Engineering probiotics to inhibit *Clostridioides difficile* infection by dynamic regulation of intestinal metabolism

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*Clostridioides difficile* infection (CDI) results in significant morbidity and mortality in hospitalised patients. The pathogenesis of CDI is intrinsically related to the ability of *C. difficile* to shuffle between active vegetative cells and dormant endospores through the processes of germination and sporulation. Here, we hypothesise that dysregulation of microbiome-mediated bile salt metabolism contributes to CDI and that its alleviation can limit the pathogenesis of CDI. We engineer a genetic circuit harbouring a genetically encoded sensor, amplifier and actuator in probiotics to restore intestinal bile salt metabolism in response to antibiotic-induced microbiome dysbiosis. We demonstrate that the engineered probiotics limited the germination of endospores and the growth of vegetative cells of *C. difficile* in vitro and further significantly reduced CDI in model mice, as evidenced by a 100% survival rate and improved clinical outcomes. Our work presents an antimicrobial strategy that harnesses the host-pathogen microenvironment as the intervention target to limit the pathogenesis of infection.
Clostridioides (or Clostridium) difficile infection (CDI) is the leading cause of healthcare-associated infectious diarrhoea globally1–3. In the United States alone, CDI annually causes over 500,000 infections and 20,000 deaths, with an annual estimated healthcare cost of $4.8 billion4. The eradication of CDI is impaired by the frequent recurrence of the disease; 20.9% of CDI cases recur once or more within 30 days post treatment5. Paradoxically, while the CDI treatment regimen involves antibiotic therapy, antibiotics have been established as a major risk factor for CDI. Antibiotics trigger and prolong a state of dysbiosis in the intestine, characterised by a loss of bacterial diversity and altered production of microbiome-derived intestinal metabolites, hindering the recovery of the normal colonic microbiome and leading to CDI and recurrent CDI (rCDI)6,7. The pathogenesis of CDI and rCDI is intrinsically related to the ability of C. difficile to shuffle between active vegetative cells and dormant endospores through the processes of germination and sporulation8. Carriers of C. difficile endospores may remain asymptomatic as the endospores remain dormant in the human gastrointestinal tract. Furthermore, endospores are inherently resistant to antibiotics6. Perturbation to the native balance of the gastrointestinal tract. Furthermore, endospores are inherently resistant to antibiotics6. Perturbation to the native balance of the microbiome, hindering the recovery of the normal colonic diversity and altered production of microbiome-derived intestinal metabolites, facilitating the colonization and infection by C. difficile. The persistence of the dysbiosis induced by antibiotic therapy involves antibiotic therapy, antibiotics have been established as a major risk factor for CDI. Antibiotics trigger and prolong a state of dysbiosis in the intestine, characterised by a loss of bacterial diversity and altered production of microbiome-derived intestinal metabolites, hindering the recovery of the normal colonic microbiome and leading to CDI and recurrent CDI (rCDI)6,7.

Results
Hydrolase-mediated bile salt deconjugation inhibits C. difficile germination and growth. To validate our hypothesis that the modulation of bile salt profiles controls C. difficile germination, we assessed the effect of taurocholate and its deconjugated form, cholate, on the germination and growth of C. difficile (Supplementary Fig. 1A). While the physiological level of bile salts varies along the intestinal tract, we have taken 2 mM of bile salts as an average representative concentration to use for in vitro assays9,10. Figure 2A, B show that cholate significantly decreased the germination of the endospores by up to 81% and the number of viable vegetative cells of C. difficile strains by up to 82%. We then chose the bile salt hydrolase Cbh of Clostridium perfringens as an enzyme to be introduced into probiotics to deconjugate taurocholate into cholate. We used this enzyme because Cbh uses taurocholate as a preferred substrate and remains more functional at physiological pH than other bile salt hydrolases21. Figure 2C shows that recombinant Cbh (Supplementary Fig. 1B, C) converted 99.2% of the taurocholate into cholate. Cbh also deconjugated glycocholate, another conjugated primary bile salt present in the gut, with similar efficiency. Glycocholate is also reported to be a germinant of C. difficile, and the deconjugating activity of Cbh against both taurocholate and glycocholate achieved >99% reduction of conjugated bile salts (Fig. 2D). Figure 2E, F show that the group with recombinant Cbh exhibited a 96% lower endospore germination rate and 89% fewer viable vegetative cells than the control group with taurocholate and no Cbh. These results suggested that recombantly expressed Cbh deconjugated taurocholate into cholate and that this enzymatic deconjugation significantly inhibited the germination of endospores and the growth of vegetative cells of C. difficile.

Engineering of dysbiosis-sensing circuits in probiotics. We chose E. coli Nissle 1917 as the probiotic host because it is an extensively studied probiotic with a long safety record in humans22,23. Furthermore, as a gram-negative bacterium, E. coli Nissle 1917 can be employed in combination with the current CDI therapy, which entails the use of antibiotics that target gram-positive bacteria. We used the previously reported auxotrophic E. coli Nissle 1917 strain as the base strain, which requires exogenous D-alanine for survival (denoted by EcN), because it allows E. coli to utilise D-alanine for survival (denoted by EcN), because it allows E. coli to utilise D-alanine for survival (denoted by EcN), because it allows E. coli to utilise D-alanine for survival (denoted by EcN), because it allows E. coli to utilise D-alanine for survival (denoted by EcN), because it allows E. coli to utilise D-alanine for survival (denoted by EcN), because it allows E. coli to utilise D-alanine for survival (denoted by EcN), because it allows E. coli to utilise D-alanine for survival (denoted by EcN), because it allows E. coli to utilise D-alanine for survival. We engineered endospores limited the germination of endospores and the growth of vegetative cells of C. difficile in vitro and, furthermore, significantly reduced CDI in murine models, as evidenced by a 100% survival rate and improved clinical symptoms. Our work presents a targeted microbiome-modulation strategy that harnesses the host-pathogen microenvironment as the intervention target to limit the pathogenesis of infection, suggesting the potential for the modulation of bile salt metabolism to serve as a mechanism of action for CDI therapy.

Based on increasing evidence that microbiome dysbiosis preludes the onset of CDI7,8,12,13,19,25, we hypothesised that a genetic sensor that regulates the expression of Cbh in response to microbiome dysbiosis might increase the efficacy of our engineered probiotics against CDI. We chose sialic acid as a proxy signal for dysbiosis because the level of sialic acid is elevated in the intraluminal space upon antibiotic treatment, which has been postulated to be a result of a dysbiosis-mediated imbalance between sialic acid-catabolising and/or sialidase-expressing members of the microbiome19. Sialic acid also supports the pathogenesis of gastrointestinal infections, including CDI, possibly because pathogens can use sialic acid as a carbon
source and decorate the cellular surface with sialic acid to evade host responses.\textsuperscript{19,26–28}

We selected the promoter pNanA as a core sensor element because it has been postulated to be responsive to sialic acid.\textsuperscript{29} We characterised pNanA with the transcriptional regulator NanR (Supplementary Fig. 2A). Supplementary Fig. 2B shows that pNanA was responsive to sialic acid when NanR was expressed, while pNanA alone resulted in a high background with a low response. Then, to improve the dynamic range of the pNanA-NanR sensor (termed ‘Sensor’), we modulated the expression level of NanR using a set of constitutive promoters and ribosome binding sites. Fig. 3A shows that the combination of J23113 (promoter) and rbs4 (RBS) led to the highest dynamic range in response to sialic acid. We also observed that the pNanA-NanR sensor dynamically regulated the expression of GFP (reporter protein), where the removal of sialic acid brought the expression back to the basal level (Supplementary Fig. 2C, D). Notably, the pNanA sequence contains a catabolite activator protein (CAP) binding site. In line with this feature, Fig. 3B shows that glucose significantly reduced the GFP expression in response to sialic acid. The level of glucose is significantly lower in the ileum, caecum and colon,\textsuperscript{30} where C. difficile primarily colonises.\textsuperscript{13} Therefore, we hypothesised that the pNanA-NanR sensor can provide another layer of expression control such that the expression of Cbh increases when the engineered probiotics reach the target sites: the ileum, caecum and colon.

**Engineering of sensor-amplifier-actuator circuits in probiotics.**

Next, we examined whether the expression of Cbh (termed ‘Actuator’), when induced by the sialic acid sensor, could inhibit the germination of C. difficile endospores. Figure 3C shows a reduction in endospore germination by up to 47%, which indicated a significantly lower efficacy than that of purified recombinant Cbh (Fig. 2D). We also observed low conversion of taurocholate into cholate (Fig. 3D). Therefore, we hypothesised that the low germination inhibition might be due to insufficient expression of Cbh. To test this hypothesis, we added an amplifier module to the sensor-actuator circuit. Specifically, the transcriptional activator gene cadC was placed under the control of the promoter pNanA so that CadC (termed ‘Amplifier’) could activate the promoter pCadBA to amplify Cbh expression. The amplifier module was evaluated for sialic acid-responsivity (Supplementary Fig. 2E). CadC regulates the cad operon and has been shown to be pH sensitive,\textsuperscript{31} providing an additional layer of expression control.\textsuperscript{32} A module with constitutive GFP expression was also evaluated for comparison (Supplementary Fig. 2F). The final sensor-amplifier-actuator circuit, where Cbh served as an actuator (Fig. 3E), resulted in significantly increased actuator (Cbh) expression of the circuit (Fig. 3F). The expression level of Cbh under sialic acid induction was comparable to that of purified recombinant Cbh (Fig. 3G), which led to significant germination and growth inhibition (Fig. 2D, E). These results suggest that the EcN harbouring the aforementioned sensor-amplifier-actuator circuit (denoted by EcN-Cbh), which comprises pNanA, NanR, CadC, pCadBA and Cbh, might significantly inhibit the germination and growth of C. difficile.

**The engineered probiotics inhibit C. difficile germination and growth.**

We then examined the extent to which EcN-Cbh could deconjugate taurocholate into cholate and reduce the germination of C. difficile endospores. In response to sialic acid, EcN-Cbh fully converted taurocholate into cholate (Fig. 4A) and caused a 98% reduction in endospore germination (Fig. 4B). Notably, the absence of sialic acid led to less but still considerable deconjugation (45%) and germination reduction (90%), likely due to the basal expression of Cbh in EcN-Cbh. Basal expression of Cbh did not significantly alter the growth of the host strains (Supplementary Fig. 3A). In addition, no extracellular deconjugation activity of EcN-Cbh was observed (Supplementary Fig. 4), suggesting that the deconjugation action of EcN-Cbh remained intracellular, although Cbh is reportedly secreted in gram-positive C. perfringens.\textsuperscript{33}

Next, we determined whether EcN-Cbh could inhibit the growth of the vegetative cells of C. difficile. Figure 4C shows that EcN-Cbh strongly reduced the number of viable vegetative cells. To further investigate the mechanism of this reduction, we evaluated the numbers of viable vegetative C. difficile cells after culture with EcN-Cbh, taurocholate and cholate. Supplementary Fig. 3B, C indicate that the growth inhibition was due to cholate. Figure 4C shows that 1-hour preincubation with taurocholate was...
sufficient to significantly inhibit the vegetative growth of C. difficile cultured with EcN-Cbh. Together, these results suggest that EcN-Cbh significantly inhibits the germination and vegetative cells of C. difficile.

The engineered probiotics reduce C. difficile toxicity. To investigate whether and the extent to which germination and growth inhibition by EcN-Cbh could lead to a reduction in the toxicity of C. difficile, we first assessed the amount of the C. difficile exotoxin TcdA when C. difficile was cocultured with EcN-Cbh. Figure 4D shows that EcN-Cbh significantly decreased the level of TcdA in the culture, which likely resulted from lowered germination and growth of C. difficile. Then, we evaluated the viability of human epithelial colorectal adenocarcinoma cells (Caco-2 cells) upon exposure to the supernatant of C. difficile. EcN-Cbh significantly improved the viability of Caco-2 cells exposed to C. difficile (Fig. 4E). These results together suggest that pre-treatment with EcN-Cbh reduced C. difficile toxicity.

The engineered probiotics inhibit CDI in a murine model. The aforementioned in vitro results prompted us to hypothesise that EcN-Cbh might inhibit CDI in vivo. To test this hypothesis, we evaluated whether and the extent to which EcN-Cbh could reduce mortality and morbidity in a murine model of CDI.
previously established\(^ {34,35}\) (Fig. 5A). The model mice were given engineered probiotics prior to being exposed to a virulent strain of \textit{C. difficile}, VPI10463, that has been reported to induce significant mortality and symptomatic displays\(^ {34,36}\). The evaluation was conducted using a treatment group (EcN-Cbh) and five control groups: (i) a no-sensor control (EcN-Cbh-S-) group, (ii) a no-amplifier control (EcN-Cbh-A-) group, (iii) a no-actuator control (EcN-Cbh-A') group, (iv) a wild-type control (EcN-WT) group and (v) an infection control (CD) group (Fig. 5B). Figure 5C shows the extent of deconjugation activities of the probiotics. Supplementary Figs. 5, 6 and 8 show further characteristics of these probiotics, such as growth, expression,
antimicrobial sensitivity and intestinal viability. We assessed the clinical symptoms, mortality and weight of the CDI model mice as previously described. Challenge with *C. difficile* (10⁷ CFU) was performed on day 0. The mortality, weight, and clinical symptoms of the mice were then monitored over the course of 9 days. The clinical symptoms were scored according to a previously established standard.

Figure 5D shows that EcN-Cbh led to a 100% survival rate in the model mice, while all the controls resulted in significantly lower survival rates ranging from 60 to 14.3%. Figure 5E indicates
that the infection model mice fed EcN-Cbh exhibited the least weight loss, especially between day 2 and day 4, when the model mice showed the most severe symptoms. Figure 5F shows that the group of model mice fed EcN-Cbh had the lowest clinical symptom score (CSS), 4.42. CSSs were used to indicate the severity of the infection and ranged from normal (0 to 2) to mild (3 to 5), moderate (6 to 8) or severe (9 to 12) based on epithelial tissue damage, mucosal oedema, cell infiltration38 (Fig. 6D). Model mice treated with EcN-Cbh exhibited the least clinical symptoms compared to the other groups, indicating that EcN-Cbh significantly reduced CDI in the model mice, as evidenced by a 100% survival rate, improved clinical symptoms and a decrease in the abundance of C. difficile.
Ecn-Cbh displayed lower HISs than the control mice. These results suggest that Ecn-Cbh treatment markedly alleviates tissue damage due to CDI in model mice.

The engineered probiotics modulate bile salt composition. Next, to determine whether and how much the sialic acid level was elevated and to assess whether Ecn-Cbh modulated bile salt profiles in vivo as hypothesised, we analysed the faeces of infection model mice as described in Fig. 7A. Figure 7B shows that the level of sialic acid was increased 6-fold upon antibiotic treatment from day -6 to -3, when Ecn-Cbh was introduced, supporting our use of a sialic acid biosensor in the probiotics. Figure 7C shows that Ecn-Cbh decreased the taurocholate level and increased the cholate level in the infection model mice from day -3 to day 0 before C. difficile was introduced. Figure 7D shows that the Ecn-Cbh and the no-sensor control resulted in significantly reduced taurocholate and correspondingly elevated cholate levels. We also quantified other bile salts, glycocholate, chenodeoxycholate, lithocholate, and deoxycholate, in the faeces of the model mice upon administration of the engineered probiotics (Supplementary Fig. 9). Supplementary Fig. 9 shows that Ecn-Cbh and the no-sensor control led to an increased level of deoxycholate, a derivative of cholate, while the Cbh-expressing probiotics (i.e. Ecn-Cbh, the no-sensor control, and the no-amplifier control) resulted in an increased level of chenodeoxycholate and its derivative lithocholate. These results suggest that Ecn-Cbh increases cholate levels and decreases taurocholate levels in model mice, as hypothesised.

Discussion

We report the engineering of probiotics to inhibit CDI through dynamic regulation of bile salt hydrolase. The engineered probiotics harbour a genetic circuit that comprises a sensor, an amplifier, and an actuator. The circuit was designed to respond to and control intestinal signals to inhibit the germination and growth of C. difficile through two actions: inhibition of germination via hydrolase-mediated deconjugation and inhibition of vegetative growth via the deconjugated product cholate. This...
work presents a targeted microbiome-modulation strategy that harnesses the host–pathogen microenvironment as the intervention target to limit the pathogenesis of infection. We based this strategy for CDI on several findings from prior studies. First, the intestinal microbiome modifies bile salt profiles\textsuperscript{9,15–17}. Second, microbiome dysbiosis caused by antibiotic treatment disrupts intestinal bile salt metabolism\textsuperscript{6,7,13}. Third, the dysregulation of microbiome-mediated bile salt metabolism results in an imbalance in the profile of bile salts, contributing to CDI and rCDI\textsuperscript{7,9,12}. This imbalance in the host–pathogen microenvironment was the primary intervention target for our work, in which probiotics were genetically modified to aid in the restoration of intestinal bile salt metabolism in response to antibiotic-induced microbiome dysbiosis.

Our work suggests that modulation of bile salt metabolism can serve as a mechanism of action for the development of therapeutics against CDI, supporting prior propositions of the use of a germination/sporulation-based strategy for CDI treatment\textsuperscript{17,18,37}. We have provided evidence that the dynamic expression of bile salt hydrolase can significantly inhibit CDI in vivo. We hypothesise that this inhibition is aided by the rebuilding of intestinal bile salt metabolism, which promotes the prevention of spore germination and the restoration of microbiome colonisation resistance\textsuperscript{9,17}. Recent clinical practice guidelines for CDI recommend faecal microbiota transplantation (FMT)\textsuperscript{39}, which facilitates the restoration of the pre-morbid state of the microbiome, for patients with rCDI\textsuperscript{25,40,41}. This recommendation signifies the importance of therapeutic mechanisms that mediate the repopulation of the normal colonic microbiome for CDI treatment. Our work substantiates the value of the regulation of bile salt metabolism among such therapeutic mechanisms.

The in vitro and in vivo efficacy of the engineered probiotics demonstrated in this work suggests the potential utility of several therapeutic regimens. First, the engineered probiotics can serve as adjuvants to standard antibiotic therapy against CDI, assisting antibiotics in inhibiting and killing vegetative, virulent C. difficile.
cells while preventing further germination of *C. difficile* spores. Notably, our engineered probiotics, which are gram-negative bacteria, can withstand the standard CDI antibiotic therapy that includes vancomycin, therefore enabling co-administration. This combination therapy may enhance the efficacy of antibiotic therapy against CDI, which disrupts the intestinal microbiome and bile salt metabolism. Second, the engineered probiotics can be administered to patients at high risk who are to undergo antibiotic therapy to prevent the potential onset of *C. difficile* infection. This probiotic administration could continue during and following antibiotic therapy, enabling the normal microbiome to repopulate the intestine, and offering protection against CDI.

It is of particular interest that the complete circuit, which comprises the sensor, amplifier, and actuator, was required for significant infection reduction (i.e., a 100% survival rate and improvement of clinical symptoms) in the model mice in the current study. From this result, we make several inferences. First, sensor-controlled regulation of bile salt hydrolase was advantageous in infection control. The genetically encoded sensor developed in our work was shown in vitro to respond dynamically to sialic acid, a proxy signal for microbiome dysbiosis whose levels were elevated upon antibiotic treatment in our murine model. We conjecture that the levels and spatial distribution of sialic acid in the model mice dynamically controlled the extent of the modulation by the hydrolase-harbouring actuator of the circuit in a spatial-specific manner. This dynamic and spatial-specific modulation might have been augmented by the sensor element (pNanA) and the amplifier element (cadC), where additional regulation by glucose and pH was applied, as stated above. The critical role of the sensor in the circuit was also evidenced by the result that the no-sensor control led to the lowest survival rate despite the constitutive expression of hydrolase, which might have imposed an additional metabolic burden on the probiotic host. This critical role is corroborated by the result that the sensor was required for hydrolase-expressing probiotics to increase the microbiome diversity in the model mice upon CDI. Second, our finding that all the control groups except the no-amplifier control group exhibited survival rates significantly lower than that of the infection control supports prior findings that probiotic administration can impair gut microbiome recovery by prolonging microbiome dysbiosis. Third, both the adequate expression level of hydrolase and its controlled regulation are strongly correlated in the model mice, neither was linearly correlated with the extent of infection inhibition except in the complete circuit group, which showed the most improved survival rate and clinical symptoms. For instance, the no-sensor control, which constitutively expressed hydrolase, led to a lower survival rate compared to that of the complete circuit but resulted in a low survival rate and a high CSS. Second, the finding that the complete circuit was a prerequisite for CDI protection indicates the need for a next-level understanding of the mechanisms of the protection and the interplay between the modules of the circuit for clinical translation. This mechanistic elucidation also entails the testing of the circuits for other *C. difficile* strains, in particular, in the spore form. Third, it is plausible that hydrolase-mediated deconjugation by our engineered probiotics shifted the profiles of other intestinal metabolites, which might have helped limit the infection. Future research could examine a complete set of intestinal metabolites and microbes that contribute to anti-germination and infection inhibition as well as determine the appropriate dosage and duration for the engineered probiotics.

In conclusion, we propose that the mechanism of action of the antimicrobial strategy presented in this work, which modulates the host–pathogen microenvironment for infection control rather than exerting direct lethality, should be considered for the development of future microbiome-based antimicrobial therapeutics.

**Methods**

**Culture and maintenance.** All *E. coli* strains were maintained in Luria broth (LB) medium at 37 °C with appropriate antibiotics and/or supplements unless stated otherwise (final concentrations: 30 μg/mL kanamycin and 50 μg/mL D-alanine). Cloning was performed with *E. coli* Top10 (Invitrogen), heterologous protein expression was performed with *E. coli* BL21 (Thermo Fisher Scientific), and characterisation was performed with a modified *E. coli* Nissle 1917 ECN strain. The *C. difficile* culture was carried out in a Coy Lab Vinyl Anaerobic Chamber and anaerobic jar to maintain anaerobic conditions. Cells were cultured in brain heart infusion-supplemented (BHI) medium supplemented with 5% v/v yeast extract and 0.03% w/v l-cysteine. Taurocholate was supplemented as necessary. The cells were incubated anaerobically at 37 °C. *C. difficile* CD630, VPI10463, BAA1870 and 9689 were obtained from the American Type Culture Collection (ATCC). The Caco-2 cell line (ATCC) was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% foetal bovine serum and 1% penicillin/streptomycin (10000 U/mL). The cells were incubated at 37 °C under a 5% CO2/95% air atmosphere. Cell passage was performed at ~70 to 90% confluence.

**C. difficile endospore germination efficiency assay and vegetative cell growth assay.** *C. difficile* endospores were extracted by resuspending *C. difficile* CD630 following 6 days of incubation in 50% v/v ethanol/PBS for 1 h. The cell pellets were then washed twice in PBS before incubation at 70 °C for 20 min and resuspended in PBS with 1% w/v bovine serum albumin (BSA). The extracted endospores were counted under anaerobic conditions for CFU determination by tenfold serial dilution on BH agar containing 2 mM taurocholate, unless otherwise stated. The endospores were diluted in PBS with 1% w/v BSA to a final concentration of 10^9/mL and then counted under anaerobic conditions for CFU determination on BH agar plates containing bile salts at concentrations ranging from 1 to 5 mM. To assess endospore germination efficiency or vegetative cell growth with Bbb-treated or engineered Ecn^-treated taurocholate, purified 10 μM Cbh or engineered Ecn at an optical density of 600 nm wavelength (OD_{600}) of 0.5 was incubated with 10 mM taurocholate for 3 h before the addition of *C. difficile* endospores or vegetative cells. The bile salt reaction mixture was diluted five times to achieve a final 2 mM bile salt concentration and incubated anaerobically with endospores at a final concentration of 10^9/mL for 1 h or with vegetative *C. difficile* at an OD_{600} of ~2 x 10^7 (CFU/mL) for 12 h. *C. difficile* were then counted for CFU determination under anaerobic conditions on BH agar. All assays were performed in triplicate unless stated otherwise.

**Molecular cloning and protein expression.** The BglBrick standard was used in the design and cloning of biological parts. Cloning was performed with a pEaaK vector. The Cbh gene was codon-optimised for expression in *E. coli* and synthesised (4x leader hexahistidine tag, final size). Cbh was expressed and purified through immobilised metal ion affinity chromatography (IMAC) with nickel-charged agarose resins followed by size exclusion chromatography on a HiLoad 16/600 Superdex 75 pg column (GE Healthcare Life Sciences). Selected fractions were then concentrated with ultrafiltration columns at 4°C, followed by molecular weight cut-off (MWCO) in protein buffer (50 mM Tris-HCl, 100 mM NaCl, 10% v/v glycerol, pH 8.0).

**Characterisation of bile salt deconjugation activity.** Bile salts were detected with a high-performance liquid chromatography (HPLC) system. To assay protein activity, 10 μM purified Cbh was incubated with 10 mM taurocholate in protein buffer at 37 °C for 3 h. Four volumes of methanol were then added to precipitate the protein. The mixture was dried by a vacuum concentrator, resuspended in 70% v/v acetonitrile, and subjected to HPLC analysis. For assays involving probiotics, the cells were pre-induced with 0.2% w/v sodium taurocholate for 3 h at 37 °C, adjusted to an OD_{600} of 3.0 and further incubated with 10 mM taurocholate for 3 h. The supernatants were then collected for quantification of the bile acids using Tripletrap® tandem MS (MS/MS) in negative ionisation mode with an Eclipse Plus C18 column (2.1 mm x 100 mm, 1.8 µm particle size) (Agilent Technologies). (See 'Liquid chromatography (LC)-mass spectrometry (MS) quantification of faecal bile salts' for further details).

**Growth and protein expression assay.** Cells or constructs were characterised with growth assays or fluorescent protein expression assays on a Synergy H1 multimode plate reader (BioTek). All assays were performed in triplicate unless stated otherwise. The absorbance of the cell culture at a 600 nm wavelength (OD_{600}) was read to indicate the cell density. Green fluorescent protein (GFP)
fluorescence (excitation: 485 nm, emission: 528 nm) in the sample medium relative to the blank medium was read to indicate GFP expression. The initial cell densities (OD600 values) of pre-cultures at the early exponential phase were adjusted to 0.05 to 0.2% v/v L-arabinose and 0.05 to 0.1% v/v taurocholate. The cultures were incubated at 37 °C for 12 h with agitation at 100 rpm. The cells were then diluted 100 times in water and sampled with a flow cytometer. Ten thousand size-gated samples were taken for each experiment, and the median fluorescence reading was quantified.

**C. difficile toxin extraction and coculture assay.** Extracted EcN-treated bile salt supernatants were collected as previously described. The supernatants were diluted 10 times in BHIS and then incubated anaerobically with C. difficile endospores (109/mL) at 37 °C. Supernatants of the coculture were collected at regular intervals and diluted 10 times with speedvac vacuum concentrator (Savant SpeedVac™, Thermo Fisher Scientific), and the buffer was exchanged for protein buffer (50 mM Tris-HCl, 100 mM NaCl, pH 8.0) with 50 kDa MWCO ultrafiltration column. The concentrated supernatants were thawed with 500 µg/mL anti-taurocholate (PCG4, ab19953, Abcam) and anti-mouse IgG conjugated to HRP at 1:2000 dilution (#7076, Cell Signaling Technology) in an immunoblot assay. Direct coculture of Caco-2 cells and C. difficile was not possible due to the differences in laboratory growth conditions. An indirect coculture assay was performed by incubating concentrated supernatants from C. difficile with Caco-2 cells. Caco-2 cells with passage numbers between 16 and 20 were used for the assay. Cells (5 x 10^4/mL) in 100 µL of culture medium were seeded in each well of a 96-well microtitre plate. The cells were allowed to proliferate for 48 h at 37 °C. Fresh complete medium without antibiotics was provided, and concentrated supernatants were added to the cultures at 20 µL/well. The cultures were incubated for 10-14 days and then diluted 10 times in water with a flow cytometer. Ten thousand size-gated samples were taken for each experiment, and the median fluorescence reading was quantified.

**CDI animal models.** All procedures were conducted under Institutional Animal Care and Use Committee (IACUC) guidelines and in conformity with institutional and federal guidelines approved by the NUS IACUC (R18-0329) and all relevant ethical regulations. The CDI mouse model has been previously reported. Male C57BL/6 mice (5–6 weeks old) were provided with an antibiotic cocktail in water (0.4 mg/mL kanamycin, 0.035 mg/mL gentamicin, 850 U/mL colistin, 0.215 mg/mL meropenem, 0.045 mg/mL vancomycin) for 3 days following acclimatisation (day -6 to -3; pre-infection). Infection with C. difficile VPI10463 (10^7 CFU of vegetative cells) was performed on day 0 through oral gavage. The mice were maintained on a complex carbohydrate-free diet (D1509702B1I, AIN-93G, Research Inc.). Probiotics (10^7 CFU) were provided through oral gavage on day –3 as a necessity. The animals were monitored up to day 9 postinfection. The survival was monitored daily until the end of the study. The body temperature was measured at the end of the 10th day. Kaplan-Meier survival curves were generated and analysed with the log-rank test using GraphPad Prism.

**Microbiome sequencing and analysis.** Metagenomic DNA was extracted from faecal samples with the Zymobiomics DNA miniprep kit (Zymo Research). Metagenomic libraries were prepared by amplifying the V3-V4 region of prokaryotes 16s rRNA, and then indexed with Illumina XT Index Kit v2. The purified indexed libraries were sequenced using KAPA Library Quantification Kit v2 and the quality of the amplicons was measured in an Agilent Tapestation (Agilent Technologies). The pooled libraries were sequenced in an Illumina Miseq platform with Miseq Reagent Kit v2. Sequencing reads were demultiplexed and were analysed using the QIIME2 workflow for 16s amplicons, the demultiplexed reads were then filtered and filtered for chimera to output the representative sequences and OTU table. The Greengenes database (v13_8) was used as a training set for the taxonomy assignment of the OTU. Microbiome 16s rRNA sequencing data generated in this study have been deposited in the Sequence Read Archive (SRA) under accession code PRJNA844050.

**Histopathological analysis of animal tissue.** Colon tissues were collected from animals, fixed with 10% formalin solution, and then processed for paraffin embedding. Haematoxylin and eosin (H&E) staining was performed on sections slides, and then the HIS was determined according to a standardised protocol by a pathologist who was blinded to the treatment information. Images were captured on Leica DMi8 Inverted Microscope.

**Derivation of salicylic acid for HPLC detection.** Faecal samples were collected and homogenised in water. The homogenised samples were filtered and resuspended in 7.0 mM 1,2-diamino-4,5-methyleneoxybenzene dihydrochloride (DMB), 0.75 M β-D-mercaptoethanol and 18 mM sodium hydrosulphite in 1.4 M acetic acid. The samples were incubated in the dark at 50 °C for 2.5 h and then analysed by reverse-phase HPLC with an octadecyl silica Inertsil ODS-3 column (4.6 mm x 250 mm, 5 µm particle size) (GL Sciences). A 5 µL aliquot was injected into the column. The run was performed at a flow rate of 0.9 mL/min with linear gradient elution at 5:88:7 to 20:73:7 v/v/v acetonitrile/water/methanol solvent until a run time of 15 min, isocratic elution at 20:73:7 until a run time of 18 min, gradient elution to 80:13:7 until a run time of 22 min, gradient elution to 58:87:7 until a run time of 25 min, and final recalculation at 58:87:7 for 3 min. The derived samples were detected under 373 nm excitation and 448 nm emission with a fluorescence detector. Salicylic acid was eluted at approximately 13.4 min. Standard curves were constructed based on the areas of the corresponding elution peaks in the elution spectra.

**Liquid chromatography (LC)-mass spectrometry (MS) quantification of faecal bile salts.** Faecal samples were collected and homogenised in Zymobiotics DNA miniprep lysis buffer (Zymo Research). Ten microlitres of the homogenised mixture was mixed with 90 µl of LC-MS grade methanol and filtered through a 0.2 µm syringe filter (FTFE filter for organic solutions (Millipore). Quantification of the bile acids was performed in a UPLC–electrospray ionisation (ESI)-tandem MS (MS/MS) in negative ionisation mode with an Eclipse Plus C18 column (2.1 mm x 100 mm, 1.8 µm particle size) (Agilent Technologies). Ten microliters of the samples were injected into the column with mobile phases A and B consisting of 0.1% formic acid in 7.5 mM ammonium acetate in water and 7.5 mM ammonium acetate in acetonitrile, respectively. The separation of the analytes was performed at a flow rate of 0.25 mL/min with a gradient at 40% B from 0 to 5 min, 100% B from 5 to 7 min and back to 40% B for 3 min. The gas temperature was set to 250 °C at a flow of 15 L/min, and the sheath temperature to 400 °C at 12 L/min. Capillary and nozzle voltages were set at 3500 and 2000 V, respectively. The bile acids were detected using the following mass spectrometric monitoring conditions: acetic acid (m/z 375 and 376), taurocholate (m/z 375–375, CE 30 V), and taurocholate (m/z 514–80, CE 64 V). The gas temperature was set to 250 °C at a flow of 15 L/min, and the sheath temperature to 400 °C at 12 L/min. Mass spectrometry data will be provided upon request.

**Statistics and reproducibility.** All in vitro experiments conducted on 96-well microtitre plates, with the exception of the flow cytometry and MTI assays, were performed in triplicate. All in vitro CFU and HPLC experiments were performed in triplicates unless stated otherwise. Immunoblot assays were repeated independently three times and representative blots were shown. Sample sizes for the animal study were selected based on a previous study. Histology samples were determined by a pathologist who was blinded to the treatment information. One image was captured for each histology slide and the representative was selected. All reported error bars represent the standard error of the mean (SEM) values unless stated otherwise. The student’s t-test was performed for statistical analysis of bile salt quantities, germination assay results, growth, and metabolite quantities. Standard curves for HPLC and LC-MS analysis were generated by the linear regression method. The log-rank Mantel–Cox test was performed to analyse animal survival, Dunnett’s multiple comparisons test was performed to analyse the animal CSS, and mixed-model ANOVA was performed to analyse the taurocholate and cholate quantification. Microsoft Excel, Graphpad Prism and SPSS were used for data analysis.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** Microbiome 16s rRNA sequencing data that support the findings of this study have been deposited in the Sequence Read Archive (SRA) under accession code PRJNA844050.
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Competing interests
E.K., I.Y.H. and M.W.C. have filed a provisional patent application (application number 17/599,998 submitted by the National University of Singapore) based on the work described in this manuscript. The remaining authors declare no competing interests.

Additional information
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