The Insect Peptide Coprisin Prevents Clostridium difficile-Mediated Acute Inflammation and Mucosal Damage through Selective Antimicrobial Activity"y

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Clostridium difficile-associated diarrhea and pseudomembranous colitis are typically treated with vancomycin or metronidazole, but recent increases in relapse incidence and the emergence of drug-resistant strains of C. difficile indicate the need for new antibiotics. We previously isolated coprisin, an antibacterial peptide from Copris tripartitus, a Korean dung beetle, and identified a nine-amino-acid peptide in the α-helical region of it (LLCIALRKK) that had antimicrobial activity (J.-S. Hwang et al., Int. J. Pept., 2009, doi:10.1155/2009/136284). Here, we examined whether treatment with a coprisin analogue (a disulfide dimer of the nine peptides) prevented inflammation and mucosal damage in a mouse model of acute gut inflammation established by administration of antibiotics followed by C. difficile infection. In this model, coprisin treatment significantly ameliorated body weight decreases, improved the survival rate, and decreased mucosal damage and proinflammatory cytokine production. In contrast, the coprisin analogue had no apparent antibiotic activity against commensal bacteria, including Lactobacillus and Bifidobacterium, which are known to inhibit the colonization of C. difficile. The exposure of C. difficile to the coprisin analogue caused a marked increase in nuclear propidium iodide (PI) staining, indicating membrane damage; the staining levels were similar to those seen with bacteria treated with a positive control for membrane disruption (EDTA). In contrast, coprisin analogue treatment did not trigger increases in the nuclear PI staining of Bifidobacterium thermophilum. This observation suggests that the antibiotic activity of the coprisin analogue may occur through specific membrane disruption of C. difficile. Thus, these results indicate that the coprisin analogue may prove useful as a therapeutic agent for C. difficile infection-associated inflammatory diarrhea and pseudomembranous colitis.

Clostridium difficile is the most common cause of antibiotic-associated diarrhea and pseudomembranous colitis in humans (33–36). C. difficile infection is highly prevalent in hospitals and nursing homes where patients frequently receive antibiotics and represents one of the most common hospital infections (2, 4, 14, 17, 23). C. difficile infection-mediated severe diarrhea and pseudomembranous colitis have been associated with preexposure to antibiotics (2, 16, 17, 23). The antibiotic-associated disruption of normal gut microbiota reportedly leads to colonization by C. difficile and the release of exotoxins (toxin A and toxin B) that mediate mucosal injuries, fluid secretion, apoptosis of surface colonocytes, and acute inflammation in the human gut (2, 14, 16–19).

To date, C. difficile-associated diarrhea has been primarily treated by the administration of vancomycin or metronidazole, both of which are effective (22). Metronidazole is more commonly used at present, as its efficacy is similar to that of vancomycin, and its cost is lower (43). However, a number of studies have reported that patients infected with C. difficile have a high relapse incidence, ranging from 15 to 50% (10). Thus, new therapeutic drug candidates are urgently required.

Recently, we isolated coprisin, a natural peptide consisting of 43 amino acids, from Copris tripartitus (a Korean dung beetle) after it had been infected with pathogenic bacteria and found that this peptide had antibacterial activity (13). Having noted that the α-helical region of the natural peptide was the activity domain, the 9 peptides (LLCIALRKK) corresponding to this domain were generated (13). The antibiotic activity of the 9 peptides was higher than that of coprisin and was effective against Escherichia coli and Staphylococcus aureus (13).

Therapeutic antibiotics intended to treat gut inflammation should have specific antimicrobial activity against pathogenic microbes while sparing the gut microbiota. In the present study, we found that the coprisin analogue exerts antibiotic activity against C. difficile but not against the gastrointestinal tract-resident organisms Lactobacillus and Bifidobacterium. Notably, clindamycin has antibiotic activity against both nor-
nal microbiota (5) and C. difficile (17); this nonselectivity may be a main cause for antibiotic-associated diarrhea and pseudomembranous colitis in both animals and humans.

The amino acid sequence of coprisin is very similar to that of the defensins, which are ∼40-amino-acid peptides that have strong antibiotic activities against Gram-positive bacteria (24) that result from their ability to disrupt the membrane or suppress cell cycle signaling (48). Here, we showed that coprisin analogue treatment caused membrane damage to C. difficile but not to species of Bifidobacterium. Finally, in an acute gut inflammation model established by exposure of mice to an antibiotic mixture and subsequent C. difficile infection, coprisin analogue treatment strongly inhibited the mucosal damage and inflammatory responses.

Collectively, our findings suggest that the coprisin analogue could prove to be a potent and specific antibiotic against C. difficile infection-induced pseudomembranous colitis and acute diarrhea in humans.

**MATERIALS AND METHODS**

**Coprisin analogue (disulﬁde dimer) synthesis, dimer peptide structure determination, and reagents.** The insect-derived coprisin peptide was synthesized by AnyGen (Gwang-ju, South Korea). The peptide was puriﬁed by reverse-phase high-performance liquid chromatography (HPLC) using a Capcell Pak C18 column (Shiseido, Japan) and eluted with a linear gradient of water-acetonitrile (0 to 80%) containing 0.1% triﬂuoroacetic acid (45% recovery). The identity of the peptide was conﬁrmed by electrospray ionization (ESI) mass spectrometry (Platform II; Micromass, Manchester, United Kingdom). To form the interchain disulﬁde bond, synthetic peptide was dissolved in acetonitrile-H2O (50:50) solution and then oxidized in 0.1 M Na2HCO3 aqueous solution (pH 6.0 to 6.5) for 24 h. To determine the disulﬁde pattern of the dimeric form of coprisin analogue, the peptide solution was analyzed by HPLC and ESI mass spectrometry. Polyclonal antibodies against caspase-3 and caspase-8 were purchased from Cell Signaling Technology (Beverly, MA). β-Actin, EDTA, kanamycin, gentamicin, colistin, metronidazole, vancomycin, clindamycin, and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO).

**Preparation of vegetative C. difficile cells.** C. difficile strain VPI 10463 (ATCC 43255) (19) was cultured in brain heart infusion (BHI) broth (Becton Dickinson, Franklin Lakes, NJ) or BHI broth supplemented with 1.5% agar at 37°C under anaerobic conditions using polystyrene incubation bags containing an oxygen-binding system (Anaerocult A; Merck, Darmstadt, Germany) at 37°C. Anaerocult A contains components that chemically bind oxygen quickly and completely, creating oxygen-free conditions that include a CO2 atmosphere. An overnight culture of C. difficile was used to generate a 24-h anaerobically produced vegetative suspension. For oral gavage infection of mice, the exact cell density of C. difficile was determined using the dilution plate method (1,000-fold dilution) after harvesting cells by centrifugation at 1,500 × g for 10 min and rinsing twice with prereduced BHI medium.

**Bacterial strains and culture conditions.** The following strains were kindly provided by the Rural Development Administration—GenBank Information Center (Southon, South Korea): Lactobacillus casei, Lactobacillus delbrueckii subsp. lactis, and Bifidobacterium thermophilum. L. casei was incubated in De Man-Rogosa-Sharp (MRS) broth (Difco Laboratories, Detroit, MI) (28) under anaerobic conditions using polystyrene incubation bags containing an oxygen-binding system (Anaerocult A; Merck, Darmstadt, Germany) at 37°C. Anaerocult A contains components that chemically bind oxygen quickly and completely, creating oxygen-free conditions that include a CO2 atmosphere. An overnight culture of C. difficile was used to generate a 24-h anaerobically produced vegetative suspension. For oral gavage infection of mice, the exact cell density of C. difficile was determined using the dilution plate method (1,000-fold dilution) after harvesting cells by centrifugation at 1,500 × g for 10 min and rinsing twice with prereduced BHI medium.

**Antibacterial activity assay.** The antibacterial activities of the coprisin analogue and various antibiotics toward the bacteria described above were determined by the disc diffusion test (25, 29), the dilution plate method (31), and a microdilution assay (1). Coprisin analogue MICs were determined using a microdilution assay (1), a C. difficile inoculum of 2 × 105 to 2 × 106 CFU per well (final volume, 100 μl of prereduced BHI broth), and a 96-well plate (Fisher, Pittsburgh, PA) (1). t-type and ϵ-enantiomeric (e-type) coprisin analogue and vancomycin were added at concentrations ranging from 0.1 to 30 μg/ml (final volume, 100 μl of BHI broth). Plates were then incubated under anaerobic culture conditions using polystyrene incubation bags containing an oxygen-binding system for 48 h at 37°C. The MIC100 was determined by visual inspection and by spectrophotometry. Experiments were repeated a minimum of three times for each organism, and tests included at least three replicate experiments for each set of assay conditions. Experiments included control serial dilutions of water alone.

The bactericidal activities of t-type or e-type coprisin analogue or vancomycin against C. difficile were simultaneously compared as survival percentages relative to untreated control results by plating dilutions of each mixture (32). After 2 days of exposure of C. difficile to the antimicrobial agents (10 μg/ml), samples were collected and suitable dilutions were plated on prereduced BHI agar to score the residual CFU values. Plates were incubated under anaerobic culture conditions using polystyrene incubation bags containing an oxygen-binding system. A culture without peptide or antibiotics was always grown in parallel as a control.

Diffusion disc tests were also performed (25, 29). Prereduced BHI agar plates were inoculated with C. difficile (106 CFU/ml). Sterile discs (6-mm diameter) were impregnated with 10 μl of reconstituted coprisin analogue (concentrations, 10 and 100 μg/μl in water) and placed on the surface of bacterium-inoculated BHI agar plates. Plates were incubated in an anaerobic chamber bag (Anaerocult A; Merck, Darmstadt, Germany) for 48 h at 37°C. Control discs contained 10 μl of water. Inhibition zone diameters at the transition point were measured in millimeters.

Comparisons of the bactericidal activities of coprisin analogue, vancomycin, kanamycin, and clindamycin against normal microbiota, expressed as survival percentages relative to untreated control results, were also carried out (32). Coprisin analogue and antibiotics (concentration range, 0 to 30 μg/ml) were added to growing cultures of L. casei, L. delbrueckii subsp. lactis, and B. thermophilum (2 × 107 CFU/ml). After 2 days at a plateau of growth, samples were collected and dilutions were plated on solid agar to score the residual CFU values. Plates were incubated under anaerobic culture conditions using polystyrene incubation bags containing an oxygen-binding system.

**Measurement of bacterial membrane damage.** In order to determine whether the coprisin analogue-induced growth inhibition was associated with lysis of bacterial membranes, C. difficile or B. thermophilum was exposed to coprisin analogue plus propidium iodide (PI) (100 ng/ml), the red fluorescent nucleic acid stain, which stained bacteria with a compromised membrane (i.e., those considered to be dead or dying). After 1 h, the bacteria were washed twice with their culture medium, and the fluorescence in the stained bacterial suspension was measured using a Max M5 fluorescent plate reader ( Molecular Devices, Sunnyvale, CA) (excitation, 470 nm; emission, 520 nm). For visualization of bacteria with compromised membranes, stained bacterial suspensions were also placed on slides and analyzed using a Bio-Rad MRC 1024 confocal scanning laser microscope (CSLM) equipped with a krypton-argon mixed-gas laser as a light source (magnification, ×1,000).

**Induction of mouse gut inflammation by administration of antibiotics plus C. difficile infection.** To test the therapeutic effects of coprisin analogue on gut inflammation following C. difficile infection, we adopted the mouse model of C. difficile-induced antibiotic-associated disease (CDAD) developed by Chen et al. (8), which more closely resembles human disease responses than other available models (8). This study was approved by the Animal Care and Use Committee of Daejin University (Pocheon, South Korea). Male CD1 mice (Duchan Biolink, Daejeon, South Korea) weighing 30 to 35 g were used. Mice had free access to food and water and were allowed to acclimate to these conditions for at least 7 days prior to experiments. For antibiotic pretreatment, mice were caged in groups of eight and given antibiotic-containing drinking water for 3 days. The antibiotic concentrations in the mixtures used (kanamycin, 0.4 mg/ml; gentamicin, 0.035 mg/ml; colistin, 850 U/ml; metronidazole, 0.215 mg/ml; vancomycin, 0.045 mg/ml) were chosen to yield appropriate daily doses of each antibiotic. The approximate values were as follows: for kanamycin, 40 mg/kg for body weight; for gentamicin, 3.5 mg/kg; for colistin, 4.2 mg/kg; for metronidazole, 21.5 mg/kg; and for vancomycin, 4.5 mg/kg. After exposure to the antibiotic mixture, all mice were given untreated autoclaved water for 1 day. Control mice then received a single dose of clindamycin (10 mg/kg) intraperitoneally, whereas experimental mice received clindamycin intraperitoneally plus coprisin analogue (1 μg/ml) orally. One day later, all mice were infected by oral gavage with 0.5 ml of a suspension of C. difficile strain VPI 10463 (5 × 105 CFU/ml). Control mice were further given drinking water alone, and experimental mice were further administered coprisin analogue (1 μg/ml) orally for 6 days and monitored for weight loss and survival. Tissues were collected on day 6, and the activation of caspase-3 and caspase-8 was assessed by immunoblot analysis. For colony counts
of *C. difficile* in infected mice, ileal tissues (3-cm sections) were isolated from conditioned mice on day 5 and suspended in 1 ml of phosphate-buffered saline (PBS). Suitable dilutions were plated on *C. difficile*-selective agar supplemented with 7% sheep blood (BD Diagnostics, Sparks, MD) and incubated for 48 h under anaerobic conditions in polyvinyl incubation bags containing an oxygen-binding system. *C. difficile* colonies on plates were counted using long-wave UV light, exploiting the greenish fluorescence of this organism.

**Evaluation of microscopic damage.** Because severe inflammatory responses were detected in both the small and large intestinal tracts following oral infection with *C. difficile* by gavage, and because numerous studies of acute inflammatory responses following *C. difficile* toxin injection in ileal tissue have been previously reported (18–20), we evaluated inflammation and mucosal damage in isolated ileal tissues. Briefly, ileal tissues of conditioned mice were removed, opened longitudinally, and washed with PBS. Full-thickness sections were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E).

**Immunoblot analysis.** Ileal tissues were washed with cold PBS and lysed in buffer (150 mM NaCl, 50 mM Tris-HCl [pH 8.0], 5 mM EDTA, 1% Nonidet P-40), and equal amounts of protein were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The appropriate antibodies were applied, and antigen-antibody complexes were detected with LumiGlo reagent (New England BioLabs, Ipswich, MA).

**Measurement of IL-6 and toxin in ileal tissues of conditioned mice.** *C. difficile* infection was previously reported to stimulate production of interleukin-6 (IL-6) from mouse mucosal extracts (20). To evaluate whether this occurred in our mouse model of gut inflammation, ileal tissues from the above-described mice were homogenized for 40 s and centrifuged at 11,000 × g for 10 min at 4°C. Supernatants were collected, and mouse IL-6 was measured by an enzyme-linked immunosorbent assay (ELISA) using kits obtained from R&D Systems (Minneapolis, MN). Next, levels of cellular toxicity of toxin released from *C. difficile* were determined in the ileal lumen of conditioned mice. Based on previous reports that *C. difficile* toxin caused cell rounding in various cell types (18), supernatants from the ileal tissue experiments described above were added to cultured HT29 human colonocytes and the resulting changes in cell shape were measured.

**Induction of mouse gut inflammation by direct toxin A injection.** Male CD1 mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). Ileal loops (2 cm in length) were prepared and injected with control buffer (PBS), toxin A (5 μg), coprisin analogue (1 μg), and toxin A plus coprisin in a volume of 10 μl of PBS (19). After 4 h of incubation, ileal tissues were collected and mucosal damage and IL-6 production levels were determined.

**Statistical analysis.** The results are presented as mean values ± standard errors of the means (SEM). Data were analyzed using the Sigma-STAT professional statistics software program (Jandel Scientific Software, San Rafael, CA). Analyses of variance with protected *t* tests were used for intergroup comparisons.

## RESULTS

**Coprisin analogue has antibiotic activity against *C. difficile*.** Since it was previously shown that coprisin analogue had antibiotic activity against *Staphylococcus aureus* and *Escherichia coli* (13), we assessed here whether it also displayed antibiotic activity against *C. difficile*, a nosocomial infectious microbe known to cause antibiotic-associated diarrhea and pseudomembranous colitis in humans (18). To accomplish this, we impregnated sterile discs with coprisin analogue and placed them on BHI agar plates inoculated with *C. difficile* (10⁸ CFU/ml). After 2 days at a plateau of growth, zone-inhibition diameters were measured. As shown in Fig. 1A, a disk diffusion test revealed that coprisin analogue increased the *C. difficile* inhibition zone diameter to 20 mm at 10 μg/ml and 30 mm at 100 μg/ml in a dose-dependent manner. However, coprisin analogue administered at 1 μg/ml did not produce a visible inhibition zone, a result similar to that obtained using control discs exposed to water alone (data not shown). Next, we compared the antibiotic activity of coprisin analogue against *C. difficile* with that of vancomycin, an antibiotic known to be effective against *C. difficile* (21). As shown in Fig. 1B, the inhibition zone following treatment with coprisin analogue at 100 μg/ml was 30 mm in diameter, which was smaller than that produced by the same concentration of vancomycin (>40 mm). Although the antimicrobial activity of coprisin analogue was slightly weaker, its inhibitory level was comparable to that seen with vancomycin.

It has been speculated that orally administered peptides may be degraded by various proteases in the gastrointestinal tract (51). Since α-enantiomeric peptides are known to be extremely protease resistant (37, 47), we synthesized D-type coprisin analogue and measured its antibiotic activity against *C. difficile*. L-type and D-type coprisin analogue (10 μg/ml), or vancomycin (10 μg/ml) was added to anaerobically growing cultures of *C. difficile* (2 × 10⁵ CFU/ml) in BHI broth at 37°C. After 2 days at a plateau of growth, suitable dilutions were plated onto BHI agar and the residual numbers of *C. difficile* bacteria were counted. Data are representative of the results obtained with three independent samples. *P < 0.05 versus water-treated control culture.

**FIG. 1.** Coprisin analogue (coprisin) has antibiotic activity against *C. difficile*. (A) Disc diffusion tests. Sterile discs containing coprisin (10 to 100 μg/ml in water) were placed on BHI agar plates inoculated with *C. difficile* (10⁸ CFU/ml) anaerobically incubated for 48 h at 37°C. Data are representative of the results obtained with three independent samples. (B) Comparison of the antibiotic activity of coprisin analogue (100 μg/ml) with that of vancomycin (100 μg/ml). (C) Water (control), L-type or D-type coprisin analogue (10 μg/ml), or vancomycin (10 μg/ml) was added to anaerobically growing cultures of *C. difficile* (2 × 10⁵ CFU/ml) in BHI broth at 37°C. After 2 days at a plateau of growth, suitable dilutions were plated onto BHI agar and the residual numbers of *C. difficile* bacteria were counted. Data are representative of the results obtained with three independent samples. *P < 0.05 versus water-treated control culture.
days later, samples were appropriately diluted and plated on BHI agar to score the residual number of *C. difficile* CFU. Interestingly, there was no significant reduction in the antibiotic activity of D-type coprisin analogue compared to that of L-type coprisin analogue. In fact, the activity of D-type coprisin analogue was higher and was only slightly less than that of vancomycin (Fig. 1C). These results indicate that coprisin analogue could be therapeutically useful against *C. difficile*. On the basis of these results, we used D-type coprisin analogue in subsequent experiments.

Next, L-type or D-type coprisin analogue or vancomycin was added to the *C. difficile* inoculum, and the lowest concentrations of coprisin analogue and vancomycin that reduced *C. difficile* growth (MIC<sub>100</sub>) were compared. The results showed that the L-type coprisin analogue MIC<sub>100</sub> against *C. difficile* (ATCC 43255) was 1.5 μg/ml, the D-type coprisin analogue MIC<sub>100</sub> was 1.0 μg/ml, and the vancomycin MIC<sub>100</sub> was 3.0 μg/ml. As shown in Fig. 1, standard broth microdilution tests also revealed that the antimicrobial activity (MIC<sub>100</sub>) of D-type coprisin analogue against *C. difficile* was similar to that of L-type coprisin analogue (D-type at 1 μg/ml versus L-type coprisin analogue at 1.5 μg/ml). Interestingly, in contrast to the results seen in disc diffusion tests, coprisin analogue at all levels was more effective than vancomycin in standard broth microdilution tests (data not shown). The fact that 1 μg of coprisin analogue was not effective in disc diffusion tests but was effective in broth culture suggests that a low concentration of coprisin analogue may fail to diffuse away from the point of application. An example of this phenomenon is provided by amyloid β-peptides, which also appear to irreversibly absorb to the cellulose disks often used as sample reservoirs in disc diffusion tests (40).

**Coprisin analogue does not show antimicrobial activity against Lactobacillus and Bifidobacterium.** Because normal microbiota such as *Lactobacillus* and *Bifidobacterium* can inhibit the growth of *C. difficile* (9, 42) and because antibiotics intended for therapeutic use against bacterial infection-mediated gut inflammation should have specific antimicrobial activity against pathogenic microbes but not commensal bacteria, we next assessed whether coprisin analogue affected the growth of normal microbiota. First, the antibacterial activity of coprisin analogue against the bacteria described above was measured and compared with that of various antibiotics (kanamycin, clindamycin, and vancomycin). An inoculum of *L. casei* (2 × 10<sup>7</sup> to 2 × 10<sup>5</sup> CFU) was treated with D-type coprisin analogue, vancomycin, kanamycin, or clindamycin (concentration range, 0 to 30 μg/ml for each) and incubated anaerobically at 37°C. After 2 days, suitable dilutions were plated on MRS agar for lactobacilli, and the residual number of CFU was scored for calculation of the survival percentage relative to those of cultures without antibiotic treatment. In cultures inoculated with *L. casei* (Fig. 2A), survival was 96% in the presence of coprisin analogue administered at up to 30 μg/ml. There was also no significant reduction in survival in cultures exposed to vancomycin, clindamycin, or kanamycin (Fig. 2A). However, survival in cultures treated with 15 μg/ml clindamycin was only 1% and decreased further at 30 μg/ml. In cultures of *L. delbrueckii* subsp. *lactis* (Fig. 2B), interestingly, survival was increased in the presence of coprisin analogue in a dose-dependent manner. Cultures exposed to kanamycin or vancomycin (each at 30 μg/ml) displayed at least 93% survival, whereas clindamycin treatment completely prevented colony growth at a concentration of 5 μg/ml (Fig. 2B). In cultures of *B. thermophilum*, survival in the presence of vancomycin at 5 μg/ml was 38%, whereas vancomycin at 15 and 30 μg/ml completely inhibited colony growth. As was the case against other strains, clindamycin also appeared to inhibit the growth of *B. thermophilum*. The colonies grown on *B. thermophilum* displayed colony formation. *P* < 0.001 versus water-treated control culture; *P* < 0.05 versus water-treated control culture.
C. difficile, leading to penetration (Fig. 3B). In contrast, exposure of *Bifidobacterium* to coprisin analogue did not significantly change the amount of PI staining relative to control culture results (Fig. 3C and D). These results suggest that the antimicrobial activity of coprisin analogue is highly specific for *C. difficile*, sparing normal microbiota such as *Lactobacillus* and *Bifidobacterium*.

The antimicrobial activity of coprisin analogue is responsible for preventing inflammation and mucosal damage in the *C. difficile*-infected mouse gut. Since coprisin analogue had membrane degradation-associated antibiotic activity against *C. difficile* but not against normal gut microbiota such as *Lactobacillus* and *Bifidobacterium*, we next assessed whether coprisin analogue could prevent the marked reductions in body weight and survival typically seen in a *C. difficile* infection-induced mouse model of gut inflammation (20, 22, 33). As shown in Fig. 4A, infection of mice with *C. difficile* caused a marked drop in body weight, but coprisin analogue treatment significantly blocked this weight loss. On day 3 of infection, infected mice that had not received coprisin analogue had lost 13% of their body weight, whereas a 5% drop in body weight was observed in coprisin analogue-treated mice. By day 6, there was no body weight difference between coprisin analogue-treated and control mice that had received antibiotics but had not been subjected to *C. difficile* infection. *C. difficile* infection also sharply decreased the survival rate, particularly on day 5 postinfection; this effect was also significantly reduced in coprisin analogue-treated mice (Fig. 4B). Similarly, mice infected with *C. difficile* for 6 days showed increased mucosal damage, but this effect was abolished by coprisin analogue treatment (Fig. 4C).

To further assess the effects of coprisin analogue on inflammation resulting from *C. difficile* infection, we measured the levels of interleukin-6 (IL-6), a marker of acute inflammation (20), in ileal tissue extracts of conditioned mice on day 6. As shown in Fig. 4D, *C. difficile*-infected mice exhibited a significant increase in IL-6 production, but this was markedly diminished by coprisin analogue treatment. Because it has been reported that *C. difficile* infection or injection of purified toxin A into the mouse ileal lumen causes epithelial cell apoptosis, which is a critical step in acute gut inflammation (18, 20), we also measured the activation of caspase-3 and caspase-8 (two critical apoptotic proteases) in the conditioned tissue extracts. Our results revealed that ileal extracts from *C. difficile*-infected mice displayed more caspase-3 and caspase-8 activation than the controls but that this effect was markedly reduced by coprisin analogue treatment (Fig. 4E). No activation of caspase-3 or caspase-8 was observed in control mice (Fig. 4E). However, in a mouse model of ileitis caused by direct injection of purified toxin A, neither toxin A-induced mucosal damage nor IL-6 production (18, 20, 50) was inhibited by coprisin analogue treatment (data not shown).

Next, we measured the number of *C. difficile* colonies in ileal tissues isolated from conditioned mice. As shown in Fig. 4F,
the number of *C. difficile* colonies in ileal tissue from mice administered water alone after oral infection with *C. difficile* was markedly higher than that in ileal tissue from mice administered coprisin analogue after *C. difficile* infection. Specifically, *C. difficile* survival in ileal tissue following in vivo coprisin analogue treatment was 28% of that seen with the controls, a degree of protection that may be capable of increasing mouse survival rates. Extracts from conditioned mice were also added to cultured human colonocytes to investigate whether the extract contained toxin that might have been released as a result of oral infection with *C. difficile*. The extracts from mice infected with *C. difficile* alone caused marked rounding of HT29 cells, whereas cellular rounding was significantly reduced in cells exposed to coprisin analogue-treated mouse extracts (Fig. 4G).

**DISCUSSION**

We previously demonstrated that coprisin analogue has antibacterial activity against various pathogenic bacterial species (13). Here, we assessed whether this peptide had antibiotic activity against *C. difficile*, the primary etiologic agent of antibiotic-associated pseudomembranous colitis and severe diarrhea in humans and animals (4, 16–19, 22). Our results revealed that coprisin analogue treatment significantly inhibited the growth rate of *C. difficile* (Fig. 1) but did not alter the growth rates of *Lactobacillus* and *Bifidobacterium* (Fig. 2). Given that normal microbiota (46), along with probiotics, have inhibitory activities against pathogenic bacteria (3) and that effective antibiotics should have specific antimicrobial activity against pathogenic but not nonpathogenic microbes, coprisin analogue may be a good candidate for use as a potential therapeutic reagent for *C. difficile*-associated acute colitis.

Although antibiotic-associated diarrhea has been linked to numerous antibiotics, including the beta-lactam antibiotics (26), clindamycin, which is usually used to treat anaerobic bacterial infections, is considered to be a primary cause of *C. difficile*-associated diarrhea and pseudomembranous colitis (16, 17). Here, we found that clindamycin treatment markedly
inhibited the growth of the tested Lactobacillus species (L. casei and L. delbrueckii subsp. lactis), as well as Bifidobacterium and C. difficile. This nonselective antibiotic activity against normal microbiota, which can inhibit the growth of pathogenic bacteria, may facilitate C. difficile colonization and subsequent damage. Compared to vancomycin, which has antimicrobial activity against Bifidobacterium but not Lactobacillus species (Fig. 2), coprisin analogue is more selective and does not appear to have antibiotic activity against the tested normal gut microorganisms.

The amino acid sequence of coprisin is very similar to that of the ~40-amino-acid defensin and defensin-like peptides, which confer antibacterial activity by disrupting the membrane or suppressing cell cycle signaling (48). In the current study, we found that coprisin analogue treatment damaged the plasma membrane of C. difficile but not that of Bifidobacterium sp. (Fig. 3C and D). Since both of those bacterial species are Gram positive and since they have biologically similar membranes (3), future work would be necessary to determine the basis for the selective antimicrobial activity of coprisin analogue. For example, a specific receptor for coprisin analogue may exist on the plasma membrane of C. difficile, or negatively charged components of the lipid membrane, such as teichoic acids (30) and peptidoglycan (30), may play a role in the interaction with coprisin analogue. Alternatively, the selectivity of coprisin analogue may arise as a result of the presence of three positively charged amino acids (NH$_2$-RKK-COOH) at the C terminus of the peptide. The defensin family peptides can interact with a wide variety of membrane components, including lipopolysaccharides (41), cardiolipin (11), and sphingolipids (44); beyond those interactions, structural features of the peptides further determine their specificity for binding to the surface of a given microorganism (e.g., via disulfide cross-linking through a cysteine) (7). Since coprisin analogue contains a cysteine in the middle position, potentially allowing it to dimerize, the peptide structure itself may affect its microbial binding capacity or selectivity.

In a mouse model of acute gut inflammation following C. difficile infection, the presence of coprisin analogue markedly ameliorated inflammatory responses and weight loss and improved survival rates. The sharp decreases in body weight on days 2 and 3 following C. difficile infection were only partially reversed by coprisin analogue treatment; it was not until day 4 that coprisin analogue-treated mice returned to control-level body weights. However, coprisin analogue did not prevent inflammation resulting from injection of purified toxin A into the ileal lumen (data not shown), suggesting that the apparent anti-inflammatory activity of coprisin analogue is associated with its antimicrobial activity rather than with inhibition of the activity of the toxins.

In summary, we report that coprisin analogue, a disulfide dimer and insect-derived peptide, has selective antibiotic activity against C. difficile but not Lactobacillus and Bifidobacterium, members of the normal bowel flora. Furthermore, coprisin treatment has a strong beneficial effect on C. difficile infection-induced mouse gut inflammation. These novel findings suggest that coprisin could be a useful candidate for therapeutic use against C. difficile-associated diarrhea and pseudomembranous colitis.