

Blocking toxin function and modulating the gut microbiota: caffeic acid phenethyl ester as a potential treatment for *Clostridioides difficile* infection

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eLife Assessment

This **valuable** study by Guo and colleagues reports the inhibitory activity of caffeic acid phenethyl ester (CAPE) against TcdB, a key toxin produced by *Clostridioides difficile*. *C. difficile* infections are a major public health concern, and this manuscript provides interesting data on toxin inhibition by CAPE, a potentially promising therapeutic alternative for this disease. The strength of the evidence to support the conclusions is **solid**, with some concerns about the moderate effects on the mouse infection model and direct binding assays of CAPE to the toxin.

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Abstract

Clostridioides difficile infection (CDI) is the leading cause of hospital-acquired diarrhea that seriously threatens public health. The disruption of normal gut microbiota by the use of broad-spectrum antimicrobial agents enables *C. difficile* to proliferate in the colon. The emergence and prevalence of hypervirulent *C. difficile* strains result in increased morbidity, mortality, and high recurrence rates of CDI, thus creating a pressing need for novel therapeutics. The multi-domain toxins TcdA and TcdB are the primary determinants of CDI pathogenesis, rendering them ideal drug targets in the anti-virulence paradigm. In this study, we identified caffeic acid and its derivatives from natural compounds library as active

inhibitors of TcdB via a cell-based high-throughput phenotypic screening. Further mechanistic investigations revealed that caffeic acid phenethyl ester (CAPE) could directly bind to TcdB, thus suppressing InsP₆-induced autoproteolysis and inhibiting glucosyltransferase activity. CAPE treatment remarkably reduces the pathology of CDI in a murine infection model in terms of alleviated diarrhea symptoms, decreased bacterial colonization, and relieved histopathological lesions. Moreover, CAPE treatment of *C. difficile*-challenged mice induces a remarkable increase in the diversity and composition of the gut microbiota and alterations of gut metabolites (e.g., adenosine, D-proline, and melatonin), which might partially contribute to the therapeutic outcomes of CAPE against CDI. Our results reveal the potential of CAPE as a therapeutic for the management of CDI, or CAPE might serve as a lead compound for the development of antivirulence drugs targeting TcdB.

Introduction

Clostridioides difficile (*C. difficile*) is an obligate anaerobic, spore-forming, gram-positive bacterial pathogen that is the most common cause of hospital-acquired and antibiotic-associated gastrointestinal diseases, including mild diarrhea and life-threatening pseudomembranous colitis ^{1,2,3}. In the United States, *C. difficile* is responsible for almost half a million infections and approximately 29,000 deaths per year, resulting in an estimated annual healthcare-associated cost of up to \$4.8 billion ^{4,5}. Importantly, the incidence, severity, mortality and healthcare costs of *C. difficile* infection (CDI) have dramatically increased in the 21st century, at least partially owing to the frequent use of antimicrobial agents and the emergence of hypervirulent strains (e.g., PCR ribotypes 027 and 078) ^{6,7,8}. Limited options are available for the treatment of CDI, while antibiotics including metronidazole, fidaxomicin and vancomycin, are the mainstays of clinical practice ^{9,10}. However, antibiotic treatment is ineffective in more than 35% of CDI patients and can lead infection recurrence ^{11,12}. Moreover, the rapid evolution of antibiotic resistance in *C. difficile* has further compromised the therapeutic efficacy of current treatment options ¹³. Therefore, the high morbidity and mortality, high recurrence rates, and emergence of multiple antibiotic-resistant isolates have made this pathogen a major threat to public health; these factors highlight the urgent need for novel therapies.

C. difficile widely exists in the environment and in the intestinal tract of humans and animals and is mainly transmitted via the fecal-oral route ¹⁴. The pathogenesis of CDI is correlated with the use of antibiotics, which impair the structure and composition of the normal gut microbiota, thus allowing *C. difficile* to proliferate in the colon ¹⁴. Therefore, previous antibiotic therapy is the major risk factor for the development of CDI. In the colon, the dormant spores of *C. difficile* germinate into oxygen-sensitive vegetative cells that produce toxins and cause symptoms of disease ¹⁵. *C. difficile* secretes two large clostridial exotoxins (TcdA and TcdB), which are directly responsible for the clinical pathology of CDI ^{16,17}. Epidemiological studies suggest that most disease-causing *C. difficile* strains have genes that encode TcdB ^{18,19}. TcdA and TcdB have identical functional domains: a N-terminal glucosyltransferase catalytic domain (GTD), a cysteine protease active domain (CPD), a transmembrane domain (DRBD), and a C-terminal receptor binding domain (CROPs) ²⁰. When secreted into the colon, both toxins are internalized by host cells through receptor-mediated, clathrin-dependent endocytosis. Following a decrease in endosomal pH, the toxins undergo conformational changes in the DRBD, resulting in insertion of the toxin into the endosome membrane, pore formation, and the release of the GTD and CPD domains into the cytosol. Then, CPD binding with cytosolic inositol hexakisphosphate (InsP₆) activates its protease activity, leading to the cleavage and release of the GTD. Eventually, the GTD utilizes UDP-glucose to glucosylate Rho and/or Ras family GTPases, thus disrupting GTPase signaling and resulting in deleterious effects, including cytopathic “rounding”, dysregulation of the actin cytoskeleton, apoptosis, and pyroptosis ¹. Although both toxins are cytotoxic to host cells, TcdB shows greater toxicity than TcdA ²¹.

Disruption of the gut microbiota by antimicrobial agents results in high recurrence rates, which limits the application of antimicrobial therapy for CDI¹⁴. Consequently, there is a critical need to develop nonantibiotic treatments for CDI, either as standalone or augmented therapies. Importantly, strategies targeting bacterial virulence factors, including toxins, are termed anti-virulence strategies and have attracted interest in the 21st century²². Owing to its essential role in *C. difficile* pathogenesis, TcdB has become an ideal target for the development of anti-virulence drugs²³. In this study, caffeic acid and a series of its derivatives were found to inhibit TcdB-mediated cell rounding via cell-based screening. Among these potential inhibitors, caffeic acid phenethyl ester (CAPE) had the greatest inhibitory effect. Mechanistically, CAPE bound to both full-length TcdB and the GTD fragment, leading to alterations in the secondary structure. This interaction suppressed the InsP₆-mediated self-cleavage of TcdB, as well as the enzymatic activity of GTD, thereby decreasing the level of glucosylated Rac1 in TcdB-treated host cells and further inhibiting the cytopathology and cytotoxicity of TcdB. Oral administration of CAPE significantly protected against CDI in models of animal infection. In addition to direct toxin inhibition, the therapeutic effect may also be attributed to CAPE-induced alteration of the gut microbiota (e.g., *Bacteroides* spp.) as well as gut metabolites (e.g., adenosine, D-proline, and melatonin). Overall, our study revealed that caffeic acid and its derivatives, particularly CAPE, may serve as promising lead compounds for the development of anti-virulence drugs to treat CDI.

Results

CAPE inhibits cell rounding induced by TcdB

TcdB can inactivate Rho GTPases via glucosylation, leading to the loss of cytoskeletal structure and cell-rounding. Consequently, we utilized this phenotype to identify potential TcdB inhibitors from 2076 chemicals, including 1515 FDA-approved drugs and 561 natural compounds. The minimal concentration of recombinant His₆-TcdB (0.2 ng/mL) that induced 100% cell rounding within 1 h was used for drug screening. This concentration of TcdB was preincubated with 8 µg/mL of the individual compounds for 1 h, the compounds that were not bound to TcdB were removed by ultrafiltration centrifugal partition and then added to Vero cell cultures. Residual compounds that have not bound to TcdB may lead to an exaggerated assessment of their efficacy and could also result in misleading positive outcomes. As the effective concentration for cellular interactions may inadvertently be increased, such carryover could distort the determination of the compound's minimum effective concentration. At 1 h post-TcdB treatment, cell morphology was visualized under a light microscope (**Figure 1A**). These analyses allowed us to identify 22 candidate compounds with potent inhibitory effects (**Figure 1B**). Interestingly, six out of the 22 compounds were caffeic acid derivatives. The natural compound library contained eight caffeic acid derivatives, of which methyl caffeic acid and ferulic acid displayed no efficacy. Caffeic acid itself only slightly inhibited TcdB-induced cell rounding, with a 50% inhibitory concentration (IC₅₀) of 527.5 µg/mL (1.86 mM) (**Table S1**). Notably, except for rosmarinic acid, the IC₅₀ values for the other screened caffeic acid derivatives were less than 6 µg/mL, suggesting that these compounds potently inhibited TcdB-mediated toxicity (**Table S1**). CAPE is the phenethyl alcohol ester of caffeic acid and a main active component of propolis with broad pharmacological properties, such as anticancer, anti-inflammatory, antiviral, antibacterial, antifungal, antioxidant, and immunomodulatory activities²⁴. As CAPE has the lowest IC₅₀ (3.0 µg/mL) among the active caffeic acid derivatives, it was selected as the representative compound for subsequent investigations (**Table S1**). After preincubation with increasing concentrations of CAPE, dose-dependent inhibition of cell rounding was observed in both Vero and HeLa cells (**Figure 1C**). The protective influence of CAPE against TcdB-induced Vero cell death remained evident even when the treatment duration was prolonged to 12 h (**Figure S1A-B**).

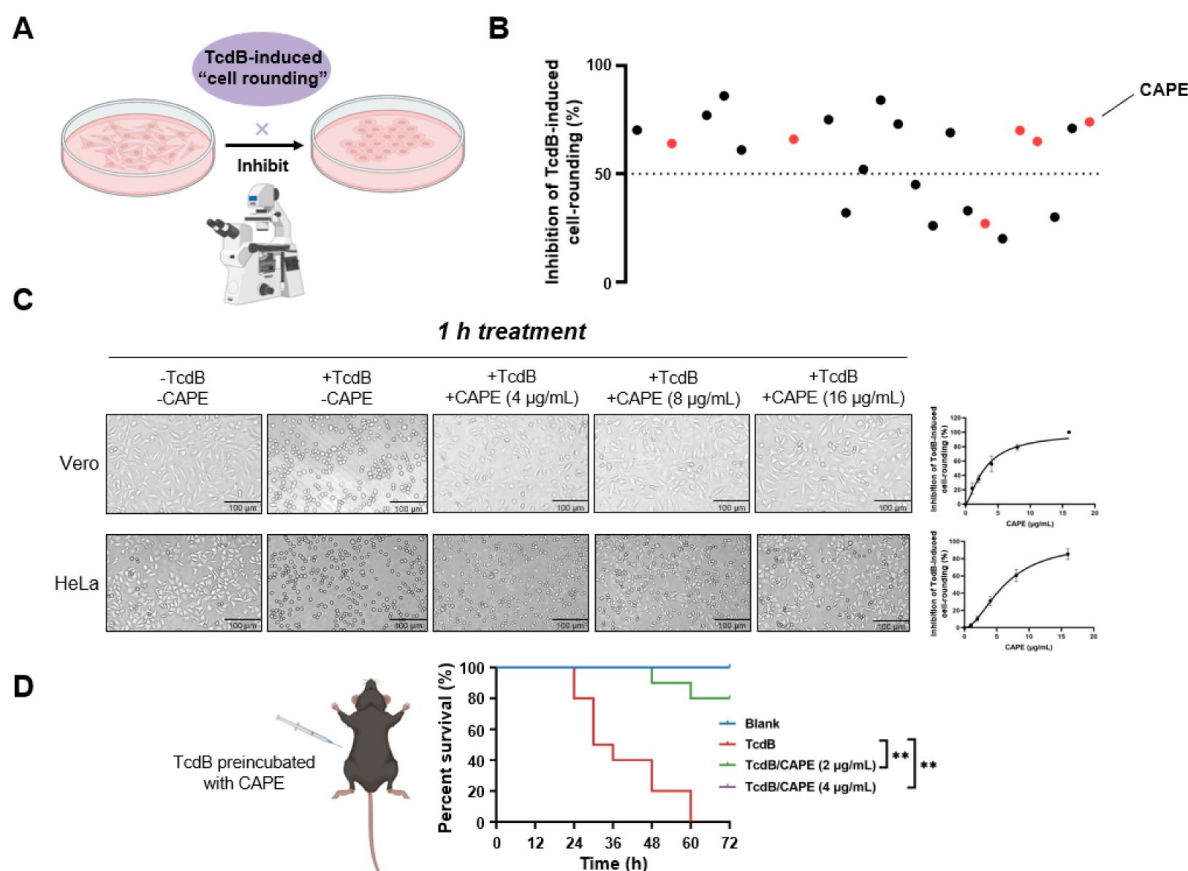


Figure 1.

CAPE suppresses TcdB-mediated cell rounding.

(A) Schematic of the process used for the identification of small molecules that inhibit the cell rounding phenotype induced by TcdB. TcdB (0.2 ng/mL) was preincubated with 8 μ g/mL of individual compounds prior to addition to the cell cultures. (B) Active compounds were identified from the chemical library consisting of 2076 chemicals. The red dots represent caffeic acid and its derivatives. (C) Dose-dependent CAPE inhibition of TcdB-induced rounding of Vero (upper) and HeLa (lower) cells. Scale bar, 100 μ m. The dose titration curves are presented on the right. (D) The survival rates of mice that were intraperitoneally injected with CAPE-pretreated or native TcdB (30 ng). * $p < 0.05$ and ** $p < 0.01$ by log-rank (Mantel-Cox) test.

In addition, we also determined whether inhibition of TcdB by CAPE could lead to loss of toxin function *in vivo* using a murine systemic intoxication model. A lethal dose of recombinant His₆-TcdB (30 ng) was preincubated with either DMSO or CAPE (2 or 4 μg/mL) for 1 h at 37 °C, followed by intraperitoneal injection into mice. Notably, all mice in the DMSO group (n=10) died within 36 h after toxin injection. However, 100% of mice pretreated with CAPE 4 (μg/mL) toxin survived at the monitoring endpoint (72 h) (n=10) (**Figure 1D** [↗](#)). Furthermore, we evaluated the efficacy of CAPE following an intraperitoneal injection by administering it 2 h prior to the injection of TcdB, in order to gauge the survival rate of the mice. The outcomes revealed that a 2 h pretreatment with CAPE at doses of 30 and 50 mg/kg significantly enhanced the mice's protection rate by 57.1% (**Figure S2** [↗](#)). Simultaneously, we assessed the impact of CAPE on *C.difficile* itself. CAPE markedly repressed *tcdB* gene expression at a concentration of 16 μg/mL (**Figure S3A** [↗](#)) and inhibited the growth and spore formation of *C. difficile* BAA-1870 at a concentration of 32 μg/mL (**Figure S3B-C** [↗](#)). Taken together, these data indicate that CAPE is a potent toxin inhibitor of TcdB and may serve as a promising antimicrobial agent.

CAPE decreases InsP₆-induced autoproteolysis of

TcdB and inhibits the enzymatic activity of GTD

TcdB is a multi-domain containing toxin that induces toxicity in host cells via an intricate and multistep mechanism. This mechanism involves (i) receptor-mediated endocytosis, (ii) pore formation and subsequent membrane translocation, (iii) autoproteolysis and release of GTD, and (iv) the glucosylation of small GTPases (**Figure 2A** [↗](#)). To determine the mechanism by which CAPE suppresses the cytotoxicity of TcdB, we first labeled full-length TcdB with Alexa Fluor 488; then, the concentration of the endocytosed toxin was determined via flow cytometry. Apparently, incubation of CAPE with TcdB did not significantly affect its entry into host cells (**Figure 2B** [↗](#)). Next, we stained CAPE-treated cells with LysoTracker, a fluorescent dye that accumulates in the acidic organelles of live cells. In contrast to the vacuolar-type H⁺-ATPase (V-ATPase) inhibitor bafilomycin A1 (Baf-A1), CAPE treatment did not alter the intensity of LysoTracker fluorescence staining in either THP-1 or Caco-2 cells, suggesting that inactivation of TcdB by CAPE is not due to changes in the pH of endosomal compartments (**Figure 2C-D** [↗](#) and **Figure S4A-B** [↗](#)).

These observations indicate that CAPE might exert its effects after membrane translocation. Indeed, preincubation of full-length TcdB with CAPE blocked the InsP₆-induced autoproteolysis of TcdB, as revealed by the gradual decrease in the production of released GTP, followed by the addition of increasing amounts of CAPE *in vitro* (**Figure 2E-F** [↗](#)). Furthermore, we determined the glucosyltransferase activity using recombinant GTD in the absence or presence of different doses of CAPE. Strikingly, incubation with 2 μg/mL CAPE resulted in a significant reduction in GTD activity, whereas 32 μg/mL CAPE compromised 87.5% of the enzymatic activity (**Figure 2G** [↗](#)). Importantly, in response to suppression of InsP₆-induced autoproteolysis and GTD function, treatment of TcdB-challenged cells with CAPE markedly decreased the level of glucosylated Rac1 in a dose-dependent manner (**Figure 2H-I** [↗](#)). Taken together, these results suggest that CAPE disrupts InsP₆-induced autoproteolysis of TcdB and inhibits the enzymatic activity of GTD, thereby decreasing the glucosylation of Rac1 and alleviating cell rounding.

CAPE alters the secondary structure of full-length TcdB and GTD

To further clarify the mechanisms used by CAPE to inactivate TcdB, we determined the secondary structures of full-length TcdB and GTD in the absence or presence of CAPE by circular dichroism spectroscopy. Apparently, incubation of CAPE with full-length TcdB resulted in a significant increase in the α-helix content (15.4% to 21.0%) and a concomitant decrease in the β-pleated sheet content (34.5% to 20.9%) (**Figure 3A-B** [↗](#)). Similarly, the secondary structure of GTD was markedly altered upon incubation with CAPE, with the proportion of α-helices decreasing from 37.9% to

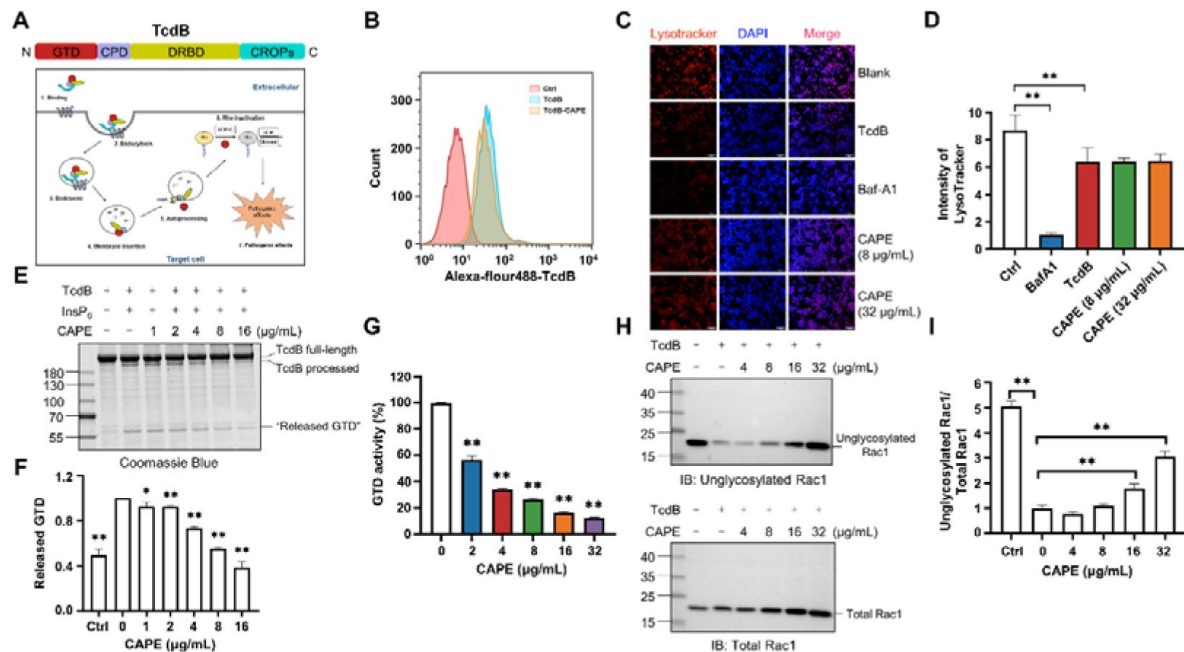


Figure 2.

Mechanism by which CAPE suppresses TcdB intoxication.

(A) Schematic illustration of the multistep mechanism of TcdB intoxication of host cells. **(B)** Determination of internalized TcdB levels. Alexa Fluor 488-labeled TcdB was incubated with 32 $\mu\text{g/mL}$ CAPE for 30 min before it was added to the cell culture. The cellular uptake of TcdB was measured by flow cytometry analysis. **(C)** Lysosomal activity in host cells treated with CAPE was measured via LysoTracker staining. The nuclei were stained with DAPI. Bar, 100 μm . **(D)** The intensities of LysoTracker staining were quantified using ImageJ. * $p < 0.05$; ** $p < 0.01$ using one-way ANOVA. **(E-F)** CAPE inhibits InsP_6 -induced autoprocessing of full-length TcdB. Two micrograms of TcdB was preincubated with increasing concentrations of CAPE for 1 h. Self-cleavage of TcdB was initiated by the addition of 3 μg of InsP_6 and the cells were incubated for 6 h at 37 $^\circ\text{C}$. Reactions were terminated with 5x SDS loading buffer, and the release of GTD was visualized by CBB staining of the SDS-PAGE gel **(E)**. The level of released GTD in panel E was quantified by ImageJ **(F)** * $p < 0.05$; ** $p < 0.01$ using one-way ANOVA. **(G)** Dose-dependent inhibition of the GTD activity of TcdB by CAPE. Recombinant GTD of TcdB was mixed with different concentrations of CAPE (0–32 $\mu\text{g/mL}$) and incubated for 15 min at 25 $^\circ\text{C}$. GTD activity was measured by the UDP-GloTM glycosyltransferase assay. **(H-I)** Intracellular Rac1 glucosylation in cells treated with TcdB. Native or CAPE-pretreated TcdB was added to the cell culture and cells were incubated for 2 h. The cells were lysed, and the glucosylation of Rac1 was analyzed by western blotting using antibodies recognizing total Rac1 and unglycosylated Rac1 **(H)**. The level of Rac1 glucosylation shown in H was quantified by ImageJ **(I)**. * $p < 0.05$; ** $p < 0.01$ using one-way ANOVA.

0.0% and the proportion of β -pleated sheets decreasing from 37.2% to 10.9% (**Figure 3C-D**). Taken together, these data suggest that CAPE may interact with full-length TcdB and GTD to induce conformational changes, thus impairing their functions.

CAPE directly interacts with full-length TcdB and GTD

To investigate the potential interaction between CAPE and TcdB or GTD, the binding constants were determined by surface plasmon resonance (SPR) analysis. The results showed that CAPE has strong binding affinity with full-length TcdB and GTD, and the K_D values were 1.269×10^{-5} M and 1.007×10^{-3} M, respectively (**Figure 4A-B**). The interaction between CAPE and TcdB exhibits a slow approach to equilibrium, which may primarily be attributed to the rapid occupation of the protein's primary binding sites by the small molecule during the initial phase. Following this, CAPE proceeds to bind to sites with secondary or reduced affinity, a process that prolongs the time required to achieve equilibrium. Furthermore, the possibility that CAPE binds to multiple sites on TcdB necessitates a duration for the exploration and occupation of these various sites before equilibrium is established. These data demonstrate the direct interaction between CAPE and full-length TcdB/GTD.

Next, we performed molecular docking and molecular simulation dynamics analyses to investigate the interaction between CAPE and GTD (**Figure 5A**). The results revealed that Leu265, Asp286, Thr465, Ile466, Leu519, and Trp520 in GTD were potential binding sites for CAPE (**Figure 5A**). The root mean square deviation (RMSD) of the GTD/CAPE complex was stable over a 100 ns period of simulation (**Figure 5B**). Further energy decomposition analysis showed that the side chains of Leu265, Thr465, Leu519, and Trp520 of GTD contributed to binding with CAPE via van der Waals interactions, among which Trp520 had the highest binding energy (**Figure 5C**). Moreover, we mutated each of the residues that were involved in binding and subsequently determined their glucosyltransferase activities in the presence or absence of CAPE. The enzymatic activities of these mutants were similar to that of wild-type GTD (**Figure 5D**). Mutations in GTD_{L265A}, GTD_{T465A}, GTD_{I466A}, and GTD_{L519A} did not affect the inhibitory effect of CAPE (**Figure 5E-I**). However, the inhibition of GTD_{W520A} and GTD_{D286A} by CAPE was notably compromised (**Figure 5J-K**).

CAPE treatment reduces the pathology of CDI in a murine model

The abovementioned observation that CAPE suppresses the function of TcdB prompted us to further evaluate the potential therapeutic effects of this compound in CDI. To this end, we employed a well-established murine model in which infected mice can develop symptoms resembling those observed in human CDIs (25). First, cytotoxicity measurements revealed that the viability of both THP-1 and Vero cells was not affected by CAPE treatment (**Figure S5A-B**), thus indicating low toxicity. Mice were preconditioned with drinking water containing 0.5 g/L cefoperazone for 5 days, followed by normal drinking water for 2 days. After challenge with *C. difficile* BAA-1870 via gavage, mice were then treated daily with either vehicle or 30 mg/kg CAPE by gavage throughout the disease course (day 0 to day 5) (**Figure 6A**). Severe weight loss in *C. difficile*-infected mice was observed on days 2 and 3 post infection and was accompanied by diarrhea (**Figure 6B**). Notably, compared to the infection control group, CAPE treatment significantly increased the dry/wet weight ratio of the mouse feces, suggesting the alleviation of diarrhea symptoms (**Figure 6C**). Correspondingly, the weight loss of mice was relieved upon receiving CAPE (**Figure 6B**). Moreover, fecal pellets collected on day 3 were used to determine the bacterial burden. The results revealed that CAPE treatment significantly decreased the number of *C. difficile* CFUs (**Figure 6D**). On day 3 post-*C. difficile* infection, the colon was collected for histopathological analysis. Remarkably, *C. difficile*-infected mice exhibited conspicuous colon edema, epithelial cell injury and inflammatory factor infiltration (**Figure 6E**). In contrast, these histopathological lesions were significantly reduced in mice given CAPE, but pathological changes were still present (**Figure 6F**). This indicates that CAPE may have a limited capacity to reverse

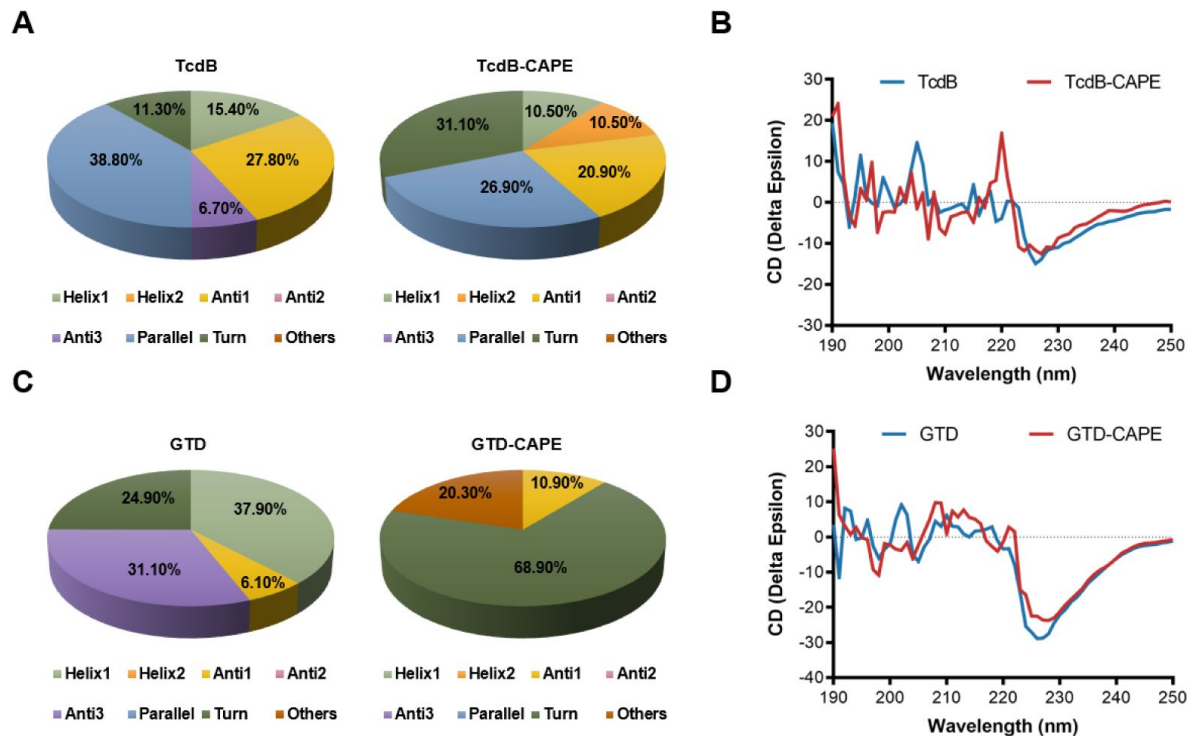


Figure 3.

CAPE alters the secondary structure of TcdB and GTD.

(A) The secondary structure of TcdB in the presence or absence of 32 $\mu\text{g/mL}$ CAPE was determined by CD spectroscopy. (B) Calculated CD spectra of CAPE-treated TcdB. (C) The secondary structure of GTD in the presence or absence of 32 $\mu\text{g/mL}$ CAPE was determined by CD spectroscopy. (D) Calculated CD spectra of CAPE-treated GTD. The wavelength for CD spectroscopy was set at 190-250 nm.

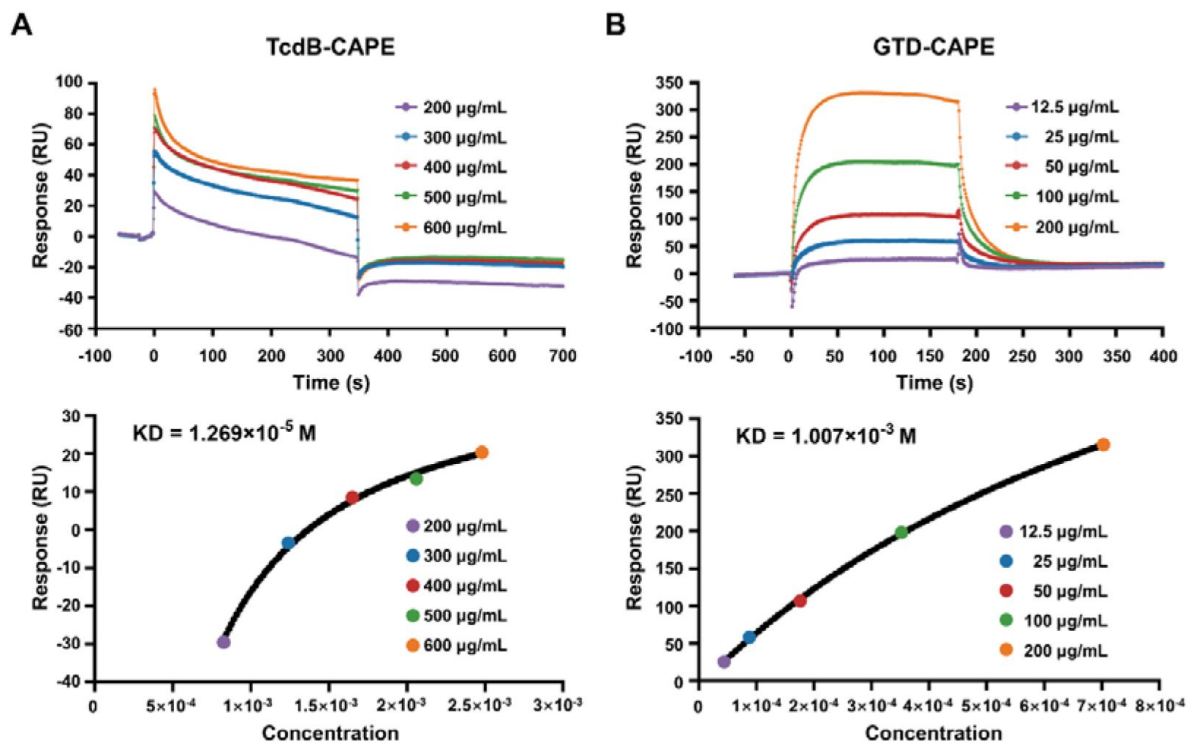


Figure 4.

Direct interaction of CAPE with TcdB and GTD.

(A-B) Full-length TcdB or GTD was immobilized on a CM5 chip, followed by the injection of various concentrations of CAPE. The response unit (RU) value and affinities of CAPE interactions with TcdB (A) and GTD (B) were determined by a Biacore T100.

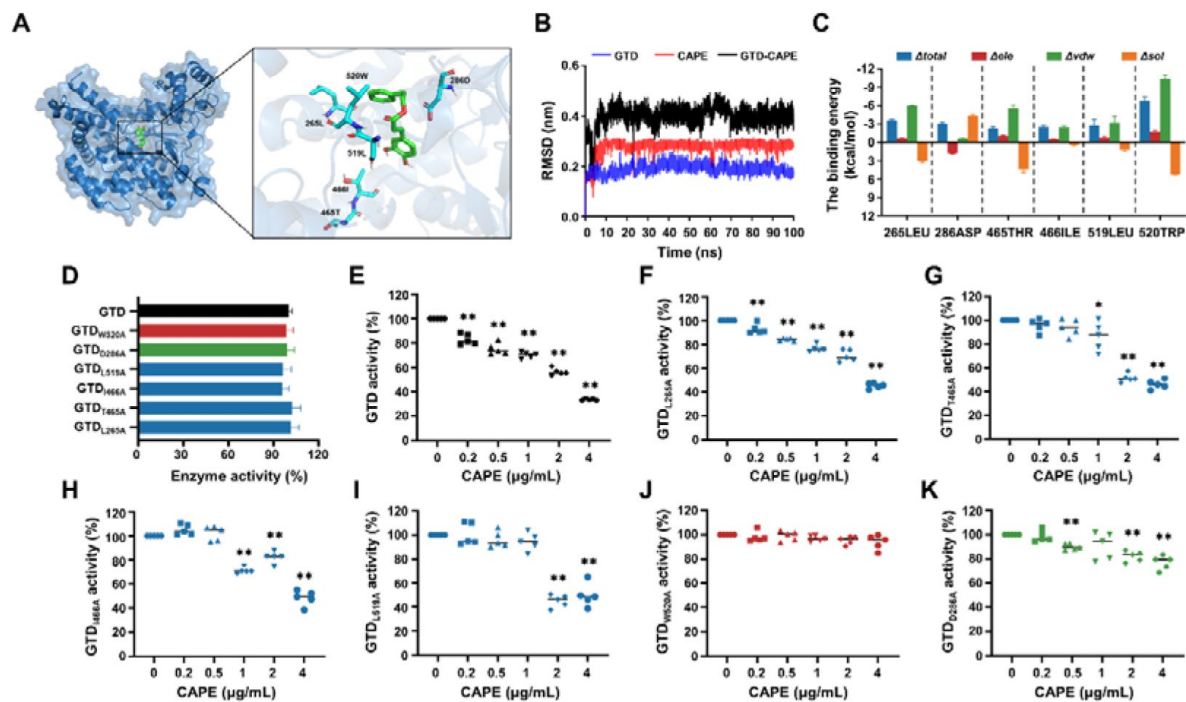


Figure 5.

Asp286 and Trp520 in the GTD are critical for CAPE-mediated inhibition of GTD.

(A) Three-dimensional structure determination of GTD with the CAPE complex was performed by molecular modeling. (B) The RMSD values of the GTD-CAPE complex. (C) Decomposition of the binding energy on a per-residue basis in the binding sites of the GTD-CAPE complex. Blue denotes the total binding energy, red signifies the electrostatic interactions, green corresponds to the van der Waals forces, and orange indicates solvation or hydration effects. The horizontal axis represents the mutation of the amino acid residue at the respective position to alanine. (D) Glucosyltransferase activity of wild-type GTD and the GTD mutants. (E-K) Residual activity of wild-type GTD (E) and its mutants GTD_{L265A} (F), GTD_{T465A} (G), GTD_{I466A} (H), GTD_{L515A} (I), GTD_{W520A} (J) and GTD_{D286A} (K) in the presence of increasing concentrations of CAPE (0-4 μ g/mL). * $p < 0.05$; ** $p < 0.01$ using one-way ANOVA.

the histological damage induced by *C. difficile*. The residual pathology could be attributed to the complex interplay between the pathogen, the host immune response, and the tissue repair process, which may not be fully mitigated by the inhibitory effects of CAPE on TcdB.

CAPE induces distinct microbiota changes in CAPE-treated mice when compared with control mice

Antibiotic-induced dysbiosis of the gut microbiota promotes the development of CDI ²⁵. Therefore, we next determined whether CAPE has any impact on gut microbial communities that may contribute to its therapeutic efficacy. To this end, on the 3rd day after *C. difficile* challenge, fecal samples from the control group and CAPE treatment group were collected and subjected to microbiota analysis via 16S rRNA gene sequencing. Remarkably, as reflected by the Venn diagram, CAPE treatment resulted in significant changes in the microbial composition compared to those in the control group, and only 16.42% of the operational taxonomic units (OTUs) were shared (**Figure 7A**). In addition, both the α - and β -diversity of the gut microbiota in CAPE-treated mice were significantly greater than those in control mice. Specifically, the increase in the Chao1 richness estimator of mice in the CAPE treatment group suggested a greater number of microbial species and increased evenness in the fecal samples (**Figure 7B-C**); the increase in the Shannon-Wiener diversity index and the Simpson diversity index reflected a significant increase in microbiota diversity (**Figure 7D-E**). Principal coordinate analysis (PCoA) revealed distinct confidence ellipses between the bacterial communities of the CAPE-treated group and the control group, thereby revealing substantial differences in their microbiota profiles (**Figure 7F**).

A comparative analysis was performed to examine changes at the phylum level following CAPE treatment. *Bacteroidota*, *Firmicutes*, and *Proteobacteria* were the major constituents in both the CAPE treatment and control groups, suggesting that the administration of CAPE did not significantly alter the mouse microbiota at the phylum level (**Figure 7G**). Next, we compared the 10 most abundant taxa at the genus level. Notably, CAPE treatment markedly elevated the abundance of *Bacteroides* and decreased the abundance of *Muribaculaceae* and *Parabacteroides* (**Figure 7H**). Leveraging linear discriminant analysis of effect sizes (LEfSe), the microbiota was examined at the genus level to identify bacteria exhibiting substantial differences in abundance between the two cohorts (LDA score > 3.5, $p < 0.05$). After CAPE treatment, the relative abundance of *Bacteroides*, *Ruminococcus_gnavus_group*, *Muribaculum* and *Faecalibaculum* OTUs notably increased, while that of *Hungatella*, *Parasutterella* and *Muribaculaceae* OTUs significantly decreased (**Figure 7I**). *Bacteroides*, *Muribaculum* and *Faecalibaculum* improve colonization resistance to *C. difficile* ²⁶, ²⁷, ²⁸. Importantly, the abundance of these bacterial species increased following CAPE treatment (**Figure 7J-L**). In contrast, the abundance of *Parasutterella*, a bacterium that facilitates *C. difficile* colonization ²⁹, was reduced in mice receiving CAPE (**Figure 7M**). Taken together, these findings suggest that the gut microbiota alteration might contribute modestly to the observed effects.

CAPE alters the levels of gut metabolites in *C. difficile*-infected mice

In addition to the crucial role of the gut microbiota in conferring colonization resistance against *C. difficile*, gut microbiota-derived metabolites significantly contribute to the disease process ³⁰. Metabolomic analysis was performed to evaluate the effects of CAPE treatment on gut metabolites in mice. On the 3rd day after infection, the feces of mice in each group were frozen by liquid nitrogen for metabolomics analysis. Specifically, utilizing mass spectrometry-based metabolomics, a simple screening of each metabolite is conducted for parameters such as retention time and mass-to-charge ratio. Subsequently, peak area calibration is performed using the first quality control (QC) sample to enhance the accuracy of identification. The peak extraction is then conducted with settings for mass deviation at 5 ppm, signal intensity deviation at 30%, minimum

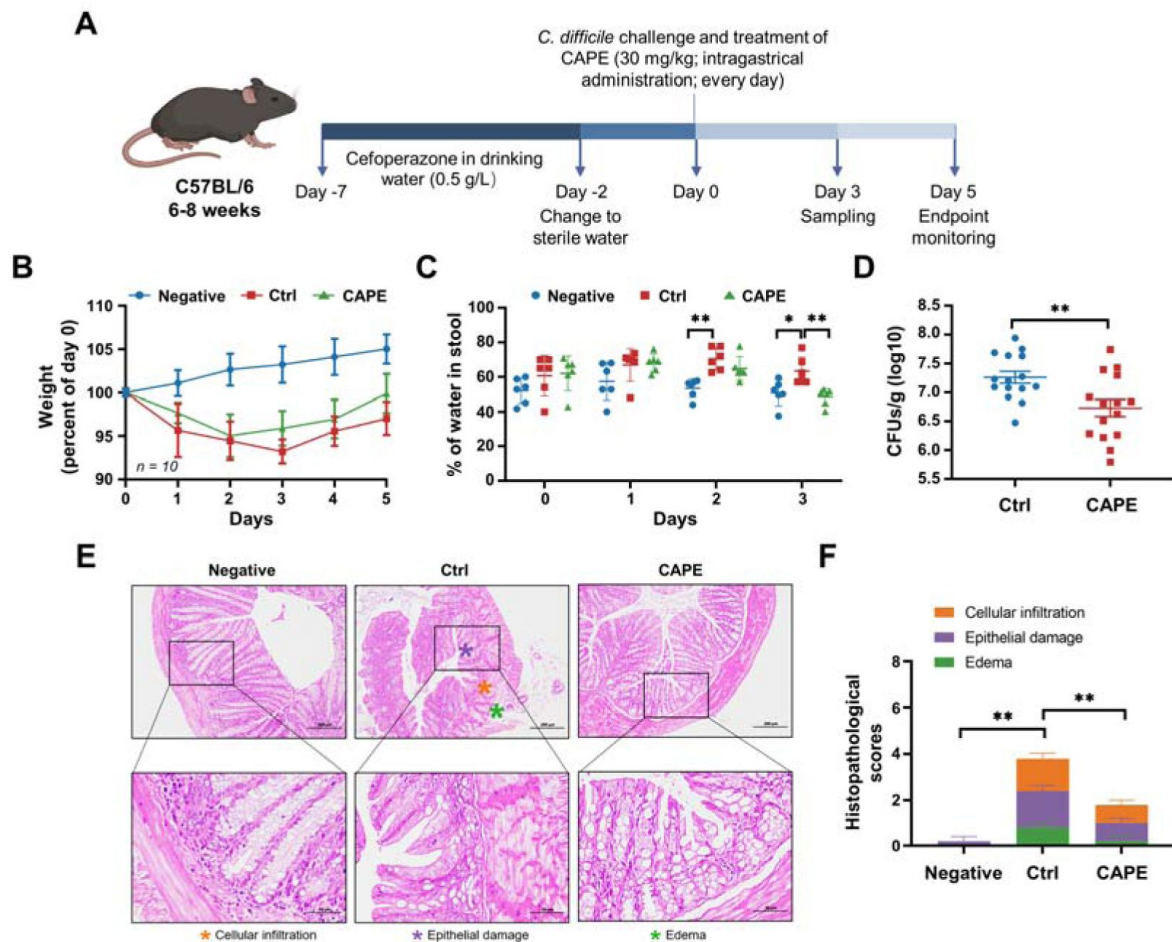


Figure 6.

CAPE treatment blocks the pathology of CDI in mice.

(A) Schematic of the process used for the establishment of the CDI mouse model. (B) Weight changes in CDI mice after CAPE or vehicle treatment (n=10). (C) The fecal dry/wet weight ratio in CDI mice treated with CAPE or vehicle (n=6). * $p < 0.05$; ** $p < 0.01$ using one-way ANOVA. (D) Bacterial colonization in the feces of CDI mice treated with CAPE or vehicle (n=15). * $p < 0.05$; ** $p < 0.01$ using the Mann Whitney U test. (E) Pathological analysis of colon sections from CDI mice treated with CAPE or vehicle. The black boxes are the regions that were magnified and are shown underneath. (F) Histopathological scores of vehicle-treated and CAPE-treated mice in terms of cell infiltration (orange), epithelial damage (purple), and edema (green). * $p < 0.05$; ** $p < 0.01$ using one-way ANOVA.

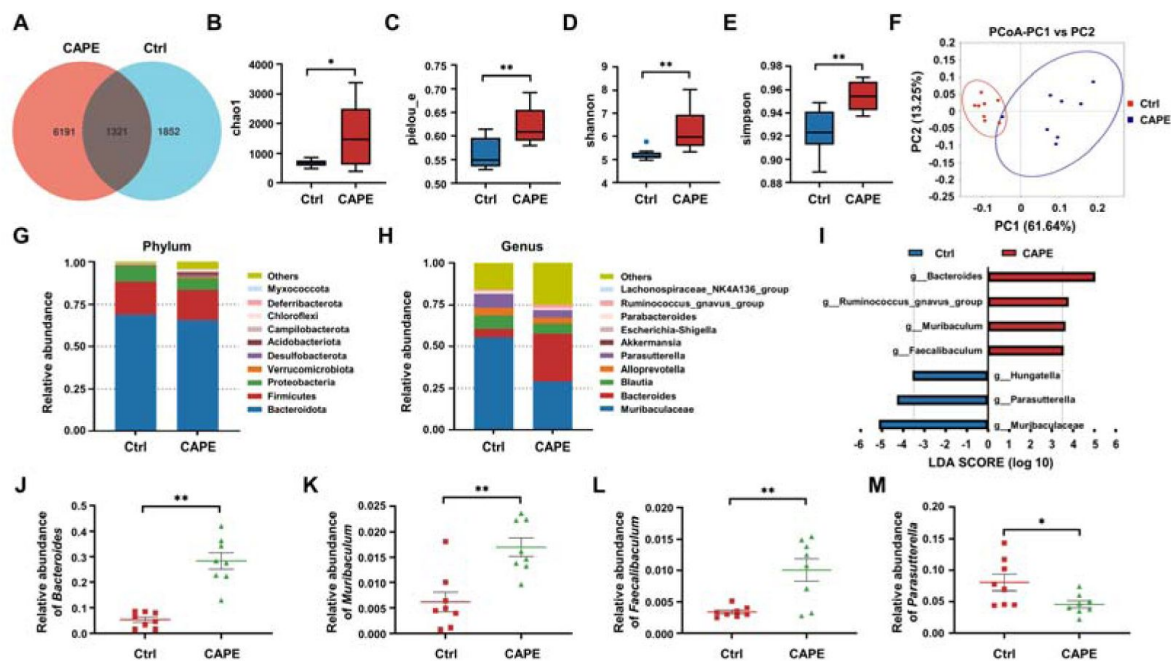


Figure 7.

The influence of CAPE treatment on the diversity and composition of the gut microbiota in CDI mice.

(A) Venn diagram illustrating the numbers of differentially abundant bacteria identified using a single model and their cooperation. (B-E) Quantification of the α diversity of the gut microbiota according to the observed species richness (B-C), Shannon index (D), and Simpson index (E). (F) PCoA showing β diversity based on the weighted UniFrac distance. (G-H) Relative abundance of the top 10 predominant bacteria classified at the phylum (G) and genus (H) levels. (I) Significant changes in flora among the control and CAPE groups, as measured by LEfSe analysis (LDA score [$\log_{10}$] > 3.5). (J-M) Relative abundances of *Bacteroides* (J), *Muribaculum* (K), *Faecalibaculum* (L), and *Parasutterella* (M) in mice in the control and CAPE treatment groups. * $p < 0.05$; ** $p < 0.01$ using an unpaired Student's t test.

signal intensity, and adduct ions. Simultaneously, quantification of peak areas is carried out, and target ions are integrated. Molecular formulas are predicted using molecular ion peaks and fragment ions, and these are compared with databases such as mzCloud, mzVault, and Masslist. Background ions are removed using blank samples. The raw quantification results are standardized using the formula: original quantification value of the sample / (total metabolite quantification value of the sample / total metabolite quantification value of QC1 sample), to obtain relative peak areas. Compounds with a coefficient of variation (CV) greater than 30% in the relative peak areas of QC samples are excluded. Finally, the identification and relative quantification results of the metabolites are obtained. The PLS-DA model exhibited significant separation of clusters between the model and CAPE treatment groups (**Figure 8A**). Among the 1599 metabolites, 109 showed substantial differences in levels between the two groups. Specifically, CAPE-treated mice exhibited 19 metabolites with upregulated expression and 50 metabolites with downregulated expression compared to those in the control group (fold change > 1.5, $p < 0.05$) (**Figure 8B**). Furthermore, KEGG enrichment analysis revealed that the differentially abundant metabolites were significantly enriched in the purine metabolism and bile secretion pathways (**Figure 8C**). Adenosine is an endogenous purine nucleoside that binds to specific cell surface receptors to exert anti-inflammatory effects at high concentrations³¹. Importantly, CAPE treatment of *C. difficile*-infected mice can increase the adenosine concentration in the gut (**Figure 8D**), which might be attributed to its inhibition of xanthine oxidase³². Similarly, the levels of uric acid, the final product of purine degradation, were markedly decreased in mice after receiving CAPE (**Figure 8D**). An earlier study reported that increasing the gut adenosine concentration with an adenosine deaminase inhibitor prevents *C. difficile* toxin A-induced enteritis in mice³³. Therefore, the increase in gut adenosine induced by CAPE might play an important role in its therapeutic efficacy in CDI.

C. difficile predominantly utilizes sugars and amino acids for energy production through various metabolic pathways, such as fermentation³⁴. Notably, Stickland fermentation, a Clostridia-specific type of amino acid fermentation, is associated with *C. difficile* pathogenesis³⁴. D-proline is one of the preferred nutrients used by *C. difficile* in the Stickland reaction and is critical for its growth^{35, 36}. Interestingly, treatment of *C. difficile*-infected mice with CAPE significantly reduced gut D-proline levels (**Figure 8D**). In addition, the metabolomics data also revealed a dramatic increase in the level of melatonin (**Figure 8D**), which is a ubiquitous indolamine that was previously shown to decrease the rate of recurrent CDI³⁷. Consistently, quantification of the melatonin content in mouse fecal supernatants using a melatonin detection kit revealed elevated levels of melatonin in CAPE-treated mice (**Figure S6**). Moreover, correlation analysis revealed a positive association between *Faecalibaculum* abundance and melatonin levels (**Figure S7**). Taken together, these results indicate that alterations in gut metabolites caused by CAPE treatment might contribute to its therapeutic efficacy in CDI. We found that the metabolites of the gut microbiota changed after CAPE treatment. While detectable, the impact of gut microbiota modifications seems to be of secondary importance.

Oral administration of melatonin alleviates the symptoms of CDI

Next, we investigated whether any of the upregulated metabolites had a direct influence on TcdB. To this end, we preincubated recombinant TcdB with each of the selected metabolites prior to addition to the cell cultures. Interestingly, treatment with TcdB and 100 µg/mL melatonin significantly alleviated the cell death induced by this toxin (**Figure 9A**). This observation prompted us to investigate the direct therapeutic potential of melatonin in CDI. Therefore, *C. difficile*-infected mice were orally administered 200 mg/kg melatonin at 24 h intervals. The weight changes and the dry/wet ratio of the stool samples were monitored daily for three days. The results showed that melatonin-treated mice displayed remarkable alleviation of diarrhea, as manifested by notable improvements in weight recovery and a significant increase in the dry/wet weight ratio on the 2nd and 3rd days after *C. difficile* challenge (**Figure 9B-C**). Consistent with these observations, histopathological assessment of colonic tissue sections also revealed attenuated colon damage upon melatonin treatment (**Figure 9D-E**). Taken together, these data

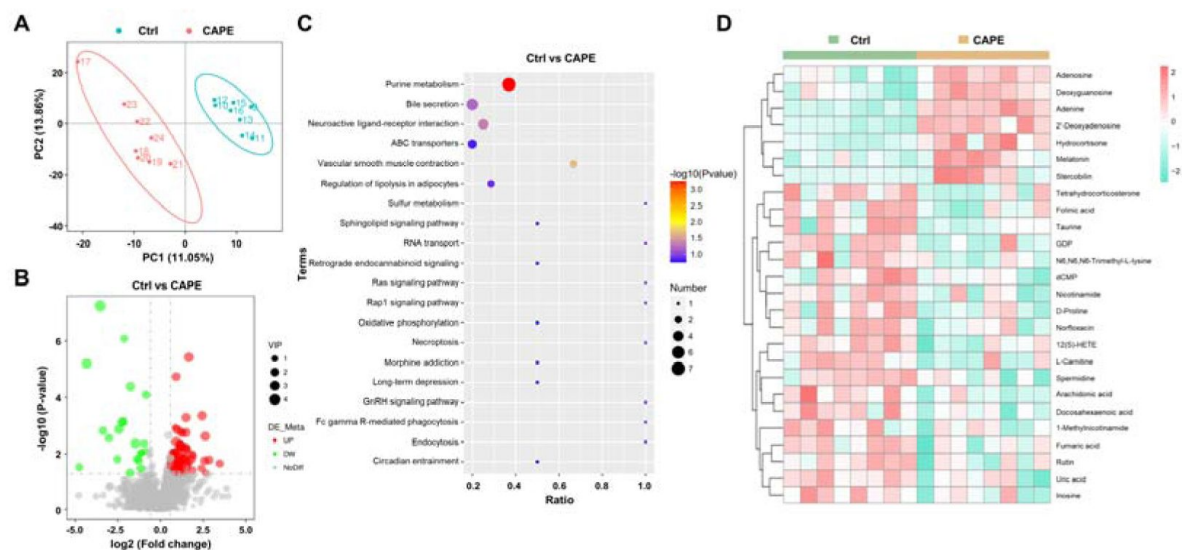


Figure 8.

Alterations in gut metabolites in CAPE-treated mice with CDI.

(A) The score plots show orthogonal partial least square discriminant analysis (PLS-DA) results. (B) Volcano plot representing the upregulated or downregulated gut metabolites in the CAPE treatment group compared to those in the control group. (C) Enrichment bubble map of the top 20 metabolic pathways in the CAPE treatment and control groups. (D) The 26 potential biomarkers are displayed in a heatmap, along with hierarchical clustering analysis.

indicate that a high dose of exogenous melatonin could protect mice from CDI. However, the CAPE treatment group induced only minor alterations in gut microbiota-derived melatonin levels, which may not reach therapeutic relevance. While existing studies suggest that melatonin exhibits beneficial effects against CDI, the primary antimicrobial activity appears to be mediated directly by CAPE rather than through melatonin-associated mechanisms.

Discussion

C. difficile has become the prevalent agent of nosocomial diarrhea and substantially threatens public health. In particular, the increase in morbidity and mortality caused by the prevalence of hypervirulent and drug-resistant strains has become a global concern⁷. In 2019, *C. difficile* was listed by the US Centers for Disease Control and Prevention as an “urgent threat” that surpassed methicillin-resistant *Staphylococcus aureus* (MRSA) and ESBL-producing *Enterobacteriales*³⁸. *C. difficile* is a commensal bacterium that colonizes the lower gastrointestinal tract³⁹. CDI typically occurs in susceptible individuals whose normal gut microbiota is disrupted by the administration of broad-spectrum antibiotics³⁹. Although CDI is considered an antibiotic-associated disease, the current treatment for CDI still relies on antimicrobial drugs, including metronidazole, fidaxomicin and vancomycin^{9, 10}. Despite antibiotic therapy, more than 35% of CDI patients are susceptible to recurrent infection, which is primarily attributable to the intrinsic effects of antimicrobial agents, which disrupt the microbiota¹². In addition, long-term administration of antibiotics promotes the selection of antibiotic-resistant subpopulations in the gut, thus increasing the risk of infections caused by antibiotic-resistant and opportunistic bacteria⁴⁰. As a result, the development of nonantibiotic therapeutics is needed for the treatment of CDI.

Indeed, numerous novel treatments for CDI are being investigated, among which the anti-virulence strategy represents one of the most promising and successful therapeutic approaches^{22, 41}. Unlike antibiotics, which target bacterial components that are critical for bacterial growth, anti-virulence drugs aim to disarm pathogenic bacteria by neutralizing their virulence factors^{42, 43}. Therefore, this strategy imposes less selective pressure that directs bacterial evolution of antimicrobial resistance⁴³. The disease progression of CDI is strictly dependent on large clostridial toxins (TcdA and TcdB), as strains lacking both toxins (i.e., A[−]B[−]) were completely avirulent and failed to induce any discernable disease symptoms in a hamster model of infection⁴⁴. Importantly, epidemic *C. difficile* strains with increased morbidity and mortality rates produce greater amounts of toxins⁴⁵. Based on these premises, TcdA and TcdB are considered ideal drug targets for CDI in the anti-virulence paradigm⁴⁶. Indeed, bezlotoxumab, a fully humanized monoclonal antibody that neutralizes TcdB, was approved by the FDA and used in CDI patients to reduce the rate of recurrence⁴⁷. This achievement has encouraged researchers to develop next-generation anti-virulence treatments. In particular, small molecule inhibitors of toxin production and/or activity have attracted increasing interest owing to their advantages in cost and the convenience of oral administration⁴¹. The multidomain architecture and mechanisms of action of clostridial toxins have provided distinct options for blocking their toxicity. Indeed, previous efforts have identified diverse inhibitors that act on distinct steps of intoxication, such as the inhibition of toxin binding to cell receptors, the suppression of endosomal maturation, and the inactivation of GTD or CPD activities^{23, 48, 49, 50, 51}. Although which approach is the best remains unknown, we believe that an inhibitor that targets multiple steps of intoxication would be superior to those that act on a single step. In this study, we identified a series of caffeic acid derivatives that were potent inhibitors of TcdB via a phenotypic screen. Further mechanistic study revealed that CAPE, the phenethyl alcohol ester of caffeic acid, could block InsP₆-induced autoproteolysis of TcdB and inactivate its GTD function (**Figure 10**). CAPE can also inhibit the growth of *C. difficile* and the production of spores. Moreover, CAPE treatment significantly protected against CDI in a mouse model of infection. Our results suggested that caffeic acid derivatives, particularly CAPE, are promising lead compounds for the development of targeted therapies against CDI. CAPE is a major component of propolis, a natural

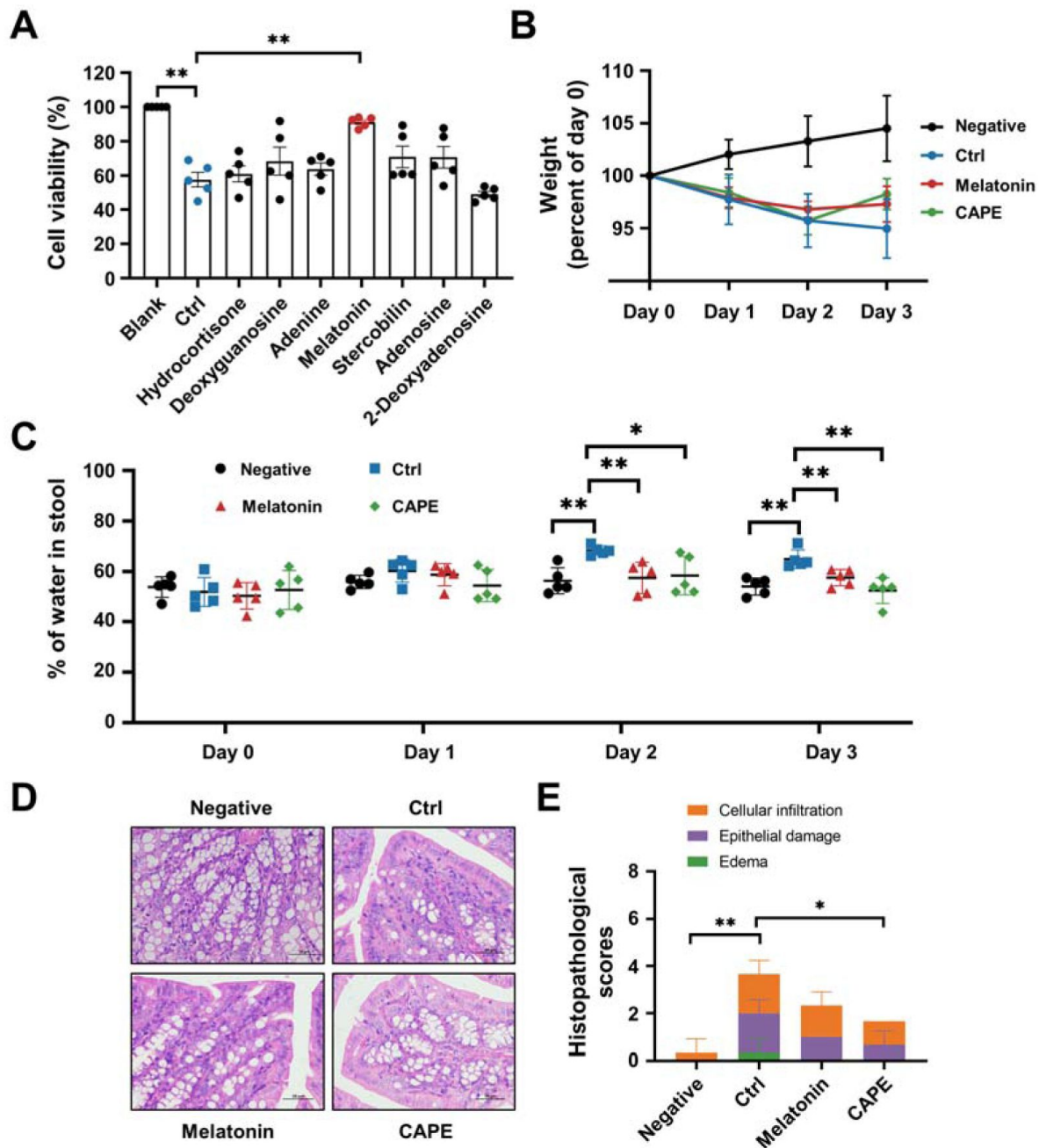


Figure 9.

Oral administration of melatonin alleviates the pathology of CDI.

(A) The impact of upregulated metabolites on the cytotoxicity of TcdB. TcdB (0.2 ng/mL) was incubated with each of the metabolites (100 µg/mL) for 1 h at 37 °C before being added to the Vero cell culture. At 3 h after TcdB treatment, cell death was evaluated with a CCK-8 kit. (B) Body weight changes in melatonin-treated CDI mice (n=5). (C) The fecal dry/wet weight ratio in CDI mice after melatonin treatment (n=5). (D) Histopathological analysis of colon tissue from CDI mice treated with melatonin. (E) Histopathological scores of melatonin-treated CDI mice. * $p < 0.05$; ** $p < 0.01$ using one-way ANOVA.

bee-produced substance that has been used as a remedy for thousands of years in Europe and Asia ⁵². As a result, further investigations on the therapeutic potential of propolis against CDI, either in combination with standard therapy or as a single therapy, would be of great interest.

Antibiotic-induced gut microbiota dysbiosis is a major risk factor for CDI ⁵³. Thus, reconstituting the gut microbiota is a promising strategy for CDI therapy. Fecal microbiota transplantation (FMT) is considered an effective CDI therapeutic strategy and is widely used in clinical practice ⁵⁴. Despite the high cure rate and increasing popularity of FMT, patients remains limited acceptance. Another prominent advantage of anti-virulence drugs is that they allow maintenance of the normal microbiota, which is of critical importance for the treatment of CDI. Importantly, CAPE treatment of *C. difficile*-infected mice restored the structure and composition of the microbiota (**Figure 10**). In particular, the abundance of *Bacteroides*, a critical gut microbiota component that is required for resistance to *C. difficile* colonization ^{26, 55, 56, 57}, was significantly elevated upon CAPE treatment. Interestingly, *Bacteroides* were also predominant in CDI patients administered RBX2660 (Rebyota), an FDA-approved fecal microbiota product ^{58, 59}. The increased diversity of the gut microbiota might contribute to the therapeutic effects of CAPE in CDI.

In addition to the microbiota, gut metabolites also play crucial roles in the pathogenesis of CDI, either by promoting or suppressing disease progression ³⁰. For example, bile acids and amino acids promote spore germination and pathogenesis, while secondary bile acid and short-chain fatty acids aid in colonization resistance and suppress spore germination ³⁰. In addition to restoration of the gut microbiota, metabolomics analysis of mouse fecal samples revealed dramatic alterations in gut metabolites upon CAPE treatment (**Figure 10**). The differentially abundant metabolites were significantly enriched in the purine metabolism and bile metabolism pathways. Remarkably, the level of adenosine, an endogenous purine nucleoside that can regulate immune responses and inflammatory processes by binding to its receptors, such as its ability to inhibit the release of certain inflammatory mediators, was dramatically increased in CAPE-treated mice ^{60, 61}. In contrast, the amount of uric acid, the final product of purine metabolism, was reduced. Previous studies showed that the increase in adenosine concentrations caused by the inhibition of adenosine deaminase via EHNA prevents *C. difficile* TcdA-mediated damage and inflammation ³³. This protection might be a result of the anti-inflammatory effects of adenosine, which rely on the activation of its receptors ⁶². In addition, we observed a reduction in the amount of D-proline, which is one of the preferred amino acids utilized by *C. difficile* to generate energy through Stickland reactions ³⁵. Melatonin is a ubiquitous indolamine that has broad biological activities ⁶³. Notably, we detected a significantly increase in the amount of melatonin in the feces of mice that were administered CAPE. Importantly, an earlier study demonstrated a decreased risk of recurrent CDI in patients exposed to melatonin, possibly owing to its antibacterial and anti-inflammatory activities ³⁷. In this study, we found that oral administration of exogenous melatonin alleviated the symptoms of CDI in a mouse model of infection. Although determining the exact mechanism will require further investigation, we propose that the melatonin-mediated inhibition of TcdB intoxication observed in this study might contribute to its therapeutic effect. Taken together, these results indicate that the alterations in gut metabolites caused by CAPE might also contribute to treatment outcomes for CDI patients.

Methods

Cell lines

All cell lines (HeLa, Caco-2, Vero, HEK293T and THP-1) were obtained from ATCC (Manassas, VA). HeLa, Caco-2, Vero and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen)

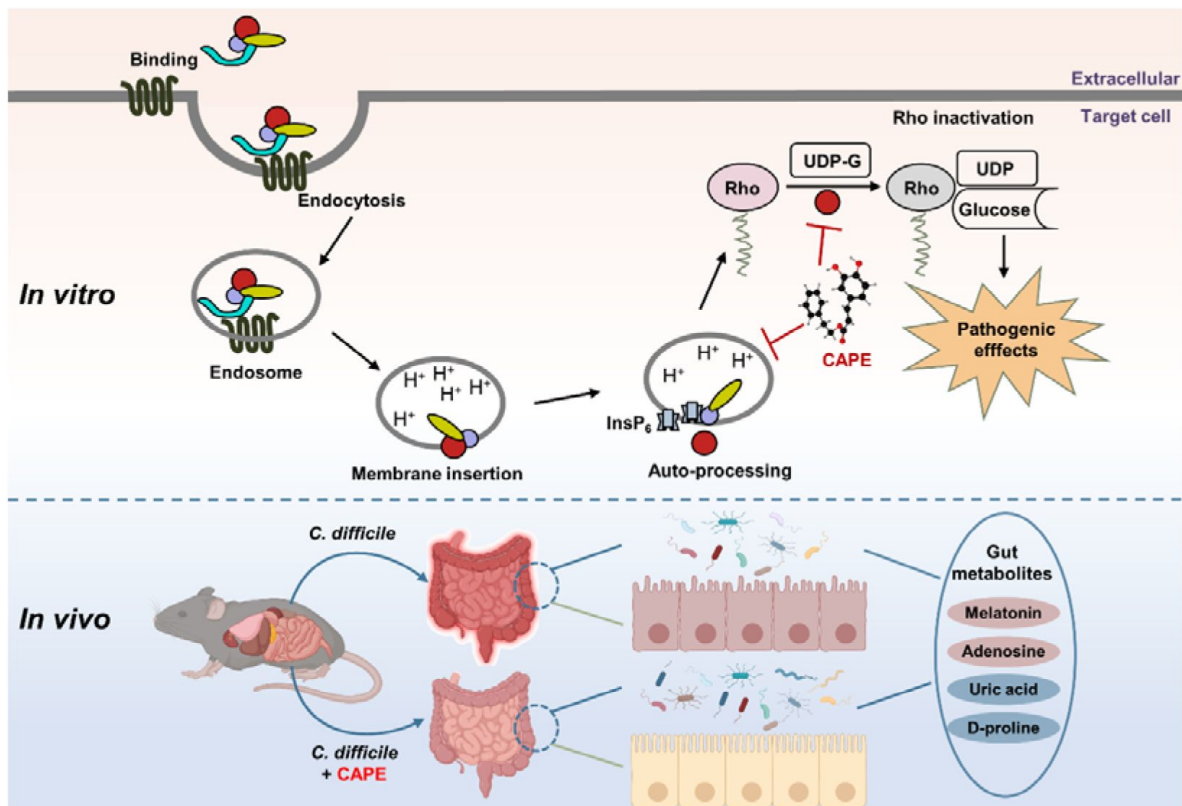


Figure 10.

A model for CAPE-mediated suppression of TcdB cytotoxicity and protection of mice from CDI.

The intoxication of cells by TcdB involves multistep mechanisms. Direct interaction of CAPE with full-length TcdB blocks InsP₆-induced autoproteolysis, resulting in decreased production of active GTD. The binding between GTD and CAPE inhibits its glucosyltransferase activity, thus decreasing the glucosylation level of Rac1. CAPE treatment of CDI mice markedly restored the diversity and composition of the gut microbiota and induced changes in gut metabolites, which might have contributed to the therapeutic outcomes of CDI following CAPE treatment.

and 1% (w/v) penicillin-streptomycin. THP-1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen) supplemented with 10% FBS and 1% (w/v) penicillin-streptomycin. All cell lines were cultured at 37 °C in a 5% CO₂ atmosphere.

Bacterial strains and reagents

C. difficile BAA-1870 (RT027/ST1) was kindly provided by Aiwu Wu (Guangzhou Medical University) and cultured in brain-heart infusion broth (BHI) supplemented with 5 g/L yeast extract and 0.1% (w/v) L-cysteine (BHIS). The compounds were identified from two main sources: FDA-approved drugs obtained from ApexBio Technology (Houston, TX, USA) and small molecule natural compounds obtained from Chengdu Ruifensi Biotechnology Co., Ltd. (Chengdu, China). LysoTracker Red DND-99, kanamycin, and cefoperazone sodium were purchased from Dalian Meilun Biotechnology Co., Ltd. (Dalian, China).

Mice

Male C57BL/6 mice aged 6–8 weeks were purchased from Liaoning Changsheng Biotechnology Co., Ltd., and housed in a specific pathogen-free (SPF) facility. The mice lived in a controlled environment with a temperature of 23±2 °C and a humidity of 55±10 %. Prior to infection, the mice were acclimated to the environment for a minimum of one week. All mouse experiments were approved by the Jilin University Institutional Animal Care Committee, Jilin University and strictly conducted according to the guidelines of this committee (SY202402301).

Protein expression and purification

The expression and purification of full-length TcdB were conducted following the method described by Yang et al. ⁶⁴[\[64\]](#). In brief, *Bacillus megaterium* harboring pHis1522-TcdB was inoculated into LB media supplemented with a final concentration of 10 µg/mL tetracycline. Once the OD_{600nm} reached approximately 0.3, xylose (0.5% w/v) was added to induce protein expression at 37 °C for 16 h. Then, 100 mL of the bacterial cells was harvested by centrifugation, resuspended in 5 mL of lysis buffer containing 20 mM imidazole, 300 mM NaCl, 20 mM NaH₂PO₄, 500 µM EDTA, and protease inhibitor cocktail (adjusted to pH 8.0), and subjected to sonication. The cell lysate was then centrifuged at 13,400 × g for 20 min to remove cell debris. The TcdB protein was purified using nickel column affinity chromatography. The purity of the protein was confirmed by SDS-PAGE, and the protein was subsequently dialyzed against buffer containing 10% glycerol, 20 mM Tris (pH 7.5), and 150 mM NaCl. After the protein concentration was determined, the purified TcdB protein was aliquoted and stored at −80 °C for further use.

For the purification of GTD, amplified DNA products were inserted into pET28a (Novagen), and the *Escherichia coli* (*E. coli*) strain BL21 (DE3) was used for the expression and purification of GTD. Transformed *E. coli* pET28a-GTD was cultured in Luria–Bertani (LB) medium supplemented with 10 µg/mL kanamycin. Once the OD₆₀₀ reached 0.6, the expression of proteins was induced with 0.2 mM isopropyl-β-D-thiogalactoside (IPTG) overnight at 18 °C. The bacterial cells were then harvested by centrifugation and subjected to sonication. The cell lysate was then centrifuged at 13,400 × g for 20 min to remove cell debris. GTD protein was purified using nickel affinity chromatography and dialyzed in buffer containing 10% glycerol, 20 mM Tris (pH 7.5), and 150 mM NaCl. The GTD mutants were introduced into pET28a-GTD using the Quick Change Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA, USA) with the primers listed in [Table S2](#)[\[65\]](#). The subsequent purification was performed using the same procedure as described for wild-type GTD.

Reverse transcription-quantitative PCR

Total RNA was extracted by TRIzol and then reverse-transcribed into complementary DNA by a NovoScript® Plus All-in-one 1st Strand cDNA Synthesis SuperMix (E047; Novoprotein, China). mRNA expression of *tcdB* was measured by quantitative PCR with NovoScript®SYBR qPCR

SuperMix Plus (E096; Novoprotein, China) using the primers shown in [Table S3](#). 16S rRNA was used as internal control. The relative mRNA expression levels were calculated by the delta-delta CT method.

Growth curve assays

Growth curve assay was performed to estimate the influence of CAPE on the growth of *C. difficile* BAA-1870. The overnight culture of BAA-1870 was transferred to fresh BHIS medium and incubated at 37°C in an anaerobic atmosphere. The culture was supplemented with different concentrations of CAPE ranging from 0 to 64 µg/mL and the optical density at 600 nm was monitored at 4 h intervals to assess growth of BAA-1870.

Inhibition of spore outgrowth

The impact of CAPE on the sporulation of *C. difficile* BAA-1870 was assessed using the protocol described by Joshua *et al*.⁶⁵ In brief, BAA-1870 cultures were treated with 8 or 32 µg/mL of CAPE for 48 h. The treated samples were then harvested, subjected to a double wash with PBS, and incubated in a 60°C water bath for 30 min. Subsequently, 20 µL of each spore suspension was plated onto BHIS agar supplemented with 0.1% taurine to facilitate germination. These plates were incubated anaerobically at 37°C for 5 d to allow for spore germination. Following this period, the number of resulting colonies on the plates was enumerated.

Cell rounding assay

Cell rounding assay was performed according to Kristina *et al*, with appropriate adjustments⁴⁸. Vero or HeLa cells were seeded in 96-well sterile plates using serum-free DMEM overnight, and the compounds were preincubated with TcdB for 1 h. After incubation, the TcdB-compound complex was added to cells for 1 h at 37 °C. The cell morphology was visualized under a light microscope.

LysoTracker assay

The pH of endosomes was determined by LysoTracker Red DND-99⁴⁹. Caco-2 or THP-1 cells in complete DMEM were seeded in 96-well sterile plates overnight. The media was changed to serum-free DMEM for 1 h, and CAPE was preincubated with TcdB for 1 h, followed by addition to the cells and incubation for 2 h. Baf-A1 served as a control. After incubation, LysoTracker and Hoechst dyes were added and the cells were incubated for 1 h. Excess dye was removed by changing the media, and alterations in lysosomal red intensity were documented using a fluorescence microscope.

In vitro TcdB autoprocessing assay

The self-cleavage ability of TcdB primarily depends on its cysteine protease activity⁴⁸. CAPE (1-16 µg/mL) was preincubated with TcdB for 1 h, followed by the addition of InsP₆ and incubation at 37 °C for 6 h. Subsequently, loading buffer was added for SDS-PAGE analysis, and the results were visualized using Coomassie Brilliant Blue staining.

Rac1 glucosylation assay

Rac1 glucosylation assay was performed following the method described by John *et al*⁵⁰. HEK293T cells were seeded into a 6-well plate. The next day, TcdB and different concentrations of CAPE (4-32 µg/mL) were added to cells. After 2 h, the cells were harvested, lysed using lysis buffer, and subjected to centrifugation at 13,400 × g for 10 min to collect the supernatant. The collected supernatant was then mixed with loading buffer and subjected to SDS-PAGE followed by western blot analysis. The glycosylation level of Rac1 was evaluated by determining the levels of non-glycosylated Rac1 (BD Biosciences, Mississauga, ON) and total Rac1 (Abcam).

UDP-Glo™ UDP-glucose hydrolase assay

GTD activity was assessed according to the manufacturer's instructions⁵⁰. A total volume of 16 μ L, consisting of 100 nM GTD, various concentrations of CAPE and glucosylation buffer, was prepared. Four microliters of UDP-glucose at a final concentration of 25 μ M was added to the mixture, which was then allowed to react at room temperature for 15 min. To stop the reaction, 10 μ L of the reaction mixture was removed and placed in a white polystyrene 96-well plate containing 10 μ L of UDP detection reagent. The reaction took place at room temperature for 1 h. The luminescence value was measured at a 750 ms integral, and the GTD activity was determined based on these measurements.

Circular dichroism (CD) spectroscopy

CD spectra of TcdB, TcdB-CAPE, GTD, and GTD-CAPE were obtained using a CD spectrophotometer (MOS-500; Bio-Logic, France). TcdB or GTD at a concentration of 0.2 mg/mL was incubated with 32 μ g/mL CAPE. Changes in the secondary structure of TcdB or GTD were assessed at room temperature using a quartz cuvette with an optical path length of 1 mm. The scan wavelength range was set from 190 to 250 nm with a resolution of 0.2 nm and a bandwidth of 1 nm. The secondary structures of TcdB, TcdB-CAPE, GTD, and GTD-CAPE were analyzed using the BeStSel web server.

Molecular docking

CAPE was obtained as a ligand from PubChem, and its topology was generated using AmberTools. The crystal structure of GTD was retrieved from the Protein Data Bank (PDB) and processed using AutoDockTools. Docking calculations were performed using AutodockVina, and molecular dynamics simulations were carried out using Gromacs 2020.6. The RMSD of the receptor and ligand was monitored to assess conformational changes during the binding process. The interaction energy was analyzed using the Poisson-Boltzmann surface area (MM-PBSA) method, which is a molecular mechanics approach.

Flow cytometry

Flow cytometry was used to quantify the cellular uptake of TcdB via endocytosis. To facilitate detection, full-length TcdB was labeled with Alexa Fluor®488 NHS Ester (Yeasen Biotechnology Co., Ltd., Shanghai, China). Prior to cell treatment, Alexa Fluor 488-TcdB was preincubated with CAPE at a concentration of 32 μ g/mL for 30 min. The treated solution was then added to Caco-2 cells and incubated for 1 h at 37 °C. Following three washes with phosphate-buffered saline (PBS), the Caco-2 cells were collected, and the fluorescence signal was measured using flow cytometry.

Cytotoxicity assay

Vero or THP-1 cells were plated into 96-well plates. The next day, different concentrations of CAPE were prepared by diluting them in DMEM and adding them to the cells. After incubation at 37 °C for 3 h, the viability of cells was assessed using a Cell Counting Kit-8 (CCK-8) (Beyotime Biotechnology Co., Shanghai, China) according to the manufacturer's instructions.

Biacore analysis

The affinity analysis of TcdB or GTD with CAPE was conducted using a Biacore T100 device (Biacore Inc., Uppsala, Sweden) according to the manufacturer's protocol. A CM5 chip (General Electric Company, GE) was utilized to immobilize the TcdB or GTD proteins. Various concentrations of CAPE were injected, and all steps were performed in running buffer consisting of 5% DMSO in PBS-P20 (GE). Evaluation version 3.1 software provided by Biacore and the 1:1 Langmuir combined model were employed for data analysis.

Mouse infection model

For the acute toxicity model, after pretreatment of TcdB with CAPE (2 or 4 µg/mL) for 1 h, the mice were intraperitoneally injected with a complex of TcdB-solvent or TcdB-CAPE (30 ng of TcdB per mouse). The survival of mice was observed for 72 h.

For the CDI model, mice were given cefoperazone (0.5 g/L) in their drinking water for 5 days (from day -7 to day -2), followed by switching to sterilized water for 2 days. On day 0, the mice were challenged with 1×10^8 colony-forming units (CFUs) of *C. difficile* BAA-1870 (Ribotype 027) by oral gavage. For the CAPE or melatonin treatment group, mice were treated with CAPE (30 mg/kg by oral gavage) or melatonin (200 mg/kg by oral gavage) every day after *C. difficile* challenge. The weight loss and fecal state of the mice were monitored daily. Fecal samples were collected on days 0-3 to evaluate the stool water content as a marker of diarrhea. Additionally, fecal samples on day 3 were collected to assess *C. difficile* colonization. Colonic tissues were collected on day 3 post infection, stained with hematoxylin and eosin (H&E) and scored as previously described [66](#).

Fecal samples were collected on day 3 post infection, frozen in liquid nitrogen and transferred to the laboratory with dry ice for 16S rRNA sequencing and untargeted metabolomics analysis (Novogene Bioinformatics Technology Co., Ltd., Tianjin, China). The melatonin level in mouse stool supernatant was determined using the Mouse MT (Melatonin) ELISA Kit (Sangon Biotechnology Co., Ltd., Shanghai, China).

Statistical analysis

Western blot densitometry and LysoTracker fluorescence intensity were measured using ImageJ software (NIH, Bethesda, MD, USA). Statistical analysis was performed using GraphPad Prism 9.0 (GraphPad Software, USA). Unpaired two-tailed Student's *t* tests were used for comparing two groups, and one-way analysis of variance (ANOVA) was used for analyzing data from more than three groups. The statistical significance was determined using the log-rank (Mantel-Cox) test (survival rates) and Mann-Whitney U test (tissue bacterial load) *in vivo*. All the data are expressed as the mean $\pm$ standard deviation (SD). * indicates $p < 0.05$ and ** indicates $p < 0.01$.

Data availability

All 16S rRNA gene sequencing data generated and analyzed during the current study were deposited in the Sequence Read Archive (SRA) under the BioProject accession number PRJNA1100298. Untargeted metabolomics data are available via Metabolights with the identifier MTBLS9938.

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Additional information

Author contributions

Y.G., L.Q.H and J.Z.Q designed the study and wrote the manuscript. Y.G., Y.Z., G.Z.W. and H.T.L. conducted experiments. J.F.W., X.M.D., L.Q.H. and J.Z.Q. supervised the study and revised the manuscript. All authors read and approved the final manuscript. J.Z.Q. and L.Q.H. are responsible for the overall content as the guarantor.

Additional files

Supplementary material [↗](#)

References

1. Smits WK, Lyras D, Lacy DB, Wilcox MH, Kuijper EJ. (2016) **Clostridium difficile infection** *Nat Rev Dis Primers* **2**:16020 [Google Scholar](#)
2. Miller BA, Chen LF, Sexton DJ, Anderson DJ (2011) **Comparison of the burdens of hospital-onset, healthcare facility-associated Clostridium difficile Infection and of healthcare-associated infection due to methicillin-resistant Staphylococcus aureus in community hospitals** *Infect Control Hosp Epidemiol* **32**:387–390 [Google Scholar](#)
3. Kelly CP, LaMont JT (2008) **Clostridium difficile--more difficult than ever** *N Engl J Med* **359**:1932–1940 [Google Scholar](#)
4. Dubberke E (2012) **Clostridium difficile infection: the scope of the problem** *J Hosp Med* **7**:S1–4 [Google Scholar](#)
5. Lessa FC, et al. (2015) **Burden of Clostridium difficile infection in the United States** *N Engl J Med* **372**:825–834 [Google Scholar](#)
6. Goorhuis A, et al. (2008) **Emergence of Clostridium difficile infection due to a new hypervirulent strain, polymerase chain reaction ribotype 078** *Clin Infect Dis* **47**:1162–1170 [Google Scholar](#)
7. He M, et al. (2013) **Emergence and global spread of epidemic healthcare-associated Clostridium difficile** *Nat Genet* **45**:109–113 [Google Scholar](#)
8. Clements AC, Magalhaes RJ, Tatem AJ, Paterson DL, Riley TV (2010) **Clostridium difficile PCR ribotype 027: assessing the risks of further worldwide spread** *Lancet Infect Dis* **10**:395–404 [Google Scholar](#)
9. Cornely OA, et al. (2012) **Fidaxomicin versus vancomycin for infection with Clostridium difficile in Europe, Canada, and the USA: a double-blind, non-inferiority, randomised controlled trial** *Lancet Infect Dis* **12**:281–289 [Google Scholar](#)
10. Bingley PJ, Harding GM (1987) **Clostridium difficile colitis following treatment with metronidazole and vancomycin** *Postgrad Med J* **63**:993–994 [Google Scholar](#)
11. Feuerstadt P, Theriault N, Tillotson G (2023) **The burden of CDI in the United States: a multifactorial challenge** *BMC Infect Dis* **23** [Google Scholar](#)
12. Hopkins RJ, Wilson RB (2018) **Treatment of recurrent Clostridium difficile colitis: a narrative review** *Gastroenterol Rep (Oxf)* **6**:21–28 [Google Scholar](#)
13. Spigaglia P, Mastrantonio P, Barbanti F (2018) **Antibiotic Resistances of Clostridium difficile** *Adv Exp Med Biol* **1050**:137–159 [Google Scholar](#)
14. Seekatz AM, Young VB (2014) **Clostridium difficile and the microbiota** *J Clin Invest* **124**:4182–4189 [Google Scholar](#)

15. Paredes-Sabja D, Shen A, Sorg JA (2014) **Clostridium difficile spore biology: sporulation, germination, and spore structural proteins** *Trends Microbiol* **22**:406–416 [Google Scholar](#)
16. Lyras D, et al. (2009) **Toxin B is essential for virulence of Clostridium difficile** *Nature* **458**:1176–1179 [Google Scholar](#)
17. Kuehne SA, Cartman ST, Heap JT, Kelly ML, Cockayne A, Minton NP (2010) **The role of toxin A and toxin B in Clostridium difficile infection** *Nature* **467**:711–713 [Google Scholar](#)
18. Carter GP, et al. (2015) **Defining the Roles of TcdA and TcdB in Localized Gastrointestinal Disease, Systemic Organ Damage, and the Host Response during Clostridium difficile Infections** *mBio* **6**:e00551 [Google Scholar](#)
19. Drudy D, Fanning S, Kyne L (2007) **Toxin A-negative, toxin B-positive Clostridium difficile** *Int J Infect Dis* **11**:5–10 [Google Scholar](#)
20. Chen P, et al. (2019) **Structure of the full-length Clostridium difficile toxin B** *Nat Struct Mol Biol* **26**:712–719 [Google Scholar](#)
21. Orrell KE, Melnyk RA (2021) **Large Clostridial Toxins: Mechanisms and Roles in Disease** *Microbiol Mol Biol Rev* **85**:e0006421 [Google Scholar](#)
22. Rasko DA, Sperandio V (2010) **Anti-virulence strategies to combat bacteria-mediated disease** *Nat Rev Drug Discov* **9**:117–128 [Google Scholar](#)
23. Tam J, Beilhartz GL, Auger A, Gupta P, Therien AG, Melnyk RA (2015) **Small molecule inhibitors of Clostridium difficile toxin B-induced cellular damage** *Chem Biol* **22**:175–185 [Google Scholar](#)
24. Balaha M, De Filippis B (2021) **Cataldi A, di Giacomo V. CAPE and Neuroprotection: A Review** *Biomolecules* **11** [Google Scholar](#)
25. Garland M, et al. (2020) **The Clinical Drug Ebselen Attenuates Inflammation and Promotes Microbiome Recovery in Mice after Antibiotic Treatment for CDI** *Cell Rep Med* **1** [Google Scholar](#)
26. Li X, et al. (2021) **A strain of Bacteroides thetaiotaomicron attenuates colonization of Clostridioides difficile and affects intestinal microbiota and bile acids profile in a mouse model** *Biomed Pharmacother* **137** [Google Scholar](#)
27. Kienesberger B, et al. (2021) **(S)-Reutericyclin: Susceptibility Testing and In Vivo Effect on Murine Fecal Microbiome and Volatile Organic Compounds** *Int J Mol Sci* **22** [Google Scholar](#)
28. Khanna S, et al. (2016) **Gut microbiome predictors of treatment response and recurrence in primary Clostridium difficile infection** *Aliment Pharmacol Ther* **44**:715–727 [Google Scholar](#)
29. Dong D, et al. (2018) **Effects of intestinal colonization by Clostridium difficile and Staphylococcus aureus on microbiota diversity in healthy individuals in China** *BMC Infect Dis* **18** [Google Scholar](#)

30. Gurung B, Stricklin M, Wang S (2024) **Gut Microbiota-Gut Metabolites and Clostridioides difficile Infection: Approaching Sustainable Solutions for Therapy** *Metabolites* **14** [Google Scholar](#)
31. Cronstein BN (1994) **Adenosine, an endogenous anti-inflammatory agent** *J Appl Physiol* **76**:5–13 [Google Scholar](#)
32. Yong T, et al. (2022) **Caffeic acid phenethyl ester alleviated hypouricemia in hyperuricemic mice through inhibiting XOD and up-regulating OAT3** *Phytomedicine* **103** [Google Scholar](#)
33. de Araujo Junqueira AF, et al. (2011) **Adenosine deaminase inhibition prevents Clostridium difficile toxin A-induced enteritis in mice** *Infect Immun* **79**:653–662 [Google Scholar](#)
34. Neumann-Schaal M, Jahn D, Schmidt-Hohagen K (2019) **Metabolism the Difficile Way: The Key to the Success of the Pathogen Clostridioides difficile** *Front Microbiol* **10** [Google Scholar](#)
35. Bouillaut L, Self WT, Sonenshein AL (2013) **Proline-dependent regulation of Clostridium difficile Stickland metabolism** *J Bacteriol* **195**:844–854 [Google Scholar](#)
36. Jackson S, Calos M, Myers A, Self WT (2006) **Analysis of proline reduction in the nosocomial pathogen Clostridium difficile** *J Bacteriol* **188**:8487–8495 [Google Scholar](#)
37. Sutton SS, Magagnoli J, Cummings TH, Hardin JW (2022) **Melatonin as an Antimicrobial Adjuvant and Anti-Inflammatory for the Management of Recurrent Clostridioides difficile Infection** *Antibiotics (Basel)* **11** [Google Scholar](#)
38. CDC (2019) **AR Threats Report** <https://www.cdc.gov/drugresistance/biggest-threats.html> | [Google Scholar](#)
39. Abbas A, Zackular JP (2020) **Microbe-microbe interactions during Clostridioides difficile infection** *Curr Opin Microbiol* **53**:19–25 [Google Scholar](#)
40. Raplee I, et al. (2021) **Emergence of nosocomial associated opportunistic pathogens in the gut microbiome after antibiotic treatment** *Antimicrob Resist Infect Control* **10** [Google Scholar](#)
41. Beilhartz GL, Tam J, Melnyk RA (2015) **Small Molecules Take A Big Step Against Clostridium difficile** *Trends Microbiol* **23**:746–748 [Google Scholar](#)
42. Ranftler C, et al. (2021) **Binding and neutralization of C. difficile toxins A and B by purified clinoptilolite-tuff** *PLoS One* **16**:e0252211 [Google Scholar](#)
43. Zucca M, Scutera S, Savoia D (2013) **Novel avenues for Clostridium difficile infection drug discovery** *Expert Opin Drug Discov* **8**:459–477 [Google Scholar](#)
44. Kuehne SA, Collery MM, Kelly ML, Cartman ST, Cockayne A, Minton NP (2014) **Importance of toxin A, toxin B, and CDT in virulence of an epidemic Clostridium difficile strain** *J Infect Dis* **209**:83–86 [Google Scholar](#)
45. Warny M, et al. (2005) **Toxin production by an emerging strain of Clostridium difficile associated with outbreaks of severe disease in North America and Europe** *Lancet* **366**:1079–1084 [Google Scholar](#)
46. Stewart D, Anwar F, Vedantam G (2020) **Anti-virulence strategies for Clostridioides difficile infection: advances and roadblocks** *Gut Microbes* **12** [Google Scholar](#)

47. Deeks ED. (2017) **Bezlotoxumab: A Review in Preventing Clostridium difficile Infection Recurrence** *Drugs* **77**:1657–1663 [Google Scholar](#)
48. Bender KO, et al. (2015) **A small-molecule antivirulence agent for treating Clostridium difficile infection** *Sci Transl Med* **7**:306ra148 [Google Scholar](#)
49. Chan H, et al. (2022) **Vitamin D(3) and carbamazepine protect against Clostridioides difficile infection in mice by restoring macrophage lysosome acidification** *Autophagy* **18**:2050–2067 [Google Scholar](#)
50. Tam J, et al. (2018) **Host-targeted niclosamide inhibits C. difficile virulence and prevents disease in mice without disrupting the gut microbiota** *Nat Commun* **9**:5233 [Google Scholar](#)
51. Tam J, et al. (2020) **Intestinal bile acids directly modulate the structure and function of C. difficile TcdB toxin** *Proc Natl Acad Sci U S A* **117**:6792–6800 [Google Scholar](#)
52. Tek NA, Senture SA, Ersoy N (2024) **Is Propolis a Potential Anti-Obesogenic Agent for Obesity?** *Curr Nutr Rep* [Google Scholar](#)
53. Schubert AM, Sinani H, Schloss PD (2015) **Antibiotic-Induced Alterations of the Murine Gut Microbiota and Subsequent Effects on Colonization Resistance against Clostridium difficile** *mBio* **6**:e00974 [Google Scholar](#)
54. van Nood E, et al. (2013) **Duodenal infusion of donor feces for recurrent Clostridium difficile** *N Engl J Med* **368**:407–415 [Google Scholar](#)
55. Bornet E, Westermann AJ (2022) **The ambivalent role of Bacteroides in enteric infections** *Trends Microbiol* **30**:104–108 [Google Scholar](#)
56. Pike CM, Theriot CM (2021) **Mechanisms of Colonization Resistance Against Clostridioides difficile** *J Infect Dis* **223**:S194–S200 [Google Scholar](#)
57. Deng H, et al. (2019) **Corrigendum: Bacteroides fragilis Prevents Clostridium difficile Infection in a Mouse Model by Restoring Gut Barrier and Microbiome Regulation** *Front Microbiol* **10** [Google Scholar](#)
58. Lee C, et al. (2023) **Safety of fecal microbiota, live-jslm (REBYOTA()) in individuals with recurrent Clostridioides difficile infection: data from five prospective clinical trials** *Therap Adv Gastroenterol* **16** [Google Scholar](#)
59. Gonzales-Luna AJ, Carlson TJ, Garey KW (2023) **Review Article: Safety of Live Biotherapeutic Products Used for the Prevention of Clostridioides difficile Infection Recurrence** *Clin Infect Dis* **77**:S487–S496 [Google Scholar](#)
60. Hasko G, Linden J, Cronstein B, Pacher P (2008) **Adenosine receptors: therapeutic aspects for inflammatory and immune diseases** *Nat Rev Drug Discov* **7**:759–770 [Google Scholar](#)
61. Shakya AK, Naik RR, Almasri IM, Kaur A (2019) **Role and Function of Adenosine and its Receptors in Inflammation, Neuroinflammation, IBS, Autoimmune Inflammatory Disorders, Rheumatoid Arthritis and Psoriasis** *Curr Pharm Des* **25**:2875–2891 [Google Scholar](#)
62. Cavalcante IC, et al. (2006) **Effect of novel A2A adenosine receptor agonist ATL 313 on Clostridium difficile toxin A-induced murine ileal enteritis** *Infect Immun* **74**:2606–2612 [Google Scholar](#)

63. Ma N, Zhang J, Reiter RJ, Ma X (2020) **Melatonin mediates mucosal immune cells, microbial metabolism, and rhythm crosstalk: A therapeutic target to reduce intestinal inflammation** *Med Res Rev* **40**:606–632 [Google Scholar](#)
64. Yang G, et al. (2008) **Expression of recombinant Clostridium difficile toxin A and B in Bacillus megaterium** *BMC Microbiol* **8** [Google Scholar](#)
65. Fletcher JR, et al. (2021) **Clostridioides difficile exploits toxin-mediated inflammation to alter the host nutritional landscape and exclude competitors from the gut microbiota** *Nat Commun* **12** [Google Scholar](#)
66. Reeves AE, Theriot CM, Bergin IL, Huffnagle GB, Schloss PD, Young VB (2011) **The interplay between microbiome dynamics and pathogen dynamics in a murine model of Clostridium difficile Infection** *Gut Microbes* **2**:145–158 [Google Scholar](#)

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Reviewer #1 (Public review):

Summary:

In this manuscript, Guo and colleagues used a cell rounding assay to screen a library of compounds for inhibition of TcdB, an important toxin produced by *Clostridioides difficile*. Caffeic acid and derivatives were identified as promising leads, and caffeic acid phenethyl ester (CAPE) was further investigated.

Strengths:

Considering the high morbidity rate associated with *C. difficile* infections (CDI), this manuscript presents valuable research in the investigation of novel therapeutics to combat this pressing issue. Given the rising antibiotic resistance in CDI, the significance of this work is particularly noteworthy. The authors employed a robust set of methods and confirmatory tests, which strengthen the validity of the findings. The explanations provided are clear, and the scientific rationale behind the results is well-articulated. The manuscript is extremely well written and organized. There is a clear flow in the description of the experiments performed. Also, the authors have investigated the effects of CAPE on TcdB in careful detail,

and reported compelling evidence that this is a meaningful and potentially useful metabolite for further studies.

Weaknesses:

Although the authors have made changes to the manuscript to address some of my comments, many of the comments were not satisfactorily addressed. Many of the changes are still superficial, and some concerns still need to be addressed. Important details are still missing from the description of some experiments. Authors should carefully revise the manuscript to ascertain that all details that could affect interpretation of their results are presented clearly.

There is still very little discussion (none, really) in the manuscript about the fact that, because the authors observed a significant effect of CAPE on both bacterial growth and spore production, some of the phenotypes observed can no longer be attributed solely to toxin inhibition.

The details about mass spectrometry are still insufficient. It is still unclear whether metabolite identifications were always based on MS1 or MS2. Instead, several details that are really secondary were included. Authors should be unequivocally clear as to how metabolite identities were obtained. They should also indicate which mass spectrometer was used, and there should be a section in the Materials and Methods describing these experiments.

About the removal of carry-over compounds, the authors stated that ultrafiltration centrifugal partition was used. However, although the authors explained this in detail in their response to reviewers file, the details were omitted from the main text. Authors should clearly state in the manuscript text that "Due to the large molecular weight of TcdB, approximately 270 kDa, we selected a 100 kDa molecular weight cutoff ultrafiltration membrane. The centrifugation was performed at 4000 g for 5 min to eliminate the compounds that did not bind to TcdB."

These are important details which need to be included.

<https://doi.org/10.7554/eLife.101757.3.sa2>

Reviewer #2 (Public review):

I appreciate the author's responses to my original review. This is a comprehensive analysis of CAPE on *C. difficile* activity. It seems like this compound affects all aspects of *C. difficile*, which could make it effective during infection but also make it difficult to understand the mechanism. Even considering the authors' responses, I think it is critical for the authors to work on the conclusions regarding the infection model. There is some protection from disease by CAPE but some parameters are not substantially changed. For instance, weight loss is not significantly different in the *C. difficile* only group versus the *C. difficile* + CAPE group. Histology analysis still shows a substantial amount of pathology in the *C. difficile* + CAPE group. This should be discussed more thoroughly using precise language.

The authors did a good job addressing my concerns regarding the infection model by providing a more accurate description in the Results section for histology. However, the weight loss improvement by CAPE does not look like a significant effect, although it is trending towards improvement. This should be more accurately described.

Another minor concern is that the current Abstract is overstating the amount of disease attenuation. I would replace "remarkably reduces the pathology" with "reduces some of the pathology"

Author response:

The following is the authors' response to the previous reviews

Public Reviews:

Reviewer #1 (Public review):

Summary:

In this manuscript, Guo and colleagues used a cell rounding assay to screen a library of compounds for inhibition of TcdB, an important toxin produced by Clostridioides difficile. Caffeic acid and derivatives were identified as promising leads, and caffeic acid phenethyl ester (CAPE) was further investigated.

Strengths:

Considering the high morbidity rate associated with C. difficile infections (CDI), this manuscript presents valuable research in the investigation of novel therapeutics to combat this pressing issue. Given the rising antibiotic resistance in CDI, the significance of this work is particularly noteworthy. The authors employed a robust set of methods and confirmatory tests, which strengthen the validity of the findings. The explanations provided are clear, and the scientific rationale behind the results is well-articulated. The manuscript is extremely well written and organized. There is a clear flow in the description of the experiments performed. Also, the authors have investigated the effects of CAPE on TcdB in careful detail, and reported compelling evidence that this is a meaningful and potentially useful metabolite for further studies.

Weaknesses:

The authors have made some changes in the revised version. However, many of the changes were superficial, and some concerns still need to be addressed. Important details are still missing from the description of some experiments. Authors should carefully revise the manuscript to ascertain that all details that could affect interpretation of their results are presented clearly. For instance, authors still need to include details of how the metabolomics analyses were performed. Just stating that samples were "frozen for metabolomics analyses" is not enough. Was this mass-spec or NMR-based metabolomics. Assuming it was mass-spec, what kind? How was metabolite identity assigned, etc? These are important details, which need to be included. Even in cases where additional information was included, the authors did not discuss how the specific way in which certain experiments were performed could affect interpretation of their results. One example is the potential for compound carryover in their experiments. Another important one is the fact that CAPE affects bacterial growth and sporulation. Therefore, it is critical that authors acknowledge that they cannot discard the possibility that other factors besides compound interactions with the toxin are involved in their phenotypes. As stated previously, authors should also be careful when drawing conclusions from the analysis of microbiota composition data, and changes to the manuscript should be made to reflect this. Ascribing causality to correlational relationships is a recurring issue in the microbiome field. Again, I suggest authors carefully revise the manuscript and tone down some statements about the impact of CAPE treatment on the gut microbiota.

Thanks for your constructive suggestion. We have carefully revised the manuscript according to your suggestions.

Reviewer #2 (Public review):

*I appreciate the author's responses to my original review. This is a comprehensive analysis of CAPE on *C. difficile* activity. It seems like this compound affects all aspects of *C. difficile*, which could make it effective during infection but also make it difficult to understand the mechanism. Even considering the authors responses, I think it is critical for the authors to work on the conclusions regarding the infection model. There is some protection from disease by CAPE but some parameters are not substantially changed. For instance, weight loss is not significantly different in the *C. difficile* only group versus the *C. difficile* + CAPE group. Histology analysis still shows a substantial amount of pathology in the *C. difficile* + CAPE group. This should be discussed more thoroughly using precise language.*

Thanks for your constructive suggestion. We have carefully revised the manuscript according to your suggestions.

Reviewer #3 (Public review):

Summary:

The study is well written, and the results are solid and well demonstrated. It shows a field that can be explored for the treatment of CDI

Strengths:

Results are really good, and the CAPE shows a good and promising alternative for treating CDI.

Weaknesses:

Some references are too old or missing.

Comments on revisions:

I have read your study after comments made by all referees, and I noticed that all questions and suggestions addressed to the authors were answered and well explained. Some of the minor and major issues related to the article were also solved. I am satisfied with all the effort given by the authors to improve their manuscript.

Thanks again for your review.

Recommendations for the authors:

Reviewer #1 (Recommendations for the authors):

*The legend of Figure 3SB is incorrect. It should read "Growth curves of *C. difficile* BAA-1870 in the presence of varying concentrations of CAPE (0-64 µg/mL)". Also, there is something wrong with the symbols in this figure. I suspect what is happening is that the symbols for the concentrations of 32 and 64 µg/mL are superimposing, but this is a problem because the lower line looks like a closed circle, which is supposed to represent the condition where no CAPE was added. The authors should change the symbols to allow clear distinction between each of the conditions.*

Thanks for your constructive suggestion. We have modified the panel and figure legend in Figure 3SB. The concentrations of 32 $\mu\text{g/mL}$ and 64 $\mu\text{g/mL}$ are quite similar, which makes it challenging to differentiate between the corresponding data points on the graph. To enhance clarity, we have utilized distinct colors to help distinguish these closely valued lines as effectively as possible.

Since the authors observed a significant effect of CAPE on both bacterial growth and spore production, their discussion and conclusions need to reflect the fact that the effects observed can no longer be attributed solely to toxin inhibition.

Thanks for your comments. We have modified the corresponding description according to your suggestions.

*In lines 43-45, authors state that "CAPE treatment of *C. difficile*-challenged mice induces a remarkable increase in the diversity and composition of the gut microbiota (e.g., *Bacteroides* spp.)". It is still unclear to this reviewer why mention *Bacteroides* between parentheses. Does this mean that there was an increase in the abundance of *Bacteroides*? If that is the case this needs to be stated more clearly.*

Thanks for your comments. Treatment with CAPE indeed significantly increased the abundance of *Bacteroides* spp. in the gut microbiota (Figure 7H-J). However, to avoid ambiguity in the abstract, we have chosen to delete the specific mention of *Bacteroides* spp. within the parentheses.

The modifications made to lines 132-135 still do not address my concern. Authors stated in the manuscript that "compounds that were not bound to TcdB were removed". But how was this done? This needs to be clearly explained in the manuscript. In the response to reviewers document, authors state that this was done through centrifugation. But given that the goal here is to separate excess of small molecule from a protein target, just stating that centrifugation was used is not enough. Did the authors use ultracentrifugation? What were the conditions employed. This is critical so that the reader can assess the degree of compound carryover that may have occurred. Also, authors need to clearly acknowledge the caveats of their experimental design by stating that they cannot rule out the contribution of compound carryover to their results.

Thanks for your comments. We employed ultrafiltration centrifugal partition to remove the unbound small molecule compounds. Due to the large molecular weight of TcdB, approximately 270 kDa, we selected a 100 kDa molecular weight cutoff ultrafiltration membrane. The centrifugation was performed at 4000 g for 5 min to eliminate the compounds that did not bind to TcdB. We have incorporated the relevant methods and discussed the potential impacts on the respective sections of the manuscript.

In line 142, authors added the molar concentration of caffeic acid, as requested. Although this helps, it is even more important that molar concentrations are added every time a compound concentration is mentioned. For instance, just 2 lines down there is another mention of a compound concentration. It would be informative if authors also added molar concentrations here and throughout the manuscript.

Thanks for your comments. In our initial test design, we have utilized the concentration unit of $\mu\text{g/mL}$. However, during the conversion to μM using the dilution method, some values do not result in neat, whole numbers. For instance, the conversion of 32 $\mu\text{g/mL}$ of caffeic acid phenyl ethyl ester yields 112.55 μM , which appears somewhat irregular when expressed in this manner.

Line 277. For the sake of clarity, I would strongly suggest that authors use the term "control mice" instead of "model mice".

Thanks for your comments. We have modified “model mice” to “control mice” throughout the manuscript.

In line 302, the word taxa should not be capitalized. I capitalized it in my original comments simply to draw attention to it.

Thanks for your comments. We have modified this word.

In the section starting in line 318, authors still need to include details of how the metabolomics analyses were performed. Just stating that samples were "frozen for metabolomics analyses" is not enough. Was this mass-spec or NMR-based metabolomics. Assuming it was mass-spec, what kind? How was metabolite identity assigned? Etc, etc. These are important details, which need to be included.

Thanks for your comments. We have added some metabolomics methods in the corresponding section.

In line 338, the authors misunderstood my original comment. This sentence should read "...the final product of purine degradation, were markedly decreased in mice after..."

Thanks for your comments. We have modified this sentence.

Panels of figure 3 are still incorrectly labeled. The secondary structure predictions are shown in A and C, not A and B as is currently stated in the legend.

Thanks for your comments. We have modified the figure legend in Figure 3.

About Figure 5C, I think the authors for the clarification, but this explanation should be included in the figure legend.

Thanks for your comments. We have added the relevant information to the figure legend.

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