega). Eluted DNA was quantified and diluted 1:100 in sterile water before 16S rRNA sequencing or qPCR analysis.

Determining bacterial abundance using quantitative PCR (qPCR)

The abundance of SFB relative to total bacteria was measured using primers SFB_F and SFB_R (for SFB) and EU_F and EU_R (for total bacteria)[23] (Extended Data Table 5). qPCR was performed using a CFX96 instrument (BioRad) and SYBR FAST universal master mix (KAPA Biosystems). Relative abundance was calculated using the ΔCq method.

Assessment of SFB sensitivity to BD-39 antibacterial activity

BD-39 cloning and purification. Mouse cDNA was used as a template, and the active *defb39* sequence was amplified using Gibson primers (Extended Table 5). Bands were gel-excised

and cloned into a pET21_NESG vector with a C-terminal His-tag for protein expression and purification.

BD-39 expression. *E. coli* (BL21) carrying pET21_NESG_defb39 (Extended Data Table 6) was inoculated into 5mL LB broth containing ampicillin and grown for 16 hours at 37 °C with shaking. The culture was diluted 200-fold in 1L LB medium containing ampicillin and grown at 37 °C with shaking to the mid-exponential phase (OD_{600} 0.6-0.8). 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce BD-39 expression. After 3 hours of induction, the culture was centrifuged, and cell pellets were collected and stored at -80 °C.

Purification, dialysis, and concentration of BD-39. Bacterial pellets were resuspended in lysis buffer [50mM Phosphate buffer, 300mM NaCl, 10mM Imidazole, 1XBugBuster buffer (Fisher, #709223), 1:3000 diluted lysonase bioprocessing reagent (Millipore, #71230) and protease Inhibitor (Thermo, #A32955)]. The lysate was incubated for 30 minutes at room temperature under mild shaking conditions. The lysate was centrifuged, and the supernatant was collected and incubated with IMAC-Ni resin (Thermo, #A50584) for 2 hours at 4°C. The mixture was then loaded onto polypropylene columns (Qiagen, #34964), washed with 20 column volumes of wash buffer (50mM Phosphate buffer, 300mM NaCl, and 34mM imidazole), and eluted with 10mL of elution buffer (50mM Phosphate buffer, 300mM NaCl, 250mM imidazole). The eluates were transferred into 3kDa Centricon columns (Sigma, #UFC700308) and dialyzed with 10mM Tris buffer (pH 8.0) three times. The concentrated protein was then stored at -80 °C in 10mM Tris buffer. The concentration of purified BD39 was measured using the BCA protein assay kit (ThermoFisher, #23225). Control buffer was prepared as above, except *E. coli* pET21_NESG (lacking the *defb39* insert) was used in the initial step.

Ex vivo SFB killing assay. All *ex vivo* steps were performed under anaerobic conditions. Cecal contents of SFB-monoassociated mice were collected, diluted 100-fold in 10mM Tris buffer, and incubated in triplicate with 10μM BD-39 or control buffer for 24 hours at 37°C anaerobically. Two groups of GF C57BL/6N mice (3 mice/group; age- and weight-matched) were orally gavaged

with 200 µL of each incubated sample. Fecal samples were collected and weighed on indicated days. Fecal DNA was extracted as described above, and SFB amounts were quantified using qPCR with SFB-specific primers. DNA from SFB-monoassociated mice was used for the standard curve and to obtain absolute SFB DNA concentration in feces.

16S rRNA sequencing

Amplification and sequencing. 16S rRNA sequencing was conducted on samples collected at the D(-2) timepoint (prior to drug/PBS administration) and the D0 timepoint (12h after the final drug dose). After DNA extraction as described above, the V4 hypervariable region of the bacterial 16S rRNA gene was amplified and sequenced as previously described[24]. Briefly, input genomic DNA was quantified (Quant-IT PicoGreen dsDNA assay kit), normalized to 5 ng/µL, and amplified using barcoded primers[25] and AccuPrime Pfx SuperMix. PCR products were cleaned and normalized (SequalPrep, Invitrogen), pooled in sets of 384 samples, and sequenced on an Illumina MiSeq instrument (2x250 bp, dual 8bp indexing, 15% PhiX spike-in) at the Yale Center for Genome Analysis.

Pre-processing. 16S rRNA sequencing analysis was performed using QIIME2[26]. Barcode extraction was performed using QIIME v1.8, and QIIME v2024.2 was used for subsequent analysis, using emp-paired for the demultiplexing step and DADA2 for truncation and denoising[27].

Alpha-diversity analysis. RESCRIPt was used to prepare a QIIME 2 compatible SSU SILVA reference database based on the curated NR99 (version 138.1) database[28–30]. The classifier was trained based on the V4-targeted 16S primers used[25] and applied to the sequences. The relative abundance of a given taxon (genus or species level, as indicated) was calculated from the feature count of that taxon in a given sample. Differential abundance analysis was

performed using Aldex2[31]. To identify significantly altered taxa, a Welch's t-test was used with Benjamini-Hochberg correction (FDR_{cutoff}<0.2).

Beta-diversity analysis. The Bray-Curtis distance matrix was used (Fig. 1d, and Extended Data Fig. 1c) for principal coordinate analyses (PCoA) of drug-treated and vehicle-treated mice within the same experimental cohort on Day 0, or between D(-2) and D0 timepoints within each mouse group. The weighted UniFrac distance matrix was also utilized to incorporate phylogenetic distance between sequences in estimates of compositional differences (Extended Data Fig. 7a-c)[32]. The amount of variation using both distance matrices was calculated using Permutational Multivariate Analysis of Variance (PERMANOVA) analysis using the Adonis function with 10,000 permutations. The effect size (R-squared) explains the magnitude of dissimilarities between groups and measures the proportion of microbiome changes that can be explained by the drug (for comparisons between PBS- and drug-treated animals within an experimental cohort) or the timepoint (for comparisons between timepoints within each mouse group). *P* values < 0.05 were considered significant. Adonis analysis and visualization were conducted using the R platform (version 4.3.0) and corresponding packages[33,34].

Scanning electron microscopy

CV C57BL/6N mice were treated with digoxin (5 mg/kg or 0.5 mg/kg) or PBS for 2 days as described above. At the D0 timepoint (12 hours after the final drug dose), mice were sacrificed, and ileum tissues were collected for scanning electron microscopy. Briefly, mice were dissected, and ~2mm ileum tissue was placed in 4% paraformaldehyde (PFA). Tissues were changed into a fresh 4% PFA solution after 30 min and incubated at 4ºC overnight, and samples were imaged at the Electron Microscopy Facility at Yale School of Medicine. The samples were pinned open onto silicone pads, and the dissected tissues were refixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.4 for 1 hour. Next, samples were rinsed in 0.1M sodium

cacodylate buffer and post-fixed in 2% osmium tetroxide in 0.1M sodium cacodylate buffer pH 7.4. These were rinsed in buffer and dehydrated through an ethanol series from 30% to 100%. The samples were dried using a Leica 300 critical point dryer with liquid carbon dioxide as transitional fluid. The samples were glued to aluminum stubs using a carbon graphite adhesive, and sputter coated with 4nm platinum/palladium using a Cressington 208HR coating unit. Digital images were acquired in Zeiss CrossBeam 550 between 1.5-2kV at a working distance of 8-12m.

RNA extraction and qRT-PCR

PBS or digoxin-treated mice were sacrificed at indicated time points. Mice were dissected, and approximately 2 cm of different tissues, including terminal ileum, cecum, and colon, were collected. Tissues were flushed to remove gastrointestinal contents, rinsed in PBS, transferred into RNAProtect (Qiagen), and stored at -80ºC. RNA was extracted using the RNeasy Plus Universal mini kit (Qiagen #73404) as per the manufacturer's instructions. RNA concentration was measured using a plate reader (Take3, Biotek). Quantitative reverse transcription-PCR (qRT-PCR) was performed as previously described[35]. Briefly, 2 µg of diluted extracted RNA was converted to cDNA with addition of Superscript II (Invitrogen), random primers (Invitrogen), DTT, and dNTPs. For qRT-PCR, validated primers (Extended Data Table 5) and SYBR FAST universal master mix (KAPA Biosystems) were used on a CFX96 instrument (BioRad). Data were collected and normalized to endogenous *Gapdh* levels. Fold change was calculated using the ΔΔCq method. A *P* value of less than 0.05 was considered significant.

RNA sequencing

Sample preparation and RNA extraction. 6-8 week old female C57BL/6N mice (n=3/group) were treated with digoxin or PBS for 2 days as described above. At the D0 timepoint, mice were sacrificed and approximately 2 cm of the distal small intestine (ileum) was collected. Tissues

were flushed and scraped to remove luminal content, stored in RNAProtect tissue reagent (Qiagen), and stored at -80ºC until RNA extraction. RNA was extracted using the QIAzol™-chloroform method and the Qiagen RNeasy Plus Universal mini kit (#73404) as per the manufacturer's instructions.

RNA-seq quality control. Total RNA quality was determined by estimating the A260/A280 and A260/A230 ratios on a Nanodrop instrument. RNA integrity was determined using an Agilent Bioanalyzer or Fragment Analyzer gel to measure the ratio of ribosomal peaks. Samples with RIN values of 7 or greater were used for library preparation.

RNA-seq library preparation. mRNA was purified from approximately 1000 ng of total RNA with oligo-dT beads and sheared by incubation at 94 ℃ in the presence of Mg^{2+} (Kapa mRNA HyperPrep). Following first-strand synthesis with random primers, second strand synthesis and A-tailing were performed with dUTP for generating strand-specific sequencing libraries. Adapters containing 3' dTMP overhangs were ligated to library insert fragments, and library amplification was used to select and amplify fragments carrying the appropriate adapter sequences at both ends. Strands marked with dUTP are not amplified. Indexed libraries that meet appropriate cut-offs were quantified by qRT-PCR using a commercially available kit (KAPA Biosystems) and insert size distribution determined with the LabChip GX or Agilent TapeStation. Samples with a yield of ≥0.5 ng/μl were used for sequencing.

Flow cell preparation and sequencing. Sample concentrations were normalized to 2.0 nM and loaded onto an Illumina NovaSeq X plus flow cell at a concentration that yields 30 million post-filtering clusters per sample. Samples were sequenced using 100bp paired-end sequencing according to Illumina protocols. The 10 bp unique dual index is read during additional sequencing reads that automatically follow the completion of read 1. A positive control (PhiX library) provided by Illumina was included in every lane at a concentration of 0.3% to monitor sequencing quality in real time.

Data analysis. Signal intensities were converted to individual base calls using Real Time Analysis (RTA) software (Illumina). Primary analysis (sample de-multiplexing and alignment to the mouse genome) was performed using Illumina's CASAVA 1.8.2 software suite. Low-quality reads were trimmed, and adaptor contamination was removed using Trim Galore v0.5.0. Trimmed reads were mapped to the mouse reference genome (GRCm38) using HISAT2 v2.1.0[36]. Gene expression levels were quantified using StringTie v1.3.3b[37] with gene models (M15) from the GENCODE project. Differentially expressed genes were identified using DESeq2[38]. Heat maps and volcano plots were generated using pheatmap v1.0.12 and EnhancedVolcano v1.16.0.

Measurement of anti-microbial peptide gene expression.

For targeted measurements, independent groups of mice (separate from those used for RNA-seq analysis) were treated with digoxin or PBS control as described above; at the D0 timepoint, ileum tissue was collected and host mRNA extracted. Expression levels of genes encoding targeted AMPs was measured by qRT-PCR using gene-specific primers (Extended Data Table 5). Genes encoding anti-microbial peptides (AMPs) were curated from the mouse REACTOME database[39] (Extended Data Table 9). Altered regulation was determined by evaluating $\log_2$ fold change ($\log_2$FC) and adjusted *P* value (p_{adj}) of digoxin-treated compared to PBS-treated animals using DESeq2[38]. Genes with $\log_2FC > 1.5$ and $p_{adj} < 0.05$ were considered significantly upregulated, and those with $\log_2FC < -1.5$ and $p_{adj} < 0.05$ were considered significantly downregulated.

Data availability

Raw and processed RNA sequencing files are available on NCBI's Gene Expression Omnibus (GEO) under accession GSE274850 (To review GEO accession GSE274850:
Go to https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE274850

Enter token itgjmwsmrhovlkx into the box). 16S rRNA sequencing files are also available on NCBI's BioProject under PRJNA1122171 (Reviewer link: https://dataview.ncbi.nlm.nih.gov/object/PRJNA1122171?reviewer=3svd5l0aiss4gibhl0m7701td0).

Statistics and reproducibility

All data were analyzed in GraphPad Prism v10.0.2 and R v4.3.0. Mice were randomized for experiments before being allocated to study groups and respective cages. Statistical significance was calculated using the non-parametric Mann-Whitney test for comparison between two groups unless otherwise indicated; for experiments involving more than two groups, a non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test was used unless otherwise stated. In some experiments, with sample sizes less than 5, an unpaired t-test was used to compare two groups, and one-way ANOVA was used to compare more than two groups. A bar was used to represent median values. All data are representative of at least two independent experiments. For survival analysis, the *P*-value was calculated using the Gehan-Breslow-Wilcoxon test. The exact number of mice used for survival analysis is indicated in the respective figures. For identifying significantly altered taxa from 16S data, a Welch's t-test was used with Benjamini-Hochberg correction (FDR_{cutoff}<0.2). In each figure, *P<0.05, ** P<0.01, ***P<0.001.

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