Precision microbiome reconstitution restores bile acid mediated resistance to Clostridium difficile

Charlie G. Buffie1,2, Vanni Bucci3,4, Richard R. Stein1, Peter T. McKenney1,2, Lilan Ling2, Asia Gobourne2, Daniel No2, Hui Liu5, Melissa Kinnebrew1,2, Agnes Viale6, Eric Littmann2, Marcel R. M. van den Brink7,8, Robert R. Jenq7, Ying Taur1,2, Chris Sander3, Justin R. Cross5, Nora C. Toussaint2,3, Joao B. Xavier2,3 & Eric G. Pamer1,2,8

The gastrointestinal tracts of mammals are colonized by hundreds of microbial species that contribute to health, including colonization resistance against intestinal pathogens. Many antibiotics destroy intestinal microbial communities and increase susceptibility to intestinal pathogens. Among these, Clostridium difficile, a major cause of antibiotic-induced diarrhoea, greatly increases morbidity and mortality in hospitalized patients. Which intestinal bacteria provide resistance to C. difficile infection and their in vivo inhibitory mechanisms remain unclear. Here we correlate loss of specific bacterial taxa with development of infection, by treating mice with different antibiotics that result in distinct microbiota changes and lead to varied susceptibility to C. difficile. Mathematical modelling augmented by analyses of the microbiota of hospitalized patients identifies resistance-associated bacteria common to mice and humans. Using these platforms, we determine that Clostridium scindens, a bile acid 7α-dehydroxylating intestinal bacterium, is associated with resistance to C. difficile infection and, upon administration, enhances resistance to infection in a secondary bile acid dependent fashion. Using a workflow involving mouse models, clinical studies, metagenomic analyses, and mathematical modelling, we identify a probiotic candidate that corrects a clinically relevant microbiome deficiency. These findings have implications for the rational design of targeted antimicrobials as well as microbiome-based diagnostics and therapeutics for individuals at risk of C. difficile infection.

Infection with C. difficile is a growing public health threat. Susceptibility to infection is associated with antibiotic use, and faecal microbiota transplant, which restores microbiota complexity, can resolve recurrent infections. However, the microbiome-encoded genes and biosynthetic gene clusters critical for infection resistance remain largely undefined, limiting mechanistic understanding and development of microbiota-based therapies. We sought to identify, interrogate, and validate sources of microbiome-mediated C. difficile resistance. We first investigated the impact of antibiotics with diverse antimicrobial spectra on the intestinal microbiota and C. difficile susceptibility (Extended Data Fig. 1a). Consistent with prior work from our group, administration of clindamycin resulted in long-lasting susceptibility to infection (Fig. 1a). In contrast, ampicillin induced transient susceptibility (Fig. 1c), whereas enrofloxacin did not increase susceptibility to C. difficile infection (Fig. 1e). C. difficile toxin expression correlated significantly with C. difficile abundance in the intestine (Extended Data Fig. 1b). The antibiotic regimens did not substantially alter bacterial density (Extended Data Fig. 1c), but 16S ribosomal RNA (rRNA) gene amplicon sequencing revealed that the three antibiotics had distinct impacts on intestinal microbiota composition (Fig. 1b, d, f).

We exploited this variance in intestinal bacterial composition and infection susceptibility to relate features of microbiota structure to C. difficile inhibition. Infection susceptibility correlated with decreased microbiota alpha diversity (that is, diversity within individuals) (Fig. 2a), consistent with previous studies. Using weighted UniFrac distances to evaluate beta diversity (that is, diversity between individuals), we found that although clindamycin and ampicillin administration induced distinct changes in microbiota structure, recovery of resistance corresponded with return to a common coordinate space shared by antibiotic-naive animals (Fig. 2b). However, these diversity metrics generally did not resolve the susceptibility status of animals harbouring microbiota with

---

1Infectious Diseases Service, Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, New York 10065, USA. 2Lucille Castori Center for Microbes, Inflammation and Cancer, Memorial Sloan Kettering Cancer Center, New York, New York 10065, USA. 3Computational Biology Program, Sloan-Kettering Institute, New York, New York 10065, USA. 4Department of Biology, University of Massachusetts Dartmouth, North Dartmouth, Massachusetts 02747, USA. 5Bone Marrow Transplant Service, Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, New York 10065, USA. 6Genomics Core Laboratory, Sloan-Kettering Institute, New York, New York 10065, USA. 7Infectious Diseases Service, Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, New York 10065, USA. 8Immunology Program, Sloan-Kettering Institute, New York, New York 10065, USA.

©2015 Macmillan Publishers Limited. All rights reserved
Intestinal microbiota alpha diversity (a) and beta diversity (weighted UniFrac distances) (b) of antibiotic-naive (n = 15) and antibiotic-exposed animals susceptible (n = 21) or resistant (n = 47) to C. difficile infection. Correlation of individual bacterial OTUs with susceptibility to C. difficile infection. Colonization (C. difficile-negative to -positive) and clearance (C. difficile-positive to -negative) events among C. difficile-diagnosed and carrier patients with low alpha diversity (Fig. 2a (red box)) or at early time points after antibiotic exposure (Fig. 2b), suggesting that recovery of more precise microbiota features (for example, individual species) contributed to infection resistance. We correlated resistance with individual bacterial species abundances, corresponding to operational taxonomic units (OTUs, ≥97% 16S sequence similarity) (Extended Data Fig. 1d), and identified 11 bacterial OTUs that correlated strongly with infection resistance (Fig. 2c). These OTUs represented a small fraction of the microbiota membership (6%) and comprised primarily Clostridium cluster XIVa, including the OTU with the strongest resistance correlation, even among animals harbouring low alpha-diversity microbiota, C. scindens (Fig. 2c).

To relate intestinal bacterial species to C. difficile resistance in humans, we extended our study to a cohort of patients undergoing allogeneic haematopoietic stem-cell transplantation (allo-HSCT). The majority of these patients were diagnosed with a haematological malignancy and received chemotherapy and/or total body irradiation as well as antibiotics during transplantation (Extended Data Table 1), incurring reduced microbiota biodiversity associated with increased risk of bacterial bloodstream infections and C. difficile infection. Compared with controlled animal studies, temporal variation in antibiotic administration and sampling times among patients complicates analysis of relationships between microbiota composition and infection susceptibility. To address these challenges, we employed a recently developed systems biology approach that integrates antibiotic delivery schedules and time-resolved microbiota data to model mathematically the microbiota dynamics and infer which bacteria inhibit C. difficile. We included 24 allo-HSCT patients: 12 diagnosed with C. difficile infection and 12 who were C. difficile carriers without clinical infection (Fig. 2d and Extended Data Fig. 2). To facilitate comparisons across data sets, we clustered murine and human microbiota together to define OTUs that together accounted for a majority of both the human and mouse microbiota structure (Extended Data Fig. 3a–c), and applied the modelling approach to the murine study in parallel. We compared the normalized interaction networks from the human (Extended Data Fig. 3d) and the murine models included in microbiota time-series inference modelling. Bacterial species with strong C. difficile interactions in human and murine microbiota models (e) that exist in a conserved subnetwork predicted to inhibit (blue) or positively associate (red) with C. difficile (f). Species interactions in bold type are common to mouse and human. **P < 0.01. In c, P < 0.0005 (‘any biodiversity’, n = 68) or P < 0.05 (‘Low biodiversity’, Shannon diversity index ≤ 1 (n = 16 animals). Centre values (mean), error bars (s.e.m.).

To evaluate causality between intestinal bacteria identified in our analyses and infection resistance, we adoptively transferred resistance-associated bacteria. We cultured a representative consortium of four intestinal bacterial isolates with species-level 16S similarity to OTUs that were conserved in the murine model (Fig. 2e, f), the strongest of which was C. scindens, corroborating our murine correlation-based analyses (Fig. 2c).

Extended Data Fig. 3e) and identified bacteria displaying strong inhibition against C. difficile. Despite some differences across host species networks, the human model identified two C. difficile-inhibiting OTUs that were conserved in the murine model (Fig. 2e, f), the strongest of which was C. scindens, corroborating our murine correlation-based analyses (Fig. 2c).
Figure 3 | Adoptive transfer of resistance-associated intestinal bacteria after antibiotic exposure increases resistance to *C. difficile* infection. Intestinal burden of *C. difficile* c.f.u. (a) and toxin (b) 24 h after *C. difficile* infection of antibiotic-exposed animals receiving adoptive transfers. Weight loss (c) and mortality (d) of animals after infection. e. Correlation of adoptively transferred bacteria engraftment (pre-infection) with *C. difficile* susceptibility. f. Microbiota biodiversity (pre-infection). ****P < 0.0001, **P < 0.01, *P < 0.05, NS (not significant). Mean (f); error bars, range (a), s.e.m. (f).

The growth in *vivo*\(^{11,12}\), but the source and contribution of such metabolites to infection resistance in *vivo* remain unclear. Noting that *C. scindens* expresses enzymes crucial for secondary bile acid synthesis\(^{13}\) that are uncommon among intestinal bacteria\(^{14}\), we hypothesized that the *C. difficile*-protective effects of *C. scindens* may derive from this rare biosynthetic capacity. Analyses of antibiotic-exposed animals (Figs 1 and 2) revealed that recovery of secondary bile acids and the abundance of the gene family responsible for secondary bile acid biosynthesis (as predicted using PICRUSt\(^{15}\)) correlated with *C. difficile* resistance (Fig. 4a, b). Targeted microbiome analysis of the gene family responsible for secondary bile acid biosynthesis indicated that abundance of the bile acid inducible (bai) operon genes correlated strongly with resistance to *C. difficile* infection (Fig. 4c) but that bile salt hydrolase (BSH)-encoding gene abundance did not. These results are consistent with reports indicating that BSH-encoding genes are distributed broadly while an extremely small fraction of intestinal bacteria possess a complete secondary bile acid synthesis pathway\(^{16}\). PCR-based assay of baiCD, which encodes the 7α-hydroxysteroid dehydrogenase enzyme critical for secondary bile acid biosynthesis, revealed that animals that had recovered *C. difficile* resistance after antibiotic exposure harboured a baiCD\(^*\) microbiome, whereas susceptible animals did not (Extended Data Fig. 6a).

Recipients of either the consortium or *C. scindens* harboured baiCD\(^+\) microbiomes with restored abundance of secondary bile acid biosynthesis genes (predicted by PICRUSt) (Extended Data Fig. 6b). Administration of either bacterial suspension also restored relative abundance of the secondary bile acids deoxycholate (DCA) (Fig. 4d) and lithocholate (LCA) (Extended Data Fig. 7a), both of which inhibit *C. difficile* in a dose-dependent fashion (Extended Data Fig. 8a, b), but abundances of primary bile acids were not significantly altered (Extended Data Fig. 7). Metagenomic inference indicated that consortia recipients harboured microbiomes with greater abundances of secondary bile acid biosynthesis genes than *C. scindens* recipients (Extended Data Fig. 6b), perhaps explaining their superior resistance to *C. difficile*. However, intestinal abundances of DCA and LCA were each comparable in the consortia

Figure 4 | *C. scindens*-mediated *C. difficile* inhibition is associated with secondary bile acid synthesis and is dependent on bile endogenous to intestinal content. Relative abundance of secondary bile acid species (a) and biosynthesis gene family abundance predicted by PICRUSt (b) in intestinal content from antibiotic-exposed *C. difficile* susceptible (n = 21), resistant (n = 47), and pre-antibiotic (n = 15) animals. c. Correlation of *C. difficile* susceptibility with the abundance of the gene family responsible for secondary bile acid biosynthesis in intestinal content (n = 6) quantified using shotgun sequencing. d. Intestinal abundance of DCA after adoptive transfer of bacteria (n = 10 per group). e. Correlation of *C. scindens* engraftment with DCA abundance and baiCD status in intestinal content of antibiotic-exposed, adoptively transferred animals (n = 30). f. Bile acid dependent *C. scindens*-mediated inhibition of *C. difficile* quantified *ex vivo* (n = 6 per group).

©2015 Macmillan Publishers Limited. All rights reserved
and C. scindens recipients pre-infection challenge (Fig. 4d and Extended Data Fig. 7a), suggesting additional mechanisms enhanced colonization resistance in consortia recipients. Indeed, of the four transferred bacteria, only C. scindens was baiCD\(^7\) (Extended Data Fig. 6a). Engraftment of C. scindens also correlated strongly with DCA relative abundance and baiCD in recipients, reaching levels observed in antibiotic-naïve animals (Fig. 4e), which indicated that precise transfer and efficient engraftment of this bacterium could restore physiological levels of secondary bile acid synthesis in antibiotic-exposed animals.

We evaluated bile acid dependent microbiota-mediated inhibition of C. difficile using an ex vivo model. Pre-incubation of intestinal content from antibiotic-naïve animals with cholesteryamine, a bile acid sequestrant\(^8\), permitted C. difficile growth (Extended Data Fig. 8c, d) comparable to intestinal content from antibiotic-exposed animals. Consistent with in vivo findings, introduction of C. scindens significantly inhibited C. difficile in the intestinal content from antibiotic-exposed animals. This effect was neutralized when intestinal content was pre-incubated with cholesteryamine (Fig. 4f), indicating that C. scindens-mediated inhibition of C. difficile is dependent upon accessing and modifying endogenous bile salts and recapitulates a natural mechanism of microbiota-mediated infection resistance.

In summary, we show that a fraction of the intestinal microbiota as precise as a single bacterial species confers infection resistance by synthesizing C. difficile-inhibiting metabolites from host-derived bile salts. Our use of a human-derived C. scindens isolate to augment murine C. difficile inhibition emphasizes the conservation of this finding across species and suggests therapeutic and diagnostic applications. The genus Clostridium is phylogenetically complex\(^7,8\), highlighting the value of integrating functional genomic and metabolomic interrogation with 16S rRNA profiling when evaluating probiotic candidates. Most bile-acid 7-dehydroxylating bacteria are clade XIVa Clostridia closely related to one another\(^14,19,20\) and resistance-associated OTUs we identified, suggesting that bai or 165 gene signatures may serve as specific, functionally meaningful biomarkers for infection resistance. The replenishment of secondary bile acids and/or biosynthesis-competent bacteria (such as C. scindens) may contribute to the therapeutic efficacy of faecal microbiota transplant\(^2\). Attempts to manipulate intestinal bile acids directly should be performed with caution since some secondary bile acids have been linked to gastrointestinal cancers\(^22\). Other bacteria may augment resistance by enhancing 7-dehydroxylating Clostridia or through additional orthogonal mechanisms, such as competition for mucosal carbohydrates\(^23\), activation of host immune defences\(^24,25\), or production of antibacterial peptides\(^26\). Knowledge of such mechanisms and the ecological context of those microbes responsible will facilitate amplification of microbiota-mediated pathogen resistance in individuals at risk of infection.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 4 May; accepted 3 September 2014.
Published online 22 October 2014; corrected online 7 January 2015 (see full-text HTML version for details).

1. Buffe, C. G. & Parmer, E. G. Microbiota-mediated colonization resistance against intestinal pathogens. Nature Rev. Immunol. 13, 790–801 (2013).
2. Buffe, C. G. et al. Profound alterations of intestinal microbiota following a single dose of cldinacymycin results in sustained susceptibility to Clostridium difficile-induced colitis. Infect. Immun. 80, 62–73 (2012).
3. Rupnik, M., Wilcox, M. H. & Gerding, D. N. Clostridium difficile infection: new developments in epidemiology and pathogenesis. Nature Rev. Microbiol. 7, 526–536 (2009).
4. van Nood, E. et al. Duodenal infusion of donor feces for recurrent Clostridium difficile. N. Engl. J. Med. 368, 407–415 (2013).
5. Cimermancic, P. et al. Insights into secondary metabolism from a global analysis of prokaryotic biosynthetic gene clusters. Cell 158, 412–421 (2014).
6. Chang, J. Y. et al. Decreased diversity of the fecal microbiome in recurrent Clostridium difficile-associated diarrhea. J. Infect. Dis. 197, 435–438 (2008).
7. Lozupone, C. & Knight, R. UniFrac: a new phylogenetic method for comparing microbial communities. Appl. Environ. Microbiol. 71, 8228–8235 (2005).
8. Taur, Y. et al. Intestinal domination and the risk of bacteremia in patients undergoing allogeneic hematopoietic stem cell transplantation. Clin. Infect. Dis. 55, 905–914 (2012).
9. Kinnebrew, M. A. et al. Early Clostridium difficile infection during allogeneic hematopoietic stem cell transplantation. PLoS ONE 9, e90158 (2014).
10. Stein, R. et al. Ecological modeling from time-series inference: insight into dynamics and stability of intestinal microbiota. PLoS Comput. Biol. 9, e1003388 (2013).
11. Wilson, K. H. Efficiency of various bile salt preparations for stimulation of Clostridium difficile spore germination. J. Clin. Microbiol. 18, 1017–1019 (1983).
12. Sorg, J. A. & Sonenshein, A. L. Bile salts and glycerol as cogerminants for Clostridium difficile spores. J. Bacteriol. 190, 2505–2512 (2008).
13. Kang, D. J., Ridlon, J. M., Moore, D. R., Barnes, S. & Hylomen, P. B. Clostridium scindens baiCD and baiH genes encode stereo-specific 7α/7β-hydroxy-3-oxo-α-4-cholenic acid oxidoreductases. Biochim. Biophys. Acta 1781, 16–25 (2008).
14. Ridlon, J. M., Kang, D. J. & Hylomen, P. B. Bile salt bioconversion by human intestinal bacteria. J. Lipid Res. 47, 241–259 (2006).
15. Langille, M. G. et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nature Biotechnol. 31, 814–821 (2013).
16. Out, C., Groen, A. K. & Brufau, G. Bile acid sequestrants: more than simple resins. Curr. Opin. Lipidol. 23, 43–55 (2012).
17. Collins, M. D. et al. The phylogeny of the genus Clostridium: proposal of five new genera and eleven new species combinations. Int. J. Syst. Bacteriol. 44, 812–826 (1994).
18. Yutin, N. & Galperin, M. Y. A genomic update on clostidial phylogeny: Gram-negative spore formers and other misplaced clostridia. Environ. Microbiol. 15, 2631–2641 (2013).
19. Kitahara, M., Takamine, F., Imaura, T. & Benno, Y. Assignment of Eubacterium sp. VPI 12708 and related strains with high bile acid 7α-dehydroxylating activity to Clostridium scindens and proposal of Clostridium hylomenae sp. nov., isolated from human faeces. Int. J. Syst. Evol. Microbiol. 50, 971–978 (2000).
20. Eyles, H. & Hylomen, B. Identification and characterization of a bile acid 7α-dehydroxylation oøyen in Clostridium sp. strain TO-931, a highly active 7α-dehydroxylation strain isolated from human feces. Appl. Environ. Microbiol. 66, 1107–1113 (2000).
21. Weingarden, A. R. et al. Microbiota transplantation restores normal fecal bile acid composition in recurrent Clostridium difficile infection. Am. J. Physiol. Gastrointest. Liver Physiol. 306, G631–G639 (2014).
22. Bernstein, H., Bernstein, C., Payne, C. M., Dvorakova, K. & Garewal, H. Bile acids as carcinogens in human gastrointestinal cancers. Mutat. Res. 589, 47–65 (2005).
23. Ng, K. M. et al. Microbiota-liberated host sugars facilitate post-antibiotic expansion of enteric pathogens. Nature 502, 96–99 (2013).
24. Jarchum, I., Liu, M., Shi, C., Equinda, M. & Pamer, E. G. Critical role for MyD88-mediated neutrophil recruitment during Clostridium difficile colitis. Infect. Immun. 80, 2989–2996 (2012).
25. Jarchum, I., Liu, M., Lipuma, L. & Pamer, E. G. Toll-like receptor 5 stimulation protects mice from acute Clostridium difficile colitis. Infect. Immun. 79, 1498–1503 (2011).
26. Rea, M. C. et al. Thuricin CD, a posttranslationally modified bacteriocin with a narrow spectrum of activity against Clostridium difficile. Proc. Natl Acad. Sci. USA 107, 9352–9357 (2010).

Acknowledgements E.G.P. received funding from US National Institutes of Health (NIH) grants R01 AI42135 and AI95706, and from the Tow Foundation. J.B.X. received funding from the NIH Office of the Director (DP200008440), NCI (U54 CA148967), and from a seed grant from the Lucille Castori Center for Microbes, Inflammation, and Cancer. C.G.B. was supported by a Medical Scientific Training Program grant from the National Institute of General Medical Sciences of the NIH (award number T32 GM07739, awarded to the Weil Cornell/Rockefeller/Sloan-Kettering Tri-Institutional MD-PhD Program).

Author Contributions C.G.B. and E.G.P. designed the experiments and wrote the manuscript with input from co-authors. C.G.B. performed animal experiments and most analyses. V.B., R.R.S., J.B.X., C.S. and C.G.B. performed microbiota time-series inference modelling and analysis. P.T.M. and C.G.B designed and performed ex vivo experiments. L.L., A.G., A.V. D.N. and M.K. performed 16S amplicon quantification and multiplexed bacterial community sequencing (454, MiSeq) and contributed to data analysis. M.R.M.x.D.B., R.R.J., Y.T., E.L., C.G.B. and E.G.P. assessed clinical parameters and supervised patient cohort analysis. N.C.T. and C.G.B performed metagenomic shotgun sequencing analysis. J.R.C. and H.L. developed the metabolomics analysis platform and performed quantification of bile acid species.

Author Information Study sequence data are deposited in the National Center for Biotechnology Information Sequence Read Archive under accession number SRP045811. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to E.G.P. (pamere@mskcc.org).

©2015 Macmillan Publishers Limited. All rights reserved
**METHODS**

Mouse husbandry. All experiments were performed with C57BL/6j female mice, 6–8 weeks old, purchased from Jackson Laboratories and housed in sterile cages with irradiated food and acidified water. Mouse handling and weekly cage changes were performed by investigators wearing sterile gowns, masks, and gloves in a sterile biosafety hood. All animals were maintained in a specific-pathogen-free facility at Memorial Sloan-Kettering Cancer Center Animal Resource Center. After co-housing for at least 2 weeks, animals (individuals or colonies, as indicated per experiment) were separately housed and randomly assigned to experimental groups. For experiments involving C. difficile infection, mice were administered 1,000 C. difficile VPI 10463 spores in PBS by oral gavage. All animal experiments were performed at least twice unless otherwise noted. Experiments were performed in compliance with the institutional animal care and use committees.

**Murine C. difficile susceptibility time-course experiments.** Mice from three separately housed colonies were kept in the same facility and administered clindamycin (administered by intraperitoneal injection, 200 μg daily), ampicillin (administered in drinking water, 0.1 g/l), or enrofloxacin (administered in drinking water, 0.4 g/l) for 3 days (days 2–0). At each time point after antibiotic cessation (days 1.6, 1, 10, 14, and 21), one mouse from each of the three colonies was randomly selected to be single-housed, infected with C. difficile, and analysed, yielding triplicate biological measurements per group, per time point. Intestinal content (faeces) was sampled before infection challenge for multiparallel 16S amplicon sequencing and microbiota structure analysis. C. difficile susceptibility was determined by selective culture and enumeration of c.f.u. from intestinal content (caecum and colon) 24 h after challenge.

**Murine in vivo adoptive transfer experiments.** Six colonies of mice (n = 30 total) were administered antibiotics as described previously27 and subsequently individually housed and assigned randomly to one of three groups. Two days before infection, groups of individually housed mice (n = 10 per group) received either 1,000,000 c.f.u. of a four-bacteria suspension (containing equal numbers of C. scindens (ATCC35704), Barnesiella intestihominis (isolated from murine faeces in-house), Pseudoflavonifractor capillosus (ATCC29799), and Blautia hansenii (ATCC27752)), a suspension containing 1,000,000 C. scindens, or vehicle (PBS) by gavage. All bacteria were provided under anaerobic conditions in reduced brain–hearts infusion media supplemented with yeast extract and cysteine except for B. intestihominis, which was grown in liquid Wilkins–Chalgren media, and re-suspended in anaerobic PBS before administration to animals. Adoptive transfers of the suspensions were performed once daily for 2 consecutive days before challenge with C. difficile VPI 10463 (1,000 spores in PBS). C. difficile bacteria and cytotoxin were quantified in faecal samples obtained from mice 24 h after infection challenge. Animals were monitored for 21 days after infection challenge and weight loss was recorded.

**Murine ex vivo adoptive transfer experiments.** Three individually housed mice were administered 200 μg of clindamycin by intraperitoneal injection and killed 24 h later. Intestinal content was harvested from the ilea of killed animals, immediately transferred to an anaerobic chamber, and re-suspended in anaerobic PBS. Fractions containing 100 mg of intestinal content from each mouse were distributed and received either C. scindens (100,000 c.f.u.) or vehicle (anaerobic PBS). A third fraction was pre-treated with cholestearmine (1.5 mg) before receiving C. scindens. After transfer, the each suspension was inoculated with vegetative C. difficile (200 c.f.u.), incubated at 37 °C for 60 h, and C. difficile bacteria were quantified by overnight culture on selective media.

**Quantitative C. difficile culture and toxin A and B.** The quantities of C. difficile c.f.u. and cytotoxin in the intestinal (caecal) contents of animals were determined as described previously27.

**Enzymatic assay for secondary bile acid abundance.** The relative abundances of primary and secondary bile acids in the intestinal content of killed animals was quantified using an enzymatic assay as described previously27.

**Sample collection and DNA extraction.** Intestinal microbiota content samples were obtained, snap-frozen, stored, and DNA extracted as described previously. Briefly, a frozen aliquot (~100 mg) of each sample was suspended, while frozen, in a solution containing 500 μl of extraction buffer (200 mM Tris, pH 8.0/200 mM NaCl/20 mM EDTA, 200 μl of 20% SDS, 500 μl of phenol:chloroform:isoamyl alcohol (24:2:1), and 500 μl of 0.1 mm diameter zirconia/silica beads (BioSpec Products). Microbial cells were lysed by mechanical disruption with a bead beater (BioSpec Products) for 2 min, after which two rounds of phenol:chloroform:isoamyl alcohol extraction were performed. DNA was precipitated with ethanol and re-suspended in 50 μl of TE buffer with 100 μg/mL RNase. The isolated DNA was subjected to additional purification with QIAamp Mini Spin Columns (Qiagen). Specimen collection from patients and analysis of the biospecimen group was approved by the Memorial Sloan-Kettering Cancer Center Institutional Review Board. All participants provided written consent for specimen collection and analysis.

Quantification 16S copy number density by rtPCR. DNA extracted from intestinal content samples (faeces) was subjected to rtPCR of 16S rRNA using 0.2 μM concentrations of the broad-range bacterial 16S primers 517F (5′-GCCAGCGAGCCCGGTGAA-3′) and 798R (5′-AGGTTACCTAATCCT-3′) and the DyNaMo SYBR green rtPCR kit (Finnzymes). Standard curves were generated by serial dilution of the PCR blunt vector (Innogen) containing one copy of the 16S rRNA gene derived from a member of the Porphyromonadaceae family. The cycling conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min.

Quantification of baicD by PCR. DNA extracted from intestinal content samples (faeces) was subjected to PCR-based detection of the 7x-HSDH-encoding baicD gene as described previously.28

16S rRNA gene amplification and multiparallel sequencing. Amplicons of the V4-V5 16S rRNA region were amplified and sequenced using an Illumina MiSeq platform for samples in the in vivo and ex vivo adoptive transfer experiments. For each sample, duplicate 50 μl PCR reactions were performed, each containing 50 ng of purified DNA, 0.2 mM dNTPs, 1.5 mM MgCl₂, 1.25 U Platinum Taq DNA polymerase, 2.5 μl of 10X PCR buffer, and 0.2 μM of each primer designed to amplify the V4-V5: 563F (5′-nnnnnnnnnnnnnn-AAYGTYGYGTAAAGN (G3′) and 926R (5′-nnnnnnnnnnnnnn-CCGTCAATTTTTR AGT-3′). A unique 12-base Golay barcode (Ns) preceded the primers for sample identification, and one or eight additional nucleotides were placed in front of the barcode to offset the sequencing of the primers. Cycling conditions were 94 °C for 3 min, followed by 27 cycles of 94 °C for 30 s, 51 °C for 30 s, and 72 °C for 1 min. A condition of 72 °C for 5 min was used for the final elongation step. Replica PCRs were pooled, and amplicons were purified using the Qiagen PCR Purification Kit (Qiagen). PCR products were quantified and pooled at equimolar amounts before Illumina barcodes and adaptors were ligated on using the Illumina TruSeq Sample Preparation protocol. The completed library was sequenced on an Illumina MiSeq platform following the Illumina recommended procedures. Samples in the murine and human C. difficile susceptibility time-course experiments were sequenced using the 454 FLX Titanium platform as described previously. Sequences from allo-HSCT patients were obtained from a previously published study.

Sequence analysis. Sequences were analysed using the mothur29 (version 1.33.3) pipeline. Potentially chimeric sequences were removed using the UChime algorithm44. Sequences with a distance–base similarity of 97% or greater were grouped into OTUs using the average neighbour algorithm and classified using the BLAST (megablast) algorithm and the GenBank 16S rRNA reference database; OTU-based microbial diversity was estimated by calculating the Shannon diversity index. Sequence abundance profiles in each sample were used for downstream statistical and modelling analysis. A phylogenetic tree was inferred using Clearcut35, on the 16s rRNA sequence alignment generated by mothur; unweighted UniFrac was run using the resulting tree, and principal coordinate analysis was performed on the resulting matrix of distances between each pair of samples. PICRUSt (version 0.9.1)33 in combination with QiIME (version 1.6.0)28 was used to predict abundances of the gene family responsible for secondary bile acid biosynthesis (KEGG pathway ko00121) for a set of 83 samples. Maximum likelihood phylogenetic trees (Kimura model, bootstrap of 100 replicates) were constructed using the MEGA 6.06 package from the original sequences of intestinal bacteria as descendant taxa. Distance data from metagenomic shotgun sequencing of six intestinal (ileal) microbiome samples were pre-processed to remove mouse-derived, duplicate, and low-quality reads as well as low-quality bases in accordance with Human Microbiome Project protocols26. The remaining reads were mapped against a set of proteins associated with the secondary bile acid biosynthesis pathway using RAPSearch version 2.07 (ref. 39). For the subsequent analysis, only hits with an E value ≤ 0.1, a minimum alignment length of 30, and a minimum similarity of 50% were considered.

**Quantification of secondary bile acid species.** Samples of murine intestinal content (faeces, ~30 mg) were homogenized using a handheld homogenizer (Omni International) in 80% aqueous methanol and corrected to a final concentration of 0.5 mg per 10 μl. Samples were then sonicated using a Daugene sonicator at high power, 6 × 30 s cycles. Four hundred microlitres of this material were removed and 20 μl of internal standard added (25 μM d4-chenoxyoic acid in 55%/45% methanol/water (v/v)). A further 1 ml methanol was added to the extract and samples were vortexed at 1,400 r.p.m. for 1 h at 37 °C (Thermomixer, Eppendorf). Remaining solid material was removed by centrifugation (21,000g for 10 min) and the supernatant transferred to a glass tube. A second extraction was performed using 1.5 ml methanol, and combined supernatants were dried under a nitrogen gas stream. Finally, samples were re-suspended in 300 μl 55%/45% methanol/water (v/v), filtered through a 3 kDa molecular weight cut-off membrane (AmiconUltra), and transferred to a mass spectrometry vial containing a reduced volume glass insert. Bile acids were separated using an Agilent 1290 HPLC and Cogent C18 column (2.1 mm × 150 mm, 2.2 μm; Microsolv Technology). Mobile phase A: water + 0.05% formic acid; mobile phase B: acetonitrile + 0.05% formic acid; flow rate 0.35 ml/min × 1. Injection volume was
5 μl and the liquid chromatography gradient was from 25% B to 70% B in 25 min. Bile acids were detected using an Agilent 6550 Q-TOF mass spectrometer with JetStream source, operating in negative ionization mode and extended dynamic range. Acquisition was from m/z: 50–1,100 at one spectrum per second; gas temperature: 275 °C; drying gas: 111 min−1; nebulizer: 30 psi; sheath gas: 325 °C; sheath gas flow 101 min−1; V_cap: 4000 V; fragmentor 365 V and Oct 1 RF 750 V. Bile acids (Extended Data Table 2) were identified by their exact mass and confirmed by chromatographic alignment to authentic standards (purchased from Steraloids or Sigma Aldrich). Abundances of the M-H and M+ formate ions were then extracted and summed using ProFinder software (Agilent Technologies) and normalized to the internal standard abundance using Mass Profiler Professional software (Agilent Technologies).

DCA and LCA C. difficile inhibition assays. Growth of C. difficile in brain–heart infusion liquid media supplemented with yeast extract and cysteine, with added DCA (0.1%, 0.01%, 0.001%, final concentration, in water vehicle) or LCA (0.01%, 0.001%, final concentration, in 100% ethanol vehicle), or vehicle alone was monitored by attenuance (D) using a spectrophotometer.

Statistics. Statistical analyses were performed using the R (v. 3.0.2) and GraphPad Prism (version 6.0c) software packages. The Mann–Whitney rank sum test (two-tailed) was used for comparisons of continuous variables between two groups with tied ranks. Inference modelling of mouse microbiota time-series.

To determine the network of bacterial–bacterial interactions and extract native resident bacteria with C. difficile inhibitory properties, we applied the Lotka–Volterra dynamics-based framework of ref. 10 to the mouse data set. This inference framework consists of a regularized least-square regression of the observed data points and the known antibiotic signal against the difference of the log-transformed total abundances in time:

$$\frac{\Delta \ln x_i(t)}{\Delta t} = (\ln x_i(t + \Delta t) - \ln x_i(t))/\Delta t$$

with i = 1, ..., N, where N is the total number of considered OTUs. This results in coefficients characterizing growth, directed interactions, and susceptibilities of each OTU to the external perturbations. The method requires temporal profiles of total abundances of each of the 36 representative OTUs, which were obtained by scaling their normalized abundance from the pyrosequencing run (fraction ranging from 0 to 1) by the total amount of bacteria DNA recovered from each gram of stool or intestinal content. The temporal profile of the C. difficile total abundance was obtained from the c.f.u. counts recovered by plating the caecal content after mouse euthanasia. The last time differences, Δln x_i(t_end), were calculated for each mouse as the difference between the total abundance in the intestinal content (faeces) on the day after C. difficile inoculation, t_end, (also the day of mouse euthanasia) minus the total abundance in the content (faeces) before C. difficile infection or Δln x_i(t_start) = ln x_i(t_start) + 1) − ln x_i(t_start). Similarly the differential profile for C. difficile was evaluated from the log-difference in the scaled colony counts for the corresponding faecal and caecal samples. Antibiotic perturbations (ampicillin, clindamycin, or enrofloxacin) were modelled as a discrete signal when administered at day −2 (Fig. 1). The inference algorithm was run on a total of 240 samples and the global model was selected with a threefold cross-validation scheme on the 75 combined time courses, validating robustness to the introduction of unseen data. In particular, the number of data points outnumbers the number of unknowns; that is, the linear system to be solved is overdetermined, ensuring a sufficient number of constraints by the data for inferring the unknown coefficients.

Inference modelling of allo-HSCT patient microbiota time-series. To determine whether commensal-C. difficile interactions observed in the mouse data were also conserved in humans, we applied the same inference-modelling framework to data from 24 allo-HSCT hospitalized patients. As above, for each of the 36 OTUs, we determined the log-differential in total abundance as the log-difference of the normalized abundance scaled by the corresponding total bacterial DNA per gram of stool at the next sampling event minus the total abundance at the current sampling time. Differential abundance in C. difficile was determined from the rPCR measurements of the C. difficile 16S rRNA gene per gram of faeces. Similarly to the above, we ran the algorithm on a total of 112 samples and the global model was selected applying a threefold cross-validation scheme on the 24 combined time courses. This choice again yields an overdetermined linear system to be solved.
Extended Data Figure 1 | Dynamics of intestinal microbiota structure and *C. difficile* susceptibility after antibiotic exposure. 

**a**, Strategy for determining *C. difficile* susceptibility duration post-antibiotic exposure (*n*=3 separately-housed mouse colonies per antibiotic arm) and relating infection resistance to microbiota structure. 

**b**, Correlation of *C. difficile* c.f.u. and toxin in intestinal content following infection.

**c**, Intestinal bacterial density of animals before and after antibiotic exposure. 

**d**, Relative abundance of bacterial OTUs (≥97% sequence similarity, >0.01% relative abundance) sorted by class (red) and corresponding *C. difficile* susceptibility (blue) among antibiotic-exposed mice (*n*=68) allowed to recover for variable time intervals prior to *C. difficile* infection challenge. Centre values (mean), error bars (s.e.m.) (c). ND, not detectable.
Extended Data Figure 2 | Allo-HSCT patient timelines and C. difficile infection status transitions. Transitions between C. difficile (tcdB-positive) colonization status in patients receiving allogeneic haematopoietic stem-cell transplantation, as measured by C. difficile 16S rRNA abundance during the period of hospitalization (light grey bars). Time points when C. difficile colonization was determined to be positive (red diamonds) and negative (blue diamonds), and when C. difficile infection was clinically diagnosed (black dots) and metronidazole was administered (dark grey bars), are displayed relative to the time of transplantation per patient.
Extended Data Figure 3 | Identification of bacteria conserved across human and murine intestinal microbiota predicted to inhibit *C. difficile.*

Identification of bacterial OTUs abundant in mice (*n* = 68) and humans (*n* = 24) (a) that account for a minority of OTU membership (b) but the majority of the structure of the intestinal microbiota of both host species after antibiotic exposure (c). Subnetworks of abundant OTUs predicted inhibit (blue) or positively associate with (red) *C. difficile* in murine (d) and human (e) intestinal microbiota.
Extended Data Figure 4 | Phylogenetic distribution of resistance-associated intestinal bacteria and isolates selected for adoptive transfer. The maximum likelihood phylogenetic tree (Kimura model, bootstrap of 100 replicates) was constructed using the MEGA 6.06 package from representative sequences of intestinal bacteria associated with resistance to *C. difficile* infection (blue), including cultured representatives subsequently used in adoptive transfer experiments (bold). The tree was rooted using intestinal bacteria associated with susceptibility to infection (red) as an outgroup.
Extended Data Figure 5 | Adoptive transfer and engraftment of four-bacteria consortium or C. scindens ameliorates intestinal C. difficile cytotoxin load and acute C. difficile-associated weight loss. a, C. difficile toxin load in antibiotic-exposed animals receiving adoptive transfers 24 h after C. difficile infection challenge. Animals’ weights 48 h after infection challenge and (b) C. difficile c.f.u. 24 h after infection challenge (c). d, Engraftment of bacterial isolates in the intestinal microbiota of antibiotic-exposed animals 2 days after adoptive transfer of B. intestihominis, P. capillosus, B. hansenii, and/or C. scindens. e, Intestinal bacterial density (faeces) from antibiotic-exposed mice administered suspensions containing four bacteria, C. scindens, or vehicle (PBS) as measured by rtPCR of 16S rRNA genes. ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05; Mann–Whitney (two-tailed) (a, b, d, e), Kruskal–Wallis with Dunn correction (c) (n = 6–10 per group). Centre values, mean; error bars, s.e.m. Results are representative of at least two independent experiments. Numbers under group columns in d denote the number of mice with detectable engraftment of the given bacterium (out of ten possible separately housed animals per group).
Extended Data Figure 6 | Adoptive transfer of consortia or C. scindens restores baiCD and the abundance of the gene family responsible for secondary bile acid biosynthesis. a, PCR-based detection of the 7α-HSDH-encoding baiCD gene in bacterial isolates, intestinal microbiomes (faeces) of animals before antibiotic exposure, and intestinal microbiomes (faeces) of animals that, after antibiotic exposure, remained susceptible to C. difficile or recovered resistance to infection spontaneously or after adoptive transfer of bacterial isolates. b, Reconstituted abundance of the gene family responsible for secondary bile acid biosynthesis, as predicted by PICRUSt, in antibiotic-exposed animals receiving adoptive transfers (n = 10 per group). ***P < 0.001; *P < 0.05; NS, not significant; Mann–Whitney (two-tailed) (b). Centre values, mean; error bars, s.e.m.
Extended Data Figure 7 | Impacts of adoptive transfers of bacteria on intestinal abundance of bile acids. Intestinal abundance of the secondary bile acids LCA (a), ursodeoxycholate (UDCA) (b), and primary bile acids (c–f) in mice after antibiotic exposure and adoptive transfer of bacteria indicated. ****p < 0.0001, *p < 0.05, NS (not significant); Kruskal–Wallis test with Dunn’s correction. Centre values, mean; error bars, s.e.m.
Extended Data Figure 8 | *C. difficile* growth inhibition by secondary bile acids and intestinal content from antibiotic-naive animals. Addition of the secondary bile acids DCA (a) or LCA (b) to culture media inhibits *C. difficile*. Bile acid dependent inhibition of *C. difficile* enumerated by recovery of c.f.u. after inoculation of vegetative *C. difficile* into cell-free (c) or whole (d) intestinal content harvested from C57BL/6J mice (*n* = 5 or 6 per group), with or without pre-incubation with cholestyramine. **P < 0.01; Mann–Whitney (two-tailed) (c, d).
| Parameter                  | No. (% of patients) |
|----------------------------|---------------------|
| Age (years)                |                     |
| ≤29                        | 2/24 (8.3%)         |
| 30-39                      | 5/24 (20.8%)        |
| 40-49                      | 2/24 (8.3%)         |
| 50-59                      | 6/24 (25.0%)        |
| ≥60                        | 9/24 (37.5%)        |
| Sex (female)               | 10/24 (41.7%)       |
| Underlying Disease         |                     |
| Leukemia                   | 11/24 (45.8%)       |
| Lymphoma                   | 5/24 (20.8%)        |
| Multiple Myeloma           | 3/24 (12.5%)        |
| Myelodysplastic Syndrome   | 3/24 (12.5%)        |
| Other                      | 2/24 (8.3%)         |
| Conditioning Intensity     |                     |
| Nonmyeloablative           | 4/24 (16.7%)        |
| Reduced intensity          | 4/24 (16.7%)        |
| Myeloablative              | 16/24 (66.7%)       |
| T-cell depletion           | 13/24 (54.2%)       |
| Stem cell source (cord vs. | 5/24 (20.8%)        |
| other)                     |                     |
| Time to engraftment (≥14d) | 5/24 (20.8%)        |
| Fever (T≥100.4°F)†         | 21/24 (87.5%)       |
| Vital Status: Dead†        | 1/24 (4.2%)         |
| Total                      | 24/24 (100.0%)      |

* Engraftment was defined as an absolute neutrophil count of more than 500 cells per microlitre for 3 consecutive days.
† Assessed during inpatient allogeneic haematopoietic stem-cell transplantation hospitalization (from 15 days before transplant to 35 days after transplant).
Extended Data Table 2 | Retention times for bile acids quantified by high-performance liquid chromatography–mass spectrometry

| Compound | Molecular formula | Accurate mass | Retention time (min) | CAS number |
|----------|-------------------|---------------|----------------------|------------|
| LCA      | C_{20}H_{32}O_{5} | 376.29775     | 24.16                | 434-13-9   |
| UDCA     | C_{20}H_{32}O_{4} | 392.29266     | 16.59                | 128-13-2   |
| DCA      | C_{20}H_{32}O_{4} | 392.29266     | 20.42                | 83-44-3    |