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Bile Acid 7 α -Dehydroxylating Gut Bacteria Secrete Antibiotics that Inhibit *Clostridium difficile*: Role of Secondary Bile Acids

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SUMMARY

Clostridium scindens, biotransforms primary bile acids into secondary bile acids, is correlated with inhibition of *Clostridium difficile* growth *in vivo*. The aim of the current study was to determine how *C. scindens* regulates *C. difficile* growth *in vitro* and if these interactions might relate to the regulation of gut microbiome structure *in vivo*. The bile acid 7 α -dehydroxylating gut bacteria, *C. scindens* and *C. sordellii*, were found to secrete tryptophan-derived antibiotics, 1-acetyl- β -carboline and turbomycin A, respectively. Both antibiotics inhibited growth of *C. difficile* and other gut bacteria. The secondary bile acids, deoxycholic acid (DCA) and lithocholic acid (LCA), but not cholic acid (CA), enhanced the inhibitory activity of these antibiotics. These antibiotics appear to inhibit cell division of *C. difficile*. The results help explain how endogenously synthesized antibiotics and secondary bile acids may regulate *C. difficile* growth and the structure of the gut microbiome in health and disease.

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AUTHOR CONTRIBUTIONS

J.D.K and P.B.H. designed the study, data interpretation, and manuscript writing; G.K., K. M., M. F., I-K. L, B-S. Y, structural determination of antibacterial compounds; C. M., and S. H. culturing bacteria and chemical synthesis of Turbomycin A; H-K. M. and H.Z. experimental/protocol design; J.S.B. experimental design and manuscript writing.

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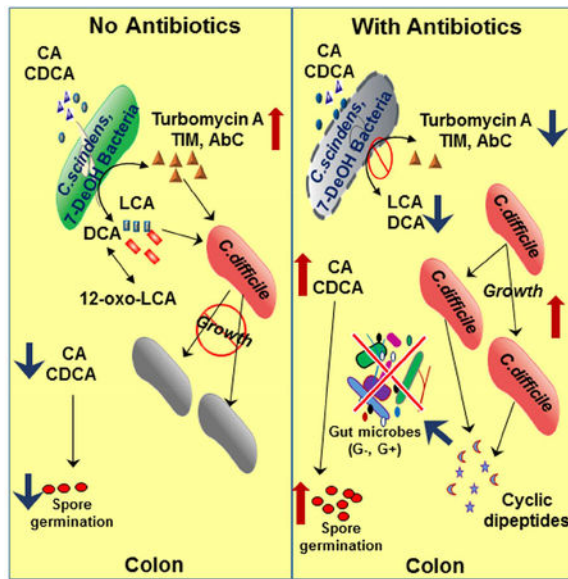
SUPPLEMENTAL INFORMATION

Supplemental information includes six figures and one table and can be found with this article online at <https://>

DECLARATION OF INTEREST

A patent application has been filed based on this study.

Graphical Abstract



ETOC Blurp

Kang et al. describes the discovery of tryptophan-derived antibiotics secreted by specific gut bacteria that inhibit *C. difficile*, a bacterial pathogen causing diarrhea and colitis. These results help explain why antibiotic treatment, which disrupts the normal protective gut microbiota, allows *C. difficile* to colonize the colon and cause disease.

Keywords

Turbomycin A; 1-acetyl- β -carboline; cyclic dipeptides; *Clostridium difficile*; *Clostridium scindens*; *Clostridium sordellii*; gut microbiome; dysbiosis

INTRODUCTION

Clostridium difficile, the cause of antibiotic associated diarrhea and colitis, is a growing health threat for patients taking broad spectrum antibiotics (Rupnik et al., 2009). Antibiotics markedly disrupt the gut microbiome homeostasis by decreasing the levels of protective gut microbiota, allowing colonization and proliferation of *C. difficile* normally found in low levels in some individuals (Loo et al., 2011). Patients treated with antibiotics, especially in hospitals, are also at risk for colonization by *C. difficile* spores, which germinate in the gastrointestinal tract (stimulated by taurocholate) producing vegetative cells that secrete toxins A and B which cause diarrhea and colitis (Kuehner et al., 2010; Sorg and Sonenshein, 2010). Patients with antibiotic associated diarrhea and/or colitis are routinely treated with either metronidazole or vancomycin to kill *C. difficile* vegetative cells colonizing the colon; however, up to 35% of all patients successfully treated with these antibiotics will relapse following cessation of antibiotic therapy.

Fecal transplants using gut microbiota from healthy donors have been highly successful in treating relapsing patients (van Nood et al., 2013; Weingarden et al., 2014). A previous study attempted to elucidate the members of the human gut microbiota most responsible for resistance to *C. difficile* infection (Buffie et al., 2015). This paper reported that *C. scindens*, a human gut bacterium that converts the primary bile acids, CA and chenodeoxycholic acid (CDCA), to secondary bile acids, DCA and LCA, respectively, is strongly associated with inhibition of *C. difficile* infections in animal models and in human patients (Buffie et al., 2015). Moreover, several clinical studies have support this association, the most recent study reported that the abundance of the baiCD gene cluster, which is a measurement of the levels of bile acid 7 α -dehydroxylating gut bacteria, are negatively correlated with *C. difficile* infection (Solbach et al., 2018). The aim of the current study was to try and elucidate the interactions between *C. difficile* and bile acid 7 α -dehydroxylating gut bacteria to help understand the possible mechanisms of regulation of *C. difficile in vivo* and the gut microbiome structure. The results of our investigation offer new insights into the how bile acid 7 α -dehydroxylating gut bacteria, which secrete both antibiotics and secondary bile acids, regulate *C. difficile* growth in the intestine and homeostasis of the gut microbiome structure.

RESULTS

***C. difficile* Growth is Inhibited by *C. scindens* in the Presence but Not Absence of Cholic Acid**

It has been reported that DCA inhibits *C. difficile* growth *in vitro* (Sorg and Sonenshein, 2008, 2010) and is hypothesized to inhibit the growth of this bacterium *in vivo* (Buffie et al., 2015). Bile acid 7 α -dehydroxylating gut bacteria, such as *C. scindens*, convert cholic acid into deoxycholic acid which increases the hydrophobicity and toxicity of the bile acid. We grew *C. difficile* and *C. scindens* in PYF medium with either CA or DCA. CA (50 μ M) had no significant effect on the growth of either bacterium. DCA (50 μ M) inhibited (~25%) *C. difficile* but had no significant effect on *C. scindens* growth (Figure 1 A and B). Next, we decided to grow *C. difficile* ATCC 9689 (type strain) and *C. scindens* ATCC 35704 in PYF medium in coculture with and without added CA (50 μ M). In order to measure the growth of each bacterium, we developed specific PCR assays to quantify a gene unique to *C. scindens* (bai CD gene encoding bile acid 3-oxo-A4-7 α -hydroxysteroid oxidoreductase) and the gene encoding Toxin A in *C. difficile* (STAR Methods). Surprisingly, *C. difficile* strongly inhibited *C. scindens* growth under co-culture conditions in the absence (Figure 1 D) but not presence of CA (Figure 1 E). The addition of a second strain of *C. scindens* ATCC 12708 further inhibited *C. difficile* growth (Figure 1 F).

***Clostridium difficile* Secretes Proline-based Cyclic Dipeptides**

How does *C. difficile* inhibit *C. scindens* growth? After filter sterilizing the spent growth medium from a pure culture of *C. difficile* ATCC 9689 grown in PYF medium and adjusting the pH to 7.2, we discovered that the spent medium inhibited several other common human gut bacteria as well as *Staphylococcus aureus* MRSA (Table 1). Moreover, three clinical isolates of *C. difficile* (BBA-1870, BBA-1801, BBA-1814) also secrete antibacterial compound(s) that inhibit *C. scindens* ATCC 35704 and *Staphylococcus aureus* MRSA under

the same culture conditions (Table 1, Table S1). Next, we decided to purify and character the major antibacterial compound(s) secreted into the culture medium by *C. difficile* ATCC 9689. Initial experiments showed that the antibacterial compound(s) were less than 3kDa in size (by ultrafiltration), hydrophobic (extracted into ethyl acetate), heat stable (autoclaving), lack a charge (did not bind to anion or cation exchange resins), and resistant to proteolytic enzymes (trypsin). Based on these traits, a protocol was developed to purify the major inhibitory compound(s) from *C. difficile* for characterization and identification. The purification started with 16 liters of PYF spent culture medium (24 hrs. incubation) from *C. difficile* ATCC 9689 and sequentially involved: molecular size ultrafiltration, extraction with ethyl acetate, Sephadex LH-20 column chromatography, and 2 different C-18 reverse-phase HPLC elution solvent systems (Figure 2, Figure S1). Two major antibacterial compounds were purified and identified by liquid chromatography-electrospray ionization mass spectrometry (LC-ESI/MS) and ¹H and ¹³C NMR chemical shifts as mixed chiral isomers of cyclo (Phe-Pro) and cyclo(Leu-Pro) (Figure 2). Proline-base cyclic dipeptides are known to be secreted by a number of bacteria (Giessen et al., 2014) but not members of the genus *Clostridium*. Proline-based cyclodipeptides have been reported to be quorum-sensing molecules for certain bacteria and to inhibit the growth of both bacteria and fungi (Belin et al., 2012; Gonzalez et al., 2017). The synthesis and secretion of these molecules by *Clostridium difficile* raises the possibility that these cyclodipeptides may enhance the ability of this bacterium to colonize the colon following antibiotic treatment by inhibiting the remaining members of the gut microbiota. In this regard, broad spectrum antibiotics also reduce the levels of bile acid 7 α -dehydroxylating gut bacteria.

Bile Acid 7 α -Dehydroxylating Bacteria Secrete Tryptophan-based Antibiotics

Because *C. scindens* ATCC 35704 was able to grow much better in co-culture with *C. difficile* ATCC 9689 in the presence, but not absence of CA, we hypothesized that CA may be inducing antibacterial compound(s) in *C. scindens* ATCC 35704 and/or enhancing their activity (Figure 1D, E). Next, we screened ten different strains and species of bile acid 7 α -dehydroxylating human gut bacteria for the presence of antibacterial compound(s) secreted into the culture medium that inhibit *C. difficile* growth. Interestingly, all species and strains of human bile acid 7 α -dehydroxylating gut bacteria tested were positive for inhibition of *C. difficile* ATCC 9689 growth to varying degrees. *C. scindens* ATCC 35704 and *C. sordellii* ATCC 9714 secrete antibacterial compound(s) that strongly inhibited *C. difficile* ATCC 9689 and a number of other bacteria, including several antibiotic resistant species (Table 1). The addition of CA to the culture medium enhanced the antibacterial activity of compounds secreted by *C. scindens*. We decided to purify and characterize the antibacterial compound(s) secreted by *C. sordellii* ATCC 9714 and *C. scindens* ATCC 35704 as these two bacteria are in different phylogenetic groups of bile acid 7 α -dehydroxylating gut bacteria. Protocols were developed to purify the major antibacterial compound(s) secreted by *C. sordellii* ATCC 9714 (red) and *C. scindens* ATCC 35704 (blue) under our culture conditions (Figure 3, Figure S2). The results show that *C. sordellii* ATCC 9714 secretes two potent antibacterial compounds that absorb at 205–215 nm and strongly at 280 nm. These compounds were identified by LC-ESI/MS and H 1 and C13 NMR chemical shifts as turbomycin A (Figure 3 A) and 1,1,1-tris(3-indolyl)-methane [TIM] (Figure 3B). These antibiotics are derived from indole and indole-3-carboxyaldehyde and have been previously

reported to be synthesized by soil microorganisms (Gillespie et al., 2002) but not anaerobic gut bacteria.

The structure of the major antibacterial compound secreted by *C. scindens* ATCC 35704 was determined by LC-ESI/MS and ^1H and ^{13}C NMR chemical shifts to be 1-acetyl- β -carboline (Figure 3 C). 1-Acetyl- β -carboline is an alkaloid synthesized from L-tryptophan and oxaloacetaldehyde and reported to be synthesized by a Gram-positive marine bacterium with algicide activity (Kim et al., 2015) and by a marine *Streptomyces albus* (Shin et al., 2010). β -Carboline alkaloids are widely distributed in nature and are known to have a variety of biological activities. However, 1-acetyl- β -carboline has not been reported to be synthesized by gut bacteria. It is of interest that antibiotics synthesized by both species of bile acid 7 α -dehydroxylating bacteria studied are derived from L-tryptophan or indole, a bacterial metabolite of L-tryptophan. Indole is found in mammalian feces in high concentrations (0.250 to 1.1 mM), is an inter-kingdom signaling molecule, and regulates the physiology of colonic epithelial cells (Lee et al., 2015).

Secondary Bile Acids Enhance the Activity of Antibiotics Secreted by Gut Bacteria

Why does bile acid 7 α -dehydroxylating gut bacteria secrete antibiotics and secondary bile acids? We decided to determine if varying concentrations of different bile acids would enhance the antibacterial effects of turbomycin A and 1-acetyl- β -carboline using *C. difficile* ATCC 9689 as the test bacterium. First, we diluted filtered sterilized and pH 7.2 adjusted spent culture medium from *C. sordellii* ATCC 9714 and added varying amounts of fresh PYF medium until growth of *C. difficile* grew to about 40% to 60% maximal. Varying concentrations of different bile acids, similar to ranges found in human fecal water (Ditscheid et al., 2009), were added to the culture medium, inoculated with 10⁶ *C. difficile* vegetative cells/ml, incubated for 24 hours, and optical density readings at 600 nm measured (STAR Methods). The results showed a strong concentration-dependent enhancement of inhibition of the growth of *C. difficile* by spent culture medium of *C. sordellii* by DCA and LCA, but not CA (Figure 4, Figure S3 A–C).

Because turbomycin A and TIM are not commercially available, we chemically synthesized turbomycin A and TIM. The chemical synthesis of these antibiotics was carried out under anaerobic conditions and optimal substrate concentrations which allowed higher yields and fewer side-products compared to the original method of chemical synthesis (Gillespie et al., 2002). LC-ESI/MS was used to demonstrate that the new chemical synthesis protocol for turbomycin A and TIM resulted in the expected product. Chemically synthesized turbomycin A inhibited the growth of *C. difficile* in a manner similar to the biologically synthesized compound (Figure 4, Figure S4).

Next, we carried out similar experiments with the spent culture medium from *C. scindens* ATCC 35704. The results also showed enhancement of inhibition of *C. difficile* by DCA and LCA, but not CA (Figure S3 D–F). We next tested the ability of different bile acids to enhance the antibacterial activity of commercially available 1-acetyl- β -carboline (Figure 4 D–F). The results showed a concentration dependent enhancement of inhibition of *C. difficile* by DCA and LCA, but not CA. We next tested the effect of different bile acids found in human fecal water on inhibition of *C. difficile* by TIM (Ditscheid, 2009).

Interestingly, iso-deoxycholic acid (3 α -DCA) and 12-oxo-lithocholic acid (12-oxo-LCA) were less effective than DCA in inhibiting *C. difficile* growth (Figure 5). The mechanism of enhancement of inhibition of these antibiotics by DCA and LCA is unknown, but DCA has been reported to be a better solubilizer of various hydrophobic drugs than CA (Enright et al., 2017).

Turbomycin A and TIM Inhibit *C. difficile* Cell Division

How does antibiotics secreted by *C. sordellii* and *C. scindens* inhibit the growth of *C. difficile*? We observed that the growth of *C. difficile* in spent culture medium of *C. sordellii* resulted in the formation of highly elongated *C. difficile* cells (Figure 6). Higher concentrations of spent culture medium increased the cell length of individual cells. Moreover, the addition of chemically synthesized turbomycin A or 1-acetyl- β -carboline to fresh PYF growth medium also resulted in inhibition of cell growth and elongated cell morphology of *C. difficile* (Figure 6, Figure S5). The type of morphological changes (2 to 7-fold longer cells in antibiotic treated cells vs controls) may be due to inhibition of the bacterial division septum (Xiao and Goley, 2015). However, additional experiments will be required to determine the mechanism(s) of growth inhibition of these antibiotics and their enhancement by DCA and LCA.

DISCUSSION

The surprising results of the current study was the discovery that human bile acid 7 α -dehydroxylating gut bacteria secrete tryptophan-derived antibiotics that are enhanced by secondary bile acids and that *C. difficile* secretes two proline-based cyclodipeptides that may function as quorum sensing molecules and/or colonization factors. The colonic ecosystem may select for bacteria that secrete antibacterial compounds giving them a competitive growth advantage in an ecosystem with 100 to 150 operational taxonomic units (OTU) at any given time and greater than 10¹¹ bacterial cells/gram wet weight feces. Interestingly, the effectiveness of these hydrophobic antibiotics to inhibit gut bacteria is enhanced by hydrophobic secondary bile acids produced by bile acid 7 α -dehydroxylating gut bacteria (Figure 4, Figure S3). The secretion of antibiotics by bile acid 7 α -dehydroxylating gut bacteria and their concentration-dependent enhancement by DCA and LCA, but not by CA, is hypothesized to be a major regulator of the structure of the gut microbiome (Graphical Abstract). The minimal inhibitory concentrations (MICs) of turbomycin A and 1-acetyl- β -carboline to inhibit *C. difficile* are in the range of 7.5 μ g/ml and 25 μ g/ml, respectively, in PYF medium (Figure 4). These are relatively high MICs to be effective antibiotics; however, if combined with DCA and/or LCA then the mixture appears to become much more potent than either alone. Because all bile acid 7 α -dehydroxylating bacteria tested are capable of secreting both secondary bile acids and antibacterial compounds, then this mixture may be very effective in controlling gut microbiome homeostasis under normal physiological conditions. Moreover, it is also possible that the antibiotics may enhance the toxicity of secondary bile acids especially if both are inhibiting the formation of the division septum. However, this possibility will have to be tested in an appropriate *in vivo* model where each antibiotic can be used alone and in combination with a secondary bile acid.

Human gut bacteria are capable of the formation of more than 20 different metabolites of cholic acid and chenodeoxycholic acid (Ridlon et al., 2006). What is the physiological purpose of these bile acid biotransformations? Bile acid hydroxyl group oxidations and epimerizations ($\alpha > \beta$) increase the hydrophilicity of the bile acid molecule making them less toxic to bacterial cells and possibly decreasing their ability to enhance hydrophobic antibiotics (Devlin and Fischbach, 2015). In this regard, iso-deoxycholic acid (3 β -hydroxyl derivative) and 12-oxoLCA, which are found in human fecal water (Ditscheid et al., 2009), were less effective in enhancing TIM than deoxycholic acid in inhibiting *C. difficile* growth (Figure 5). Therefore, the hydrophilic-hydrophobic balance of the colonic bile acid pool may be a key factor in determining the structure of the gut microbiome

Bile acids have been hypothesized to play an important role in the regulation of the gut microbiome structure by a variety of mechanisms. As detergents, bile acids directly inhibit gut bacteria growth by membrane-damage related on their hydrophobicity (Devlin and Fischbach, 2015; Ridlon et al., 2006). Unconjugated secondary bile acids are generally more hydrophobic than corresponding primary bile acids. During their enterohepatic circulation, bile acids can activate the bile acid activated nuclear receptor, farnesoid X receptor (FXR), to induce genes that are enteroprotective by inhibiting bacterial overgrowth (Inagaki et al., 2006). However, these FXR-dependent antibacterial effects may be more relevant to mucosal associated bacteria than those in the lumen of the intestine.

There is evidence that bile acids may be important regulators of the human gut microbiome in cirrhotic liver disease. In this regard, cirrhotic patients have a decreased bile acid pool size due to loss of bile acid synthesis in the liver, resulting in a marked reduction in bile acid 7 α -dehydroxylating gut bacteria, and a shift to a more “toxic” Gram-negative gut microbiota (Bajaj et al. 2012; Chen et al. 2011; Kakiyama et al., 2014). In contrast, when cirrhotic patients are transplanted with a new liver, there is increased primary bile acids synthesis, increased fecal secondary bile acid levels and a return to a more diverse fecal microbiome (Bajaj et al., 2018). Moreover, in animal model studies, feeding CA to rats markedly shifts the bacterial gut composition from a ~1:1 ratio of *Firmicutes/Bacteroidetes* to greater than 98% *Firmicutes* (Islam et al., 2011) while feeding CA to mice has been reported to increase the levels of bile acid 7 α -dehydroxylating bacteria by ~1000-fold as these bacteria use primary bile acids as an electron acceptor (Ridlon et al., 2013). In addition, animals fed high-fat diets increase the *Firmicutes/Bacteroidetes* ratio possibly because of increased enterohepatic cycling of bile acids and increased loss into feces (Yokata et al., 2012; Turnbaugh et al. 2008; Hildebrandt et al. 2009). The concentration of various secondary bile acids found in human fecal water samples (unbound bile acids) appear not to adequately explain the marked shift in the microbiome structure with changes in fecal bile acid pool composition and concentration (Ditscheid et al., 2009). However, the combination of secondary bile acids and tryptophan-derived antibiotics may work as a powerful “cocktail” to regulate the structure of the gut microbiome.

STAR METHODS

KEY RESOURCES TABLE

CONTACT FOR REAGENT AND RESOURCE SHARING

“Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Phillip Hylemon (Phillip.hylemon@vcuhealth.org)”

EXPERIMENTAL MODE AND SUBJECT DETAILS

Bacterial Strains and Culture Conditions—The following bacterial strains were obtained from the American Type Culture Collection (ATCC): *Clostridium difficile* ATCC 9689; *Clostridium difficile* clinical isolates from the ATCC include: BAA-1870 (ribotype 027); BAA-1801 (ribotype 010); BAA-1814 (ribotype 251); *Clostridium scindens* ATCC 35704; *Clostridium sordelli* ATCC 9714; *Bacteroides vulgatus* ATCC 8482. *Clostridium scindens* VPI 12708 was obtained from the Virginia Tech Anaerobe Laboratory. Bacteria were cultured in peptone-yeast extract-fructose (PYF) medium and growth monitored at 600 nm.

METHODS DETAILS

Light Microscopy—*Clostridium difficile* cultures were smeared, fixed on glass slides, stained using Remel Gram Staining Kit according to manufacturer’s directions and allowed to air dry. Bacteria were covered with DPX Mountant and a cover glass. Bacteria were examined under bright field microscopy using a Zeiss AxioImager microscope with computer controlled stage using the NeuroLucida 2017 software. Images were obtained using x63 and x100 oil-immersion objectives using a Zeiss MRC camera as .jpg files.

DNA Purification—One ml of a pure culture of *C. difficile* ATCC 9689, *C. scindens* ATCC 35704 and *C. scindens* VPI 12708, respectively, were subjected to genomic DNA isolation using a Fast DNA Spin Kit, according to the manufacturer’s instructions. The final elution volume was 100 μ l and the nucleic acid concentration was measured fluorimetrically using a Nano Spectrophotometer.

PCR Quantitation of Bacterial Strains—Target DNA (1 μ g) was added and the copy number of the *tcdA* and *baiCD* genes were determined based on the molecular weight of 3.8- to 4.18-mbp-sized genome of *C. difficile* ATCC 9689, *C. scindens* ATCC 35704, and *C. scindens* VPI 12708, respectively. 1 ng of DNA was calculated to equal to 6×10^5 times the entire genomes and that the *tcdA* and *baiCD* are single-copy genes. Gene specific primers were synthesized for *C. difficile* ATCC 9689 toxin A(*tcdA*) gene as follows: (CDF9689TcdA-1F, Forward: T GGGCT GAT ATT AAT GCAGAA; CDF9689TcdA-1R, Reverse: TTCCCAACGGTCTAGTCCAA) and *baiCD*s of *C. scindens* ATCC 35704 and *C. scindens* VPI 12708 (CS357N127BaiCDWhole-F, Forward: T GGCT GTT CAGGCT GGATTT GA; CS357N127VaiCDWhole-R, Reverse: TCGTAGCATCCCTGGTCACA). The PCR assay was performed at 95°C for 4 min., followed by 35 cycles of 95°C for 30 sec., 52°C for 1 min, 72°C for 2 min with a final 10 min extension step 72°C. PCR products from conventional PCR assays were detected on 1% Agarose gels and the size of the PCR products (588 bp for Toxin A and 595 bp for *baiCD* genes) were confirmed.

Quantitative PCR of Species Specific Genes—Gene specific primers were used to quantitate *C. difficile* and *C. scindens* in co-culture using a designed target region of the toxin A gene (*tcdA*) and *baiCD* genes that are highly conserved in *C. difficile* ATCC 9689, *C. scindens* ATCC 35704, and *C. scindens* VPI 12708. The primers sets specific for the individual bacteria are as follows: *C. difficile* ATCC 9689 toxin A gene (CDF9689NtxA-1F, Forward: TCTACCACTGAAGCATTAC; CDF9689NtxA-1R, Reverse, TAGGTACTGTAGGTTTATTGj, *baiCD* for *C. scindens* ATCC 35704 (CS35704*baiCD*-1F, Forward: CTT CATTGCAACT GT AT AT GA, CS35704*baiCD*-1R, Reverse: T AACCGGGAT ATT GAT CT GCTT) and for *C. scindens* VPI 12708 (CS12708*baiCD*-1F, Forward: CAACCGT AT AT GAAGTTCC, CS12708*baiCD*-1R, Reverse: ATCCCGGTTATGGGAGTTGG). Amplification and detection of DNA by real-time PCR was performed with the IQ5 Realtime PCR system (BioRad, CA) using optical grade 96-well plates. Triplicate samples were routinely used for the determination of DNA by real-time PCR, and the mean values calculated. The PCR reaction was performed in a total volume of 25 μ l. *C. difficile* ATCC 9689, *C. scindens* ATCC 35704, and *C. scindens* VPI 12708 were detected by using the Sso Advanced Universal SYBR Green PCR Master Mix (Bio-Rad, CA), with 100 nmol l⁻¹ of each of the forward and reverse primers and 1 ng DNA for each reaction. The PCR reaction conditions for amplification of DNA were 50°C for 2 min, 95°C for 10 sec. and 40 cycles of 95°C for 15 sec. and 60°C for 1 min. A melting curve analysis was done after amplification. The threshold cycle (CT) values and baseline settings were determined by automatic analysis settings. Data analysis was performed using iQ5 Optical System Software supplied by Bio-Rad. The relative abundance of *C. difficile* ATCC 9689 (toxin A gene copies), *C. scindens* ATCC 35704 (*baiCD* genes), and *C. scindens* VPI 12708 (*baiCD* genes) were related to the cycle threshold (Ct) which is the number of PCR cycles needed to obtain a detectable fluorescent signal above background and typically was the point on the amplification curve where it becomes linear. Ct was experimentally confirmed and determined to be related to copy number of the strains by amplifying a series of serial 10-fold dilutions of the genomic DNA from each bacterium.

Purification of Antibacterial Compounds from *C. difficile*, *C. scindens*, and *C. sordellii*—The purification protocols for the cyclic dipeptides from *C. difficile*, turbomycin A and TIM from *C. sordellii*, and 1-acetyl- β -carboline from *C. scindens* are described in Figures S1 and S2, respectively.

Identification of the Antibacterial Compounds Secreted by *C. difficile*, *C. sordellii* and *C. scindens*—Characterization and identification of turbomycin A, 1,1,1-tris(3-indolyl)-methane and cyclic dipeptides was carried out by Drs. In-Kyoung Lee and Bong-Sik Yun at Chonbuk National University, Republic of Korea. Antibacterial compounds were characterized and identified by spectroscopic methods. ESI/MS data were obtained on a QTRAP-3200 mass spectrometer (Applied Biosystems, USA) in positive and negative modes. NMR spectra were obtained on a JNM-ECA600 600MHz FT-NMR spectrometer (JEOL, Japan) with ¹H NMR at 600 MHz and ¹³C NMR at 150 MHz in CD₃OD, CDCl₃ or DMSO-*d*₆. Chemical shifts are given in ppm (δ) using tetramethylsilane as internal standard. Chemical structures were determined by one- and two-dimensional NMR experiments including ¹H-¹H COSY, HMQC, HMBC, and NOESY.

Chemical Synthesis of Turbomycin A and 1,1,1-tris(3-indolyl)-methane—

Because turbomycin A and 1,1,1-tris(3-indolyl)-methane are not commercially available, we chemically synthesized these two antibiotics using a modification of the protocol reported by Gillespie D.E. et al. 2002. Briefly, we optimized the concentration of reactants, incubation times, and carried out synthesis under anaerobic conditions. The reaction was carried out in 100 ml stoppered bottles flushed with N₂ gas. Two hundred μmoles of indole-3-carboxyaldehyde and 600 μmoles of indole were dissolved in 450 μL ethanol and 50 μL acetic acid under N₂ gas. In order to maintain anaerobic condition, the bottle was flushed with N₂ gas, immediately sealed and placed at 65°C water bath for 12 hours or overnight with mixing. Then, 600 μl of 10 N NaOH was added to achieve a pH of ~7.2. After which 4 mmoles of tetrachloro-1,4-benzoquinone was added and the bottle was immediately resealed and contents mixed. The bottle was placed in a water bath at 65°C for 2 hrs with mixing. Then, 50 ml of distilled water was added and the crude product, extracted 3 times with an equal volume of ethyl acetate and allowed to air dry. The crude product was then dissolved in 100% acetonitrile, run through a Sep-Pak column, and fractions collected. Five μl of each fraction was loaded onto TLC plates and reaction products separated using (chloroform:methanol:acetone, 80:15:2, vol/vol/vol) for turbomycin A and (hexane:chloroform:acetone, 20:60:10, vol/vol/vol) for 1,1,1-tris-(3-indolyl)-methane as solvents, respectively (Figure S4). TLC spray reagent was used for the detection of the products on the TLC (Ehmann, 1977). Active fractions were assayed for bacteria inhibition activity and positive fractions purified using a preparative reversed-phase HPLC column (Kinetex C18, 5 μm, 21.2 mm inner diameter and × 150 mm length) with linear gradient elution from 50% to 70% acetonitrile and a flow rate (3.0 ml/min) for 35 min for the separation of turbomycin A and 1,1,1-tris-(3-indolyl)-methane. Active fractions were subjected to an analytical C18 HPLC column (Agilent, Eclipse XDB-C18, 4.6 mm inner diameter × 150 mm length) to confirm identification of compound by mass spectrometry analysis.

STATISTICAL ANALYSIS

All experiments were performed in triplicate and repeated at least three times. The values were expressed as Means ± Standard deviation (SD) in all graphs. The statistical analysis of the results was performed by one-way analysis of variance (ANOVA) to compare tests and control using Graphpad Prism (Graphpad software, San Diego, CA, U.S). Symbols * and ** indicate statistical significance, p<0.05 and p<0.01, respectively.

DATA AVAILABILITY

The software used in this study are listed in the Key Resources Table. Additional experimental data are provided as supplementary information and are available from the corresponding author upon request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

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Highlights

- Bile acid 7 α -dehydroxylating gut bacteria secrete tryptophan-derived antibiotics
- Secondary bile acid enhanced the activity of these antibiotics
- Tryptophan-derived antibiotics appear to inhibit the division septum of bacteria
- *Clostridium difficile* secretes proline-based cyclic dipeptides

SIGNIFICANCE

This work reports that bile acid 7 α -dehydroxylating bacteria secrete tryptophan-derived antibiotics and their antibacterial activity enhanced by secondary bile acids. These discoveries appear relevant to how the structure of the gut microbiome is regulated by bile acids and bile acid 7 α -dehydroxylating gut bacteria and how this ecosystem may be disrupted by antibiotics or in liver diseases where there is a decrease in secondary bile acids. Moreover, we also report that *Clostridium difficile* secretes proline-based cyclic dipeptides which are known quorum sensing molecules for some bacteria. They have been reported to have antibacterial activity and may be important colonization factors in *C. difficile* infection in the absence of bile acid 7 α -dehydroxylating bacteria.

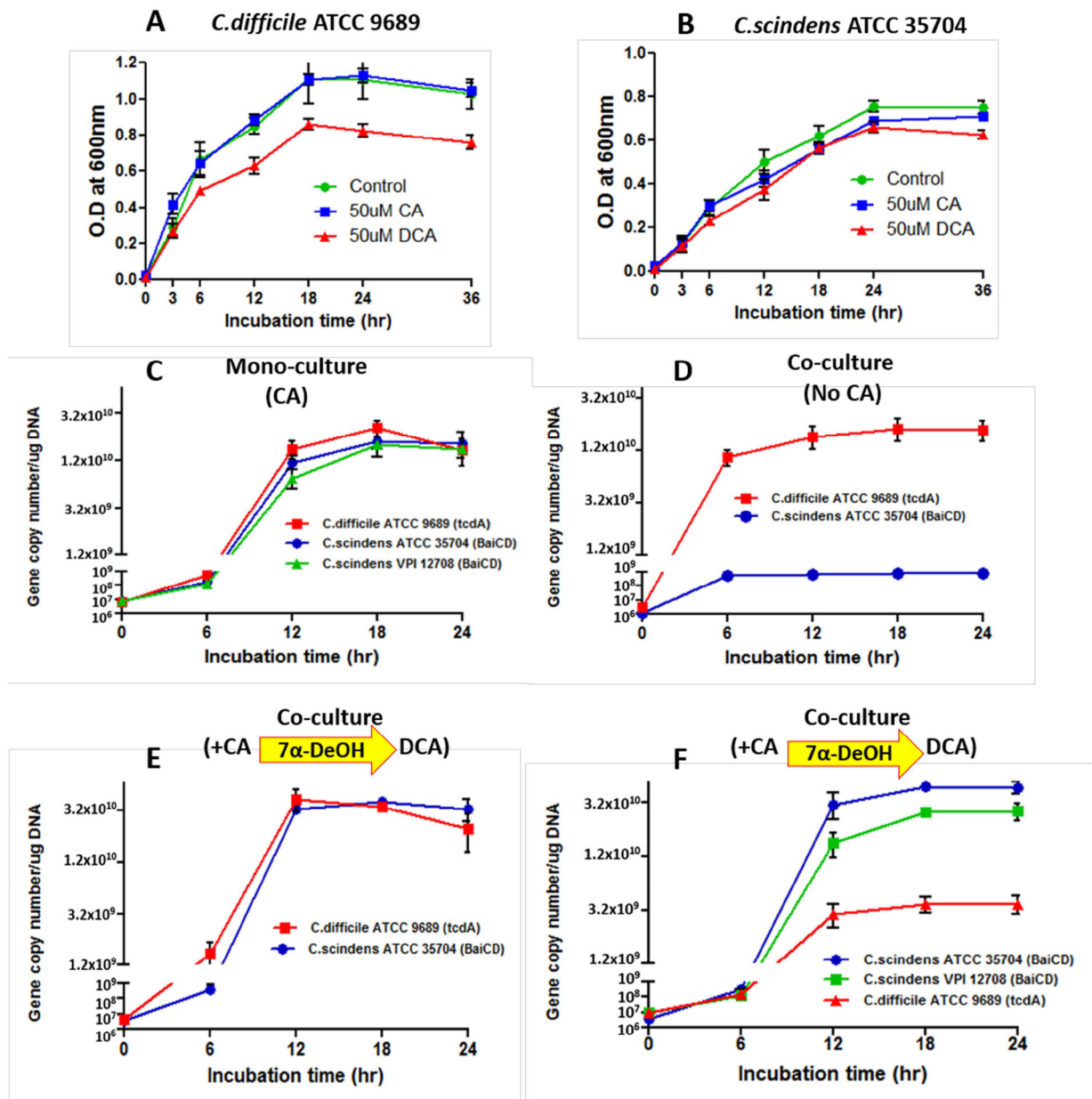
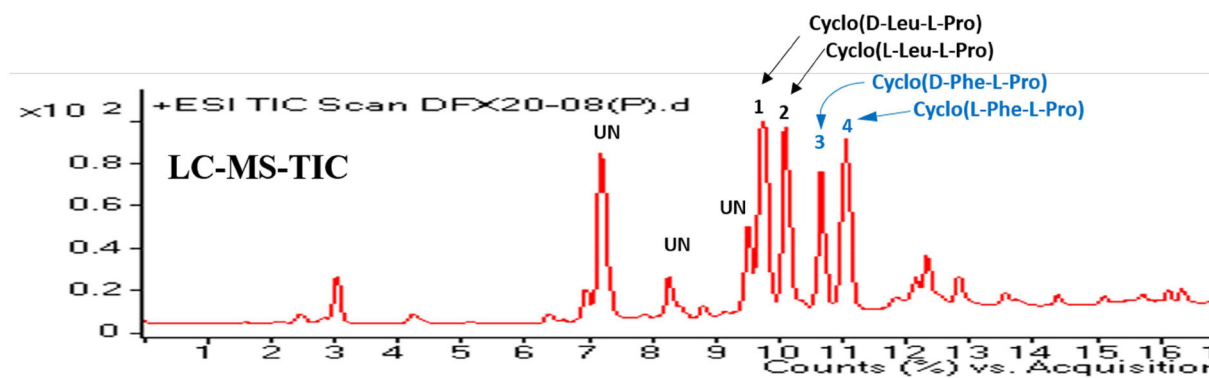
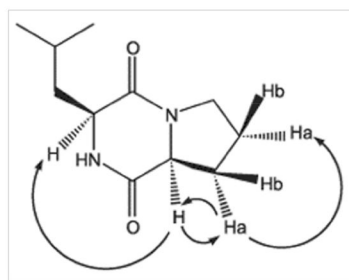


Figure 1. *C. difficile* Growth is Inhibited by *C. scindens* in the Presence but Not Absence of Cholic Acid

PYF medium containing 50 μ M cholic acid (CA) or 50 μ M deoxycholic acid (DCA) was inoculated with 10⁶ vegetative cells/ml of *C. difficile* (Panel A) or *C. scindens* (Panel B), incubated for a 24 hr. time course, and growth determined at indicated time points by measuring optical density (OD) at 600 nm. PYF medium was inoculated with 10⁶ vegetative cells/ml of *C. difficile* and *C. scindens* grown in either mono culture with 50 μ M CA (Panel C) or co-culture without CA (Panel D) or with 50 μ M CA (Panels E and F), incubated over a 24 hour time course and levels of bacteria estimated at indicated time points by measuring gene dosage of Toxin A gene for *C. difficile* and *baiCD* gene for *C. scindens* (STAR Methods).

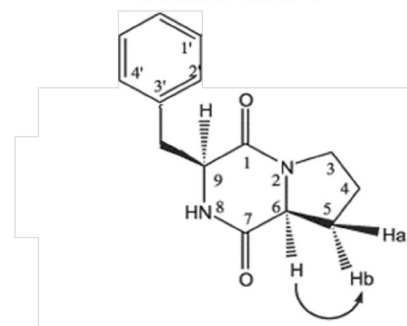


Peak 1 and 2



D/L Cyclo(Leu-Pro)

Peak 3 and 4



D/L Cyclo(Phe-Pro)

Figure 2. Identification of antibacterial compounds secreted by *C. difficile*

The purification protocol for antibacterial compounds secreted by *C. difficile* is described in Figure S1. The mass spectra and 1H and ^{13}C NMR chemical shifts of peaks 1 through 4 were used to identify compounds. Antibacterial compounds were identified as chiral isomers of cyclo(Leu-Pro) for peaks 1 and 2 and chiral isomers of cyclo (Phe-Pro) for peaks 3 and 4.

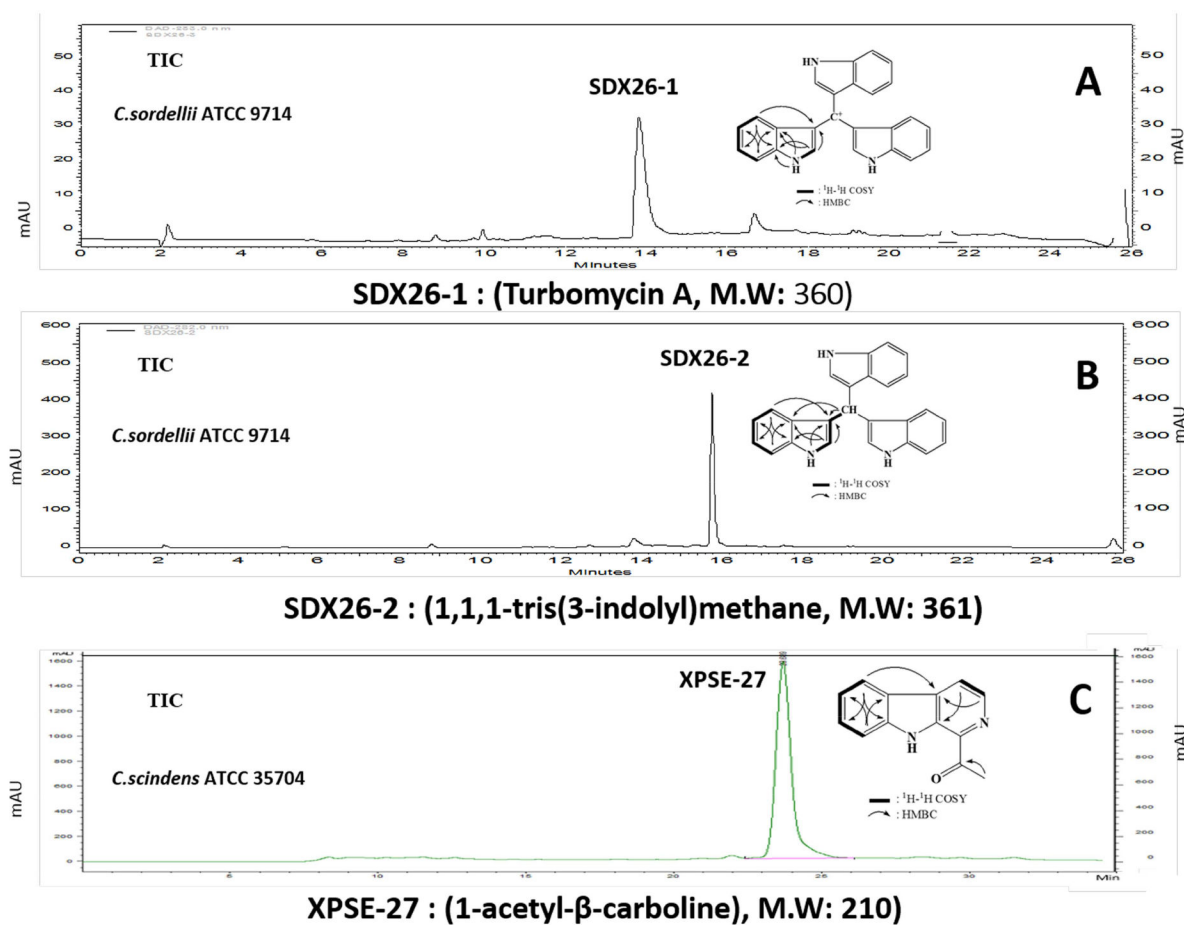


Figure 3. Identification of antibacterial compounds secreted by *C. sordellii* ATCC 9714 and *C. scindens* ATCC 35704.

The purification protocols for antibacterial compounds secreted by *C. sordellii* and *C. scindens* are described in Figure S2. The LC-ESI/MS and ^1H and ^{13}C NMR chemical shifts of SDX26-1 and SDX26-2 secreted by *C. sordellii* were positively identified as turbomycin A and 1,1,1-tris(3-indolyl)-methane, respectively. The LC-ESI/MS and ^1H and ^{13}C NMR chemical shift assignments of antibacterial compound (XPSE-27) was positively identified as 1-acetyl- β -carboline.

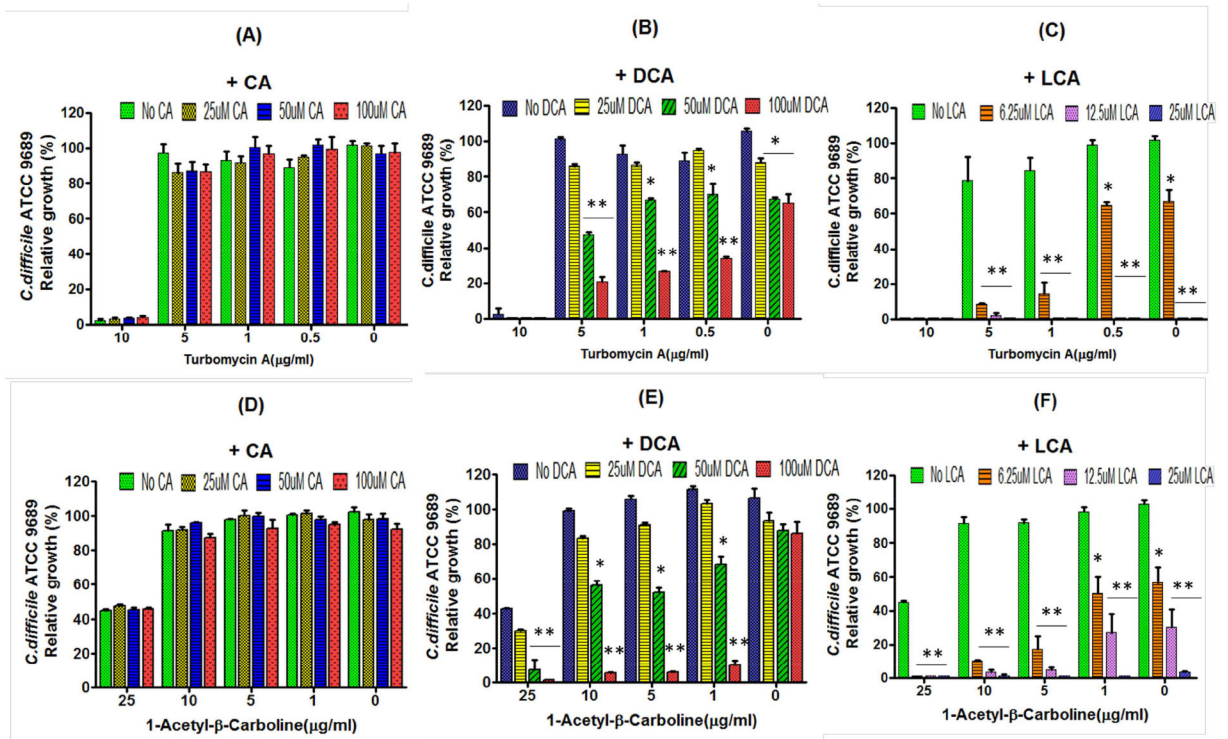


Figure 4. Effects of different bile acids on inhibition of growth of *C. difficile* by turbomycin A and 1-acetyl-β-carboline.

Varying concentrations of Turbomycin A (0, 0.5, 1, 5 and 10 μg/ml) or 1-acetyl-β-carboline (0, 1, 5, 10 and 25 μg/ml) and either cholic acid (CA) (0 to 100 μM), deoxycholic acid (DCA), (0 to 100 μM) or lithocholic acid (LCA), (0 to 25 μM) were added to PYF medium, inoculated with 10⁶ vegetative cells/ml of *C. difficile*, incubated for 24 hrs. and optical density reading determined at 600 nm. * p < 0.05, ** p < 0.01

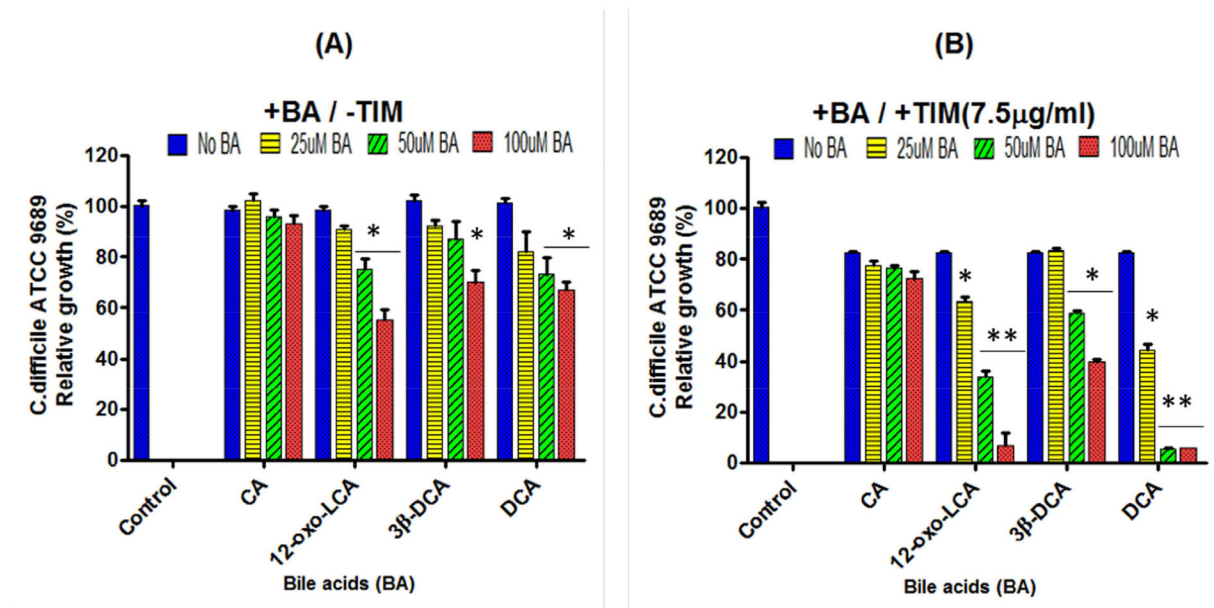


Figure 5. Effect of 1,1,1-tris(3-indolyl)-methane (TIM) and different bile acids (BA) on inhibition of *C. difficile* ATCC 9689 growth.

A. Varying concentrations of cholic acid (CA) (0 to 100 μ M), 12-oxo-lithocholic acid (12-oxo-LCA) (0 to 100 μ M), iso-deoxycholic acid (3 β -DCA) (0 to 100 μ M) or deoxycholic acid (DCA) (0 to 100 μ M) were added to PYF medium, inoculated with 10^6 vegetative cell/ml of *C. difficile*, incubated for 24 hrs, and O.D. readings at 600 nm determined. **B.** Experiments were repeated with the addition of TIM (7.5 μ g/ml) added to the culture medium. * $p < 0.05$, ** $p < 0.01$.

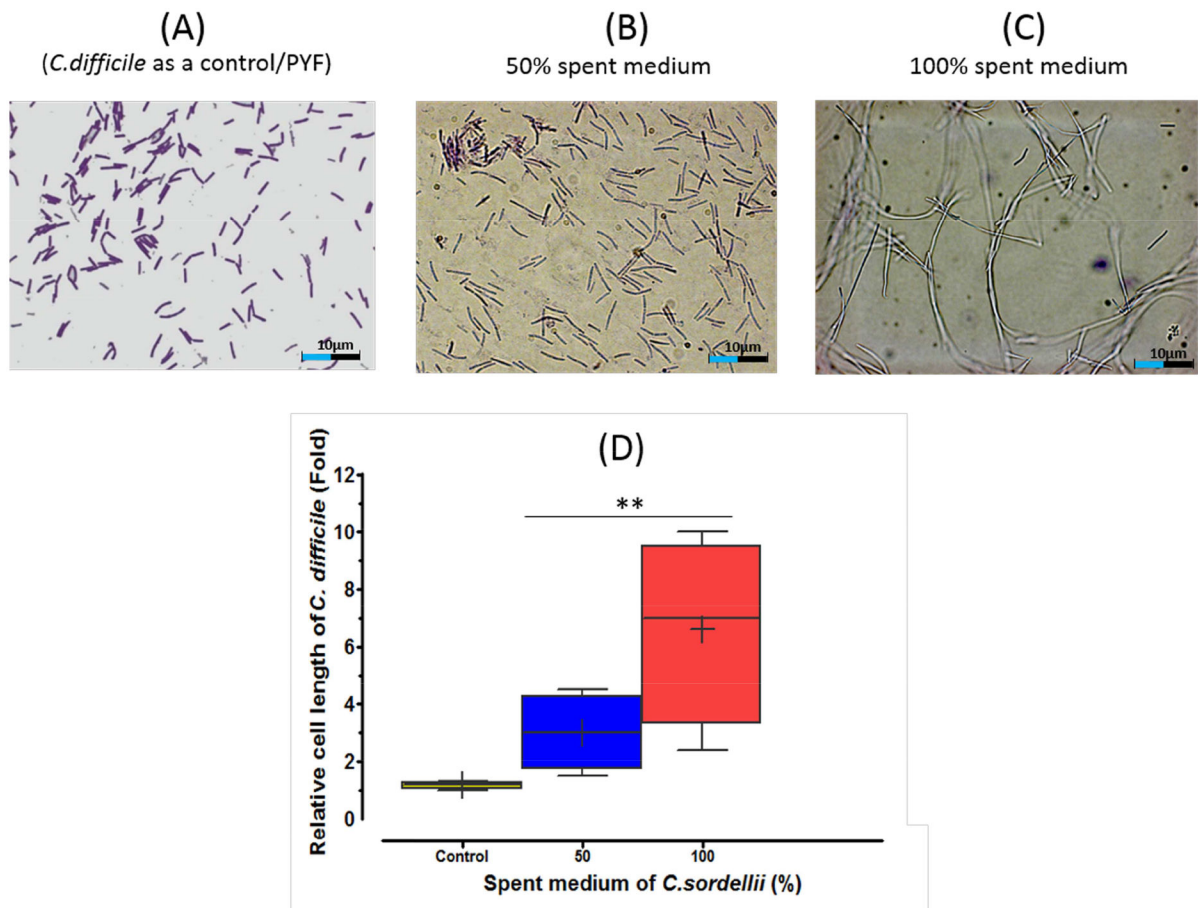


Figure 6. Changes in *C. difficile* ATCC 9689 cell morphology induced by antibacterial compounds secreted by *C. sordelli* ATCC 9714

A) PYF medium control; **B)** *C. difficile* grown in 50% *C. sordellii* spent culture medium; **C)** *C. difficile* incubated in 100% *C. sordellii* spent culture medium. Each culture (1ml) was concentrated to 100 μl by centrifugation, 5 μl of cell suspension loaded on to a slide for Gram staining and photo images taken (STAR Methods). **D)** Cell length was determined using a scale bar generated by NeuroLucia 1027 software in several microscopic fields. (Magnification 1000x). ** P<0.01

Table 1.

Inhibition of different gut bacteria by compounds secreted by *C. difficile* ATCC 9689, *C. scindens* ATCC 35704, and *C. sordellii* ATCC 9714

Test bacteria strains	Cell growth of bacteria(O.D at 600nm) in PYF for 24hr							
	<i>C.difficile</i> ATCC 9689	<i>C.scindens</i> ATCC 35704	<i>C.sordellii</i> ATCC 9714	<i>Staph.aureus</i> (MRSA)	<i>Ent.faecium</i> ATCC 2316	<i>E.coli</i> ATCC 2340	<i>B.vulgatus</i> ATCC 8482	
<i>C.scindens</i> ATCC 35704 spent culture medium	UN [‡]	0.81	0.78	0.85	0.69	0.71	0.98	
	IN [‡]	0.54±0.07*		0.31±0.11**	0.30±0.01**	0.41±0.03*	0.47±0.04*	
<i>C.sordellii</i> ATCC 9714 spent culture medium	UN [‡]	0.26±0.05**		0.10±0.02**	0.25±0.03**	0.38±0.04*	0.24±0.11**	
	IN [‡]	0.08±0.01**	0.08±0.01**	0.08±0.03**	0.10±0.02**	0.07±0.03**	0.10±0.03**	
<i>C.difficile</i> ATCC 9689 spent culture medium	-	0.12±0.04**		0.32±0.03**	0.33±0.05**	0.28±0.03**	0.53±0.05*	

[‡]: No addition of CA,

[‡]: Induced by 100µM CA.

*, ** : Significant growth inhibition p<0.05 and p< 0.01, respectively.