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Activity of Species-specific Antibiotics Against Crohn's Disease-Associated Adherent-invasive *Escherichia coli*

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Background: Crohn's disease (CD) is associated with bacterial dysbiosis that frequently includes colonization by adherent-invasive *Escherichia coli* (AIEC). AIEC are adept at forming biofilms and are able to invade host cells and stimulate the production of proinflammatory cytokines. The use of traditional antibiotics for the treatment of CD shows limited efficacy. In this study, we investigate the use of species-specific antibiotics termed colicins for treatment of CD-associated AIEC.

Methods: Colicin activity was tested against a range of AIEC isolates growing in the planktonic and biofilm mode of growth. Colicins were also tested against AIEC bacteria associated with T84 intestinal epithelial cells and surviving inside RAW264.7 macrophages using adhesion assays and gentamicin protection assay, respectively. Uptake of colicins into eukaryotic cells was visualized using confocal microscopy. The effect of colicin treatment on the production of proinflammatory cytokine tumor necrosis factor alpha by macrophages was assessed by an enzyme-linked immunosorbent assay.

Results: Colicins show potent activity against AIEC bacteria growing as biofilms when delivered either as a purified protein or through a colicin-producing bacterial strain. In addition, colicins E1 and E9 are able to kill cell-associated and intracellular AIEC, but do not show toxicity toward macrophage cells or stimulate the production of proinflammatory cytokines. Colicin killing of intracellular bacteria occurs after entry of colicin protein into AIEC-infected macrophage compartments by actin-mediated endocytosis.

Conclusions: Our results demonstrate the potential of colicins as highly selective probiotic therapeutics for the eradication of *E. coli* from the gastrointestinal tract of patients with CD.

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Key Words: adherent-invasive *E. coli*, Crohn's disease, biofilms, colicins, antibiotics

I ncreased understanding of the relationship between health and the gut microbiome is changing our fundamental understanding of the pathogenesis of chronic disease. In some conditions, such as Crohn's disease (CD), a link with dysbiosis has been established in multiple populations and is widely believed to be relevant to disease pathogenesis.^{1–3} For other conditions such as diabetes, obesity, rheumatoid arthritis, and asthma, associations between disease and the composition of the microbiome have also been observed.^{4–6}

The ability to manipulate the microbiome in a targeted and predictable manner is therefore a potentially powerful tool to treat a range of chronic conditions.

The use of highly selective species- or genus-specific antibiotics such as the colicin-like bacteriocins provides a route through which specific bacteria can be targeted within a complex microbial community, for example, to perform “precision surgery of the microbiome.” This diverse family of bacteriocins is produced by a range of gram-negative bacteria and includes the S-type pyocins from *Pseudomonas aeruginosa* and the well-characterized colicins from *Escherichia coli*.^{7,8} Colicin-like bacteriocins are highly potent multidomain protein antibiotics that are often capable of killing susceptible cells at subnanomolar (nM) concentrations. They kill cells through either a nuclease activity that specifically targets DNA (colicins E2, E7, E8, and E9), rRNA (colicins E3 and E6), tRNA (colicin D and E5), or pore-forming activity (colicin E1, S4, N, A, and B) or through the inhibition of cell wall synthesis (colicin M).⁹ The specificity of colicins is due to their high affinity for a specific outer membrane protein and the ability to use the Tol and Ton cell envelope complexes to mediate their active transport across the outer membrane.¹⁰

A potential application of colicins is in the treatment of CD, for which there is evidence implicating *E. coli* in disease

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pathogenesis.^{11–13} CD is a chronic and incurable form of inflammatory bowel disease of complex etiology that is characterized by granulomatous inflammation and intestinal dysbiosis. Dysbiosis in patients with CD frequently involves decreased diversity in intestinal bacterial microflora.¹⁴ Previous studies analyzing luminal samples and mucosal biopsies have shown a reduction in the major classes of commensals such as Firmicutes and Bacteroidetes and increased numbers of the Proteobacteria, especially *E. coli*.^{3,15} *Escherichia coli* resides as a nonpathogenic commensal organism in the human gut and accounts for less than 1% of the fecal microbiota.¹⁶ However, studies in multiple populations have shown that a novel pathotype of *E. coli* termed adherent-invasive *E. coli* (AIEC) are more frequently isolated from the ileal mucosa of patients with CD relative to healthy individuals.^{13,17,18} AIEC comprise approximately 3.58% and 0.9% of ileal and colonic *E. coli*, respectively, of healthy subjects; however, in this setting, it is not shown to translocate the intestinal mucosa barrier.^{18,19} In patients with CD, AIEC prevalence ranges from the absence of to 15.8% of colonic samples and from 6.2% to 18% of ileal samples. Additionally, recent work reported a prevalence of approximately 25% for AIEC in patients with ileal CD.²⁰

AIEC strains have the ability to adhere to and invade epithelial cells and produce extensive biofilms that protect bacteria from the host immune system and antibiotic therapy.^{19,21,22} For the ileal isolates, this opportunism is linked to abnormally increased expression of CEACAM6 receptors in patients with CD in which AIEC bacteria are able to adhere through FimH of the bacterial type I pilus.²³ Furthermore, expression of long polar fimbriae by AIEC allows the bacteria to interact with Peyer's patches and to translocate across M cells.²⁴ AIEC have also been shown to survive and replicate within host macrophages.^{25,26} The invasion of macrophages by AIEC stimulates the production of several proinflammatory cytokines such as tumor necrosis factor alpha (TNF- α) and interleukin 6.^{25,26} Furthermore, in the recent work of Small et al,²⁷ persistent AIEC infection was established in streptomycin-treated mice, which caused the development of CD-like symptoms. A plausible model for the development of CD is that alteration of the gut microbiota through an event such as inflammation induced by aberrant immune responses or the administration of broad-spectrum antibiotics creates a niche for the proliferation of the AIEC population. This is both consistent with the genetic loci associated with CD making the carrier deficient in the detection and clearance of bacteria and also with the observations linking increased antibiotic use with an increased risk of developing CD.^{28–30}

To begin to determine the potential of colicins as therapeutics for the treatment of AIEC infection associated with CD, we tested the ability of colicins to kill AIEC in the biofilm, host cell-associated, and intracellular states. Our data show that colicins have potent activity against AIEC in the biofilm state and are able to kill both host cell-associated and intracellular AIEC. Thus, colicins are potentially useful therapeutics for the

specific targeting of pathogens such as AIEC, in which maintenance of a "healthy microbiome" is desirable.

MATERIALS AND METHODS

Antimicrobials

The following antibiotics were obtained from Sigma-Aldrich (Poole, Dorset, UK): ampicillin, ciprofloxacin, kanamycin, metronidazole, penicillin–streptomycin, and gentamicin. Colicins E1, E3, E9, and D were expressed and purified as previously described.³¹ Measurement of the LPS content of purified colicins was performed using the E-TOXATE kit (Sigma-Aldrich) as previously described.³² Removal of LPS from purified colicins was performed using polymyxin B-agarose columns (Detoxi Gel, Pierce, Leicestershire, UK) as previously described.³³ Briefly, 1 mL of purified colicin E9 (3 mg/mL) was loaded onto each 0.5-mL Detoxi column and incubated at room temperature for 60 minutes. The protein was then eluted with endotoxin-free water in 1 mL fractions.

Bacterial Strains, Plasmids, and Protein Purification

The AIEC reference strain LF82, an ileal CD mucosa associated isolate¹² and the additional AIEC strains HM95, HM419, and HM615,¹⁷ colonic CD mucosa-associated isolates were grown on Luria Bertani (LB) agar plates or in LB broth with shaking at 37°C with the addition of ampicillin for LF82. For the creation of GFP-expressing LF82, competent cells were transformed with pDM15³⁴ by electroporation as previously described.³⁵ Transformants were selected by plating on LB agar supplemented with kanamycin (50 μ g/mL) and isolating those that exhibited green fluorescence under UV light. The colicin E9-H575A variant was created by site-directed mutagenesis using the Stratagene Quik-Change kit (Stratagene, Cheshire, UK) with the plasmid pCS4³¹ and mutagenesis primers E9H575AF (ACT ACA CCT AAG CGA GCT ATC GAT ATT CAC CGA GGT AAG) and E9H575AR (CTT ACC TCG GTG AAT ATC GAT AGC TCG CTT AGG TGT AGT. pAR5, encoding RFP-tagged colicin (colicin E9-RFP), was constructed by amplification of the RFP gene using plasmids RFPF (GTT GAC GTC CAT GGC CTC CAC CGA GGA CGT CAT CAC CGA) and RFPR (CCA CTG TGC TCG AGC AGG AAC AGG TGG TGG CGG CCC TCG), digestion of the polymerase chain reaction product with *Nco*I and *Xho*I, and ligation into the corresponding sites of pCS4. Colicin E9-RFP and colicin E9-H575A were purified by nickel affinity chromatography and gel filtration as described for colicin E9.³¹

Colicin Spot Test Assay

The antimicrobial spectrum of purified colicins was tested against AIEC strains by the overlay spot plate method.³⁶ Briefly, 50 μ L of log phase bacterial culture ($OD_{600} = 0.6$) was added to 5 mL of molten 0.8% agar and overlaid on an LB agar plate. A 5- μ L drop of each colicin was spotted onto the overlay plate. Plates were dried and incubated for 18 hours at 37°C and examined for zones of inhibition.

Biofilm Formation by *E. coli* Isolates

AIEC LF82 biofilms were formed on an MBEC 96-peg plate platform (Innovotech, Edmonton, AL, Canada). Briefly, each well of a 96-well flat bottom plate was inoculated with 150 μ L of LB containing a 1:100 dilution of an overnight culture of *E. coli* that had been grown at 37°C with shaking at 200 rpm. Bacteria were added to the plate in triplicate, and control wells containing only LB media were included on every plate. The 96-peg plate was placed on top of the microtiter plate, submerging all the pegs in the bacterial culture. The isolates were incubated for 24 hours at 37°C on a rocking platform at 20 rpm, to allow mature biofilms to establish. The 3-dimensional structure of AIEC LF82 mature biofilms was examined by scanning electron microscopy. For antibiotic treatment of *E. coli* biofilms, 24-hour biofilms were formed as described above and challenged for 1 hour with the antibiotics ciprofloxacin and metronidazole and colicins E1 and E9 in sterile phosphate-buffered saline (PBS) in a volume of 150 μ L in the concentration range of 0.002–200 μ g/mL. All measurements were performed in triplicate, and control antimicrobial-free biofilms were treated with 150 μ L of sterile PBS. After treatment, the viability of biofilm-associated cells was tested in comparison with untreated control biofilms using the metabolic dye 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), as previously described.³⁷ To assess the inhibition of LF82 biofilms by *E. coli* W3110 harboring the colicin E1-encoding plasmid, pColE1-K53 (pColE1),³⁸ competition assays were performed. LF82 biofilms were grown for 24 hours as described above, and *E. coli* W3110 carrying or lacking pColE1-K53 were grown in LB broth for 18 hours at 37°C in the presence of ciprofloxacin (0.001 μ g/mL) to induce colicin production. At 1, 2, 4, 6, and 24 hours, MBEC pegs were extracted and sonicated to remove biofilm-forming cells. The number of surviving LF82 cells was determined by counting colony-forming units (CFUs) on LB containing ampicillin (50 μ g/mL) to select for growth of LF82. All competition assays were repeated with biofilms in triplicate.

Eukaryotic Cell Culture

T84 human colonic adenocarcinoma cells were cultured as previously described.³⁹ Briefly, cells were cultured in a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich) and Ham's F-12 medium (Invitrogen, Leicestershire, UK) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 2% penicillin-streptomycin, 2% sodium pyruvate, and 10 mM HEPES (Sigma-Aldrich). Confluent T84 monolayers were subcultured by trypsin (0.25%) in Ethylenediaminetetraacetic acid (EDTA) treatment. Cells were grown for 7 days in tissue culture dishes until a cell monolayer was formed before being used in assays. Experiments that required infection of T84 cells were performed using antibiotic-free medium. RAW264.7, a murine macrophage-like cell line, was cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% FBS (Invitrogen), 2% penicillin-streptomycin, and 2% L-glutamine (Gibco, Leicestershire, UK). All tissue culture cell lines used

in this study were grown at 37°C, 5% CO₂. RAW264.7 cells and T84 cells were seeded as 2 × 10⁵ cells per well in 24-well plates and on glass coverslips in 6-well plates (Corning, Birmingham, UK). For infections, RAW264.7 macrophages reached 70% confluency after 18 hours, and for T84 cells, a confluent monolayer was grown after 5 to 7 days.

Endocytosis Inhibitor Treatments

The following inhibitors were purchased from Sigma-Aldrich and used as described previously.⁴⁰ Cytochalasin B was used at 10 μ g/mL, polyinosinic acid was used at 50 μ g/mL, dynasore was used at 80 μ M, dimethylamiloride dimethylamiloride (DMA) was used at 500 μ M, and mannan was used at 1 mg/mL.

Bacterial Adhesion Assays

To assess the efficacy of colicin killing against AIEC adhered to intestinal epithelial cells, bacterial adhesion assays were performed. Briefly, T84 intestinal epithelial cells were grown in 24-well plates at 37°C in 5% CO₂ until an epithelial monolayer was formed. Before adhesion assays, T84 cell monolayers were washed with sterile PBS. LF82 bacteria were grown to OD₆₀₀ = 0.6 in LB broth and back diluted in DMEM culture media to a concentration of approximately 10⁸ cells per milliliter. Cells were infected with LF82 at a multiplicity of infection (MOI) of 10 for 3 hours at 37°C, 5% CO₂. To assess antimicrobial killing of adherent bacteria, colicin E9 and antibiotic ciprofloxacin were diluted in DMEM culture media to concentrations of 100 μ g/mL and added to separate wells at 15, 30, 45, and 60 minutes after infection. After 3 hours, monolayers were washed 3 times with sterile PBS to remove nonadherent bacteria and treated with trypsin (0.25%) in EDTA for 10 minutes at 37°C. The cell suspension was removed by aspiration and homogenized by passing through a 22-gauge needle. Cells were lysed by sonication, and cell-associated LF82 were serially diluted and plated on LB agar plates containing ampicillin (50 μ g/mL). Plates were incubated overnight at 37°C and CFUs were counted. All adhesion assays were repeated in triplicate. For photomicroscopy, T84 cell monolayers were grown on glass coverslips in 6-well plates for 6 to 8 days. Cells were infected with LF82 (MOI of 10), and when required, were treated with purified colicin E9 (100 μ g/mL) 15 minutes after infection. After 3 hours, monolayers were washed 3 times with sterile PBS and fixed with 10% methanol in PBS for 10 minutes. Cells were washed again in sterile PBS as described above and were then Giemsa stained (20% Giemsa in dH₂O) for 20 minutes at room temperature. Coverslips were washed and mounted on slides using clear nail varnish solution. Images were captured using a Zeiss Axioskop microscope attached to a QImaging MicroPublisher 3.3 RTB camera at ×40 magnification.

Gentamicin Protection Assay

Investigation of colicin activity against intramacrophagic LF82 was measured by the gentamicin protection assay.⁴¹ Before infection, RAW264.7 cell monolayers were washed with

sterile PBS. RAW264.7 macrophages grown in 24-well plates were infected with LF82 at an MOI of 50. After 2 hours, infected macrophages were washed with PBS and exposed to culture media (RPMI-1640, FBS, L-glutamine) containing gentamicin (100 $\mu\text{g}/\text{mL}$) to kill extracellular bacteria that had failed to invade macrophages. After 1 hour, the media was removed and macrophages were then treated with culture media containing antimicrobial treatments in the presence of gentamicin (20 $\mu\text{g}/\text{mL}$) at 37°C, 5% CO₂. For LF82 growth curves, media was free from antimicrobial treatments and was replaced every 24 hours. Antimicrobial treatments used were colicin E9, colicin E1, and antibiotic ciprofloxacin (100 $\mu\text{g}/\text{mL}$). After incubation with antibiotic for the desired time point, RAW264.7 cells were washed 3 times with sterile PBS. If colicin protein was added, macrophages were also treated with trypsin (0.25%) in EDTA for 5 minutes at 37°C. This was to ensure that all macrophage-bound colicin protein was inactivated. The macrophages were then scraped from the surface of the plate and lysed with 2% Triton X-100 in PBS for 5 minutes. Recovered intracellular bacteria were quantified by plating serial dilutions on LB agar containing ampicillin. The plates were incubated overnight at 37°C, and CFUs were counted. Bacterial survival in RAW264.7 cells (% survival) were calculated as a percentage of viable internalized bacteria relative to the number recovered from untreated cells infected with LF82, considered as 100%.

Fluorescence Microscopy

RAW264.7 macrophages were seeded on glass coverslips in 6-well plates and grown to confluency for 18 hours at 37°C. Culture media used for infection contained 50 $\mu\text{g}/\text{mL}$ kanamycin to select for growth of GFP-expressing LF82 strain. Where required, macrophages were infected with LF82-GFP (MOI of 50) for 2 hours and exposed to the culture medium (RPMI-1640, FBS, L-glutamine, kanamycin) containing gentamicin (100 $\mu\text{g}/\text{mL}$) for 1 hour to ensure killing of extracellular bacteria. Cells were then treated with culture media containing colicin E9-RFP (100 $\mu\text{g}/\text{mL}$) and gentamicin (20 $\mu\text{g}/\text{mL}$) for 24 hours. Infected cells were washed 3 times in sterile PBS and treated with 0.25% trypsin in EDTA for 5 minutes at 37°C to inactivate extracellular colicin E9-RFP. RAW264.7 macrophages were washed as described above to remove macrophage-bound protein and fixed with 2% paraformaldehyde in PBS and 0.5 M sucrose for 10 minutes at room temperature. Fixed cells were permeabilized with 0.5% Triton X-100 for 3 to 5 minutes at room temperature. Actin cytoskeleton was stained using Alexa-647-labeled phalloidin (Life Technologies, Leicestershire, UK) and nuclear DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies). Coverslips were air dried and mounted on slides with Dako Mounting Medium (Dako, Cambridgeshire, UK). For colicin uptake experiments, macrophages were pretreated with each inhibitor for 30 minutes and then further incubated with colicin E9-RFP in the presence of inhibitor for 4 hours. Slides were examined using a Zeiss LSM410 Laserscan Microscope equipped with a peripheral argon-UV laser. Image

processing was performed using LSM Image Browser software (Zeiss, Cambridgeshire, UK).

Detection of TNF- α and LDH Cytotoxicity Assay

Macrophage culture supernatants were harvested from LF82-infected and antimicrobial-treated RAW264.7 macrophages at 24, 30, and 40 hours after infection. Supernatants were assayed for the production of TNF- α using an enzyme-linked immunosorbent assay (BioLegend, Cambridge, UK). Optical density was determined at 450-nm wavelength, and cytokine concentration was determined as by manufacturer's instructions. For RAW264.7 macrophages, cytotoxicity was determined by lactate dehydrogenase (LDH) release. LDH release was measured colorimetrically (BioLegend) according to the manufacturer's protocol. % cytotoxicity = (compound-treated LDH activity – spontaneous LDH activity) / (maximum LDH activity – spontaneous LDH activity) \times 100.

RESULTS

Colicins Show Activity Against Planktonic and Biofilm Associated AIEC

The high frequency of recovery of AIEC isolates from lesions in patients with CD suggests that AIEC may be involved in the pathogenicity of this disease. To begin to determine whether colicins could be used as therapeutics to specifically target AIEC, we tested the sensitivity of the AIEC reference strain LF82 to colicins E1, E3, E9, and D. All tested colicins displayed killing activity against planktonic LF82, with colicin E1 and E9 displaying potent activity down to a concentration of 0.32 $\mu\text{g}/\text{mL}$ (Fig. 1A). However, as AIEC colonizing the mucosa of patients with CD exist as multilayered biofilms, which generally show enhanced tolerance to a range of antibiotics, we investigated the killing activity of colicins against biofilm-associated AIEC bacteria.⁴² To determine whether colicins were active against AIEC growing in the biofilm state, mature LF82 biofilms were grown using the MBEC platform (Fig. 1B).⁴³ Biofilms grown for 24 hours were treated with colicins E1, E9, or the antibiotics ciprofloxacin and metronidazole at a range of concentrations. Colicin E1 displayed a killing activity against LF82 biofilms superior to that of both ciprofloxacin and metronidazole, and the activity of colicin E9 was similar to that of ciprofloxacin but superior to that of metronidazole (Fig. 1C). After colicin E1 treatment (200 $\mu\text{g}/\text{mL}$ for 1 h), LF82 biofilms showed only 5% survival relative to untreated controls.

The activity of colicins E1 and E9 was also tested against additional AIEC strains isolated from patients with CD in the planktonic and biofilm states (see Fig., Supplemental Digital Content 1, <http://links.lww.com/IBD/A971>). Strains HM95, HM419, and HM615 have been described previously and were originally isolated from patients with colonic, ileocolonic, and ileal CD, respectively.¹⁷ Strains HM95, HM419, and HM605 showed high levels of susceptibility to colicins E1 and E9 when

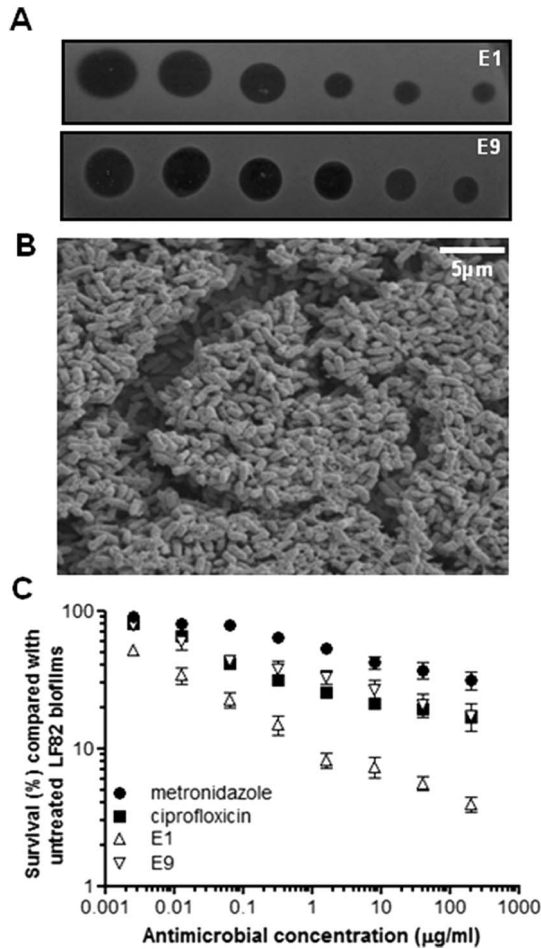


FIGURE 1. Colicins show activity against CD-associated AIEC bacteria. **A**, Colicins show activity against AIEC LF82 in the planktonic state. AIEC reference strain LF82 was grown in planktonic culture, and purified colicin E9 and colicin E1 protein were serially diluted 5-fold (1 mg/mL–0.32 µg/mL) and spotted on molten agar overlay. Zones of inhibition show killing of LF82 bacteria by colicin. **B**, Scanning electron microscopy image of AIEC LF82 multilayered biofilm grown for 24 hours. Scale bar shown is 50 µm. **C**, Colicins show activity against AIEC biofilm-forming cells in vitro. LF82 biofilms were grown on MBEC platform for 24 hours and treated with antimicrobials ciprofloxacin and metronidazole and colicin E1 and colicin E9 at different concentrations for 1 hour. Data shown represent mean and SD. Tests for significant differences between antibiotic treatments were performed using data from an antibiotic concentration of 1.6 µg/mL. At this concentration, significant differences between antibiotic groups were observed by the Kruskal–Wallis test ($P < 0.05$). Pairwise comparisons using the Mann–Whitney test with a significance threshold of $P < 0.05$, adjusted for multiple comparisons using the Bonferroni correction, were used to compare killing by colicins E1 and E9 with ciprofloxacin and metronidazole at this concentration. At this significance, threshold killing of LF82 by colicin E1 was better than by either ciprofloxacin or metronidazole. Killing by colicin E9 was significantly better than by metronidazole but was not significantly different from killing by ciprofloxacin.

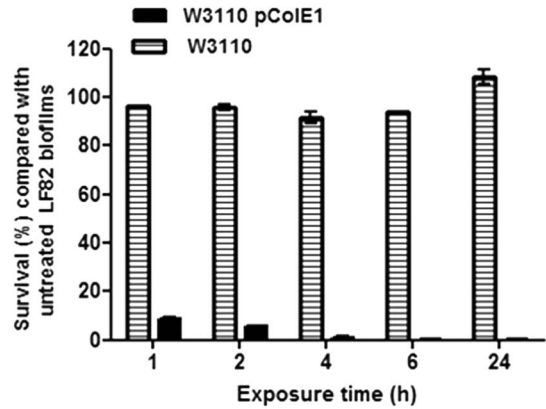


FIGURE 2. Colicin-producing commensal *E. coli* showed killing activity against AIEC biofilms. Mature biofilms (24 h) of LF82 were treated with an *E. coli* strain W3110, which naturally produces the colicin E1 protein. Biofilms were treated for 1, 2, 4, 6, and 24 hours, and biofilm-associated cells were removed from the surface by sonication and CFUs were counted. As a control, LF82 biofilms were also exposed to a non-E1 producing *E. coli* strain W3110 for the same time points. Each experiment was performed in triplicate. Error bars represent the SD between replicates. Significant differences in killing by W3110 and W3110 pColE1 were observed at all time points ($P < 0.05$, unpaired Student's *t* test).

growing in the planktonic state and a similar pattern of susceptibility to colicins E1 and E9, ciprofloxacin, and metronidazole in the biofilm state with the exception of HM615, which showed reduced sensitivity to colicin E9 and increased sensitivity to metronidazole (see Fig., Supplemental Digital Content 1, <http://links.lww.com/IBD/A971>).

Colicins could potentially be delivered to patients with CD to reduce or eliminate AIEC colonization in the form of a colicin-producing probiotic. To test the ability of a nonpathogenic *E. coli* colicin-producing strain to kill LF82 biofilms, we added a colicin E1-producing strain (*E. coli* W3110 pColE1) to established LF82 biofilms. Viable LF82 cells were counted after 1, 2, 4, 6, and 24 hours of exposure to the E1-producing strain. At 1 hour after addition of W3110 pColE1, close to 90% of LF82 biofilm-associated cells were killed, and this rose to 99.6% after 24 hours (Fig. 2). Addition of *E. coli* W3110 lacking the colicin E1-encoding plasmid failed to reduce the survival of the LF82 biofilm population at any of the time points tested (Fig. 2). These data show that the concentration of colicin produced by naturally occurring colicin-producing bacteria is sufficient to eradicate mature *E. coli* biofilms.

Colicins Show Activity Against AIEC LF82 Cells Adhered to T84 Intestinal Epithelial Cells

It has been proposed that the initial stages of chronic AIEC infection involve receptor-mediated bacterial adhesion to epithelial cells.²³ To determine whether colicins are active against AIEC that are adhered to host cells, we infected monolayers of the T84 human intestinal epithelial cell line with LF82

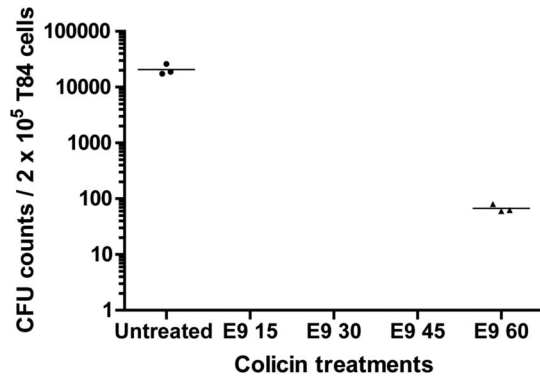


FIGURE 3. Colicins show activity against AIEC strain LF82 adhered to intestinal epithelial cells. T84 intestinal epithelial cell monolayers were infected with AIEC LF82 (MOI of 10 for 3 h), and when required, colicin E9 (100 μ g/mL) was added in the presence of AIEC bacteria at 15, 30, 45, and 60 minutes after infection. Cell-associated LF82 numbers were determined by CFU counts. Data shown represent mean and SE of 3 independent experiments. No CFU counts were detected for E9 15, E9 30, and E9 45 by this method. Comparison of CFUs for untreated and colicin E9 treated showed a significant reduction on colicin treatment at 60 minutes ($P < 0.05$, unpaired Student's *t* test).

and tested the ability of colicin E9 to kill cell-adhered bacteria when added 15, 30, 45, and 60 minutes after infection. On addition of colicin E9 at 15, 30, and 45 minutes after infection, live and adhered LF82 could no longer be detected by CFU counts (Fig. 3). Colicin treatment at 1 hour after infection caused a 2-log unit reduction in CFU counts of cell associated LF82 compared with untreated controls (Fig. 3). These data show that colicin E9 can effectively kill AIEC bacteria adhered to human intestinal epithelial cells.

To determine whether changes in the morphology of intestinal epithelial cells were induced by AIEC infection and subsequent colicin treatment, T84 cells were grown on glass coverslips, infected with LF82, and treated at 15 minutes after infection with colicin E9, where required. At 3 hours after infection, T84 monolayers were Giemsa stained and visualized. Light micrographs showed that LF82 infection of T84 cell monolayers caused alterations in the cell morphology (Fig. 4). LF82-infected monolayers displayed high levels of cell detachment and increased production of abnormal cell protrusions and lamellipodia, indicative of actin remodeling (Fig. 4B).¹³ Colicin

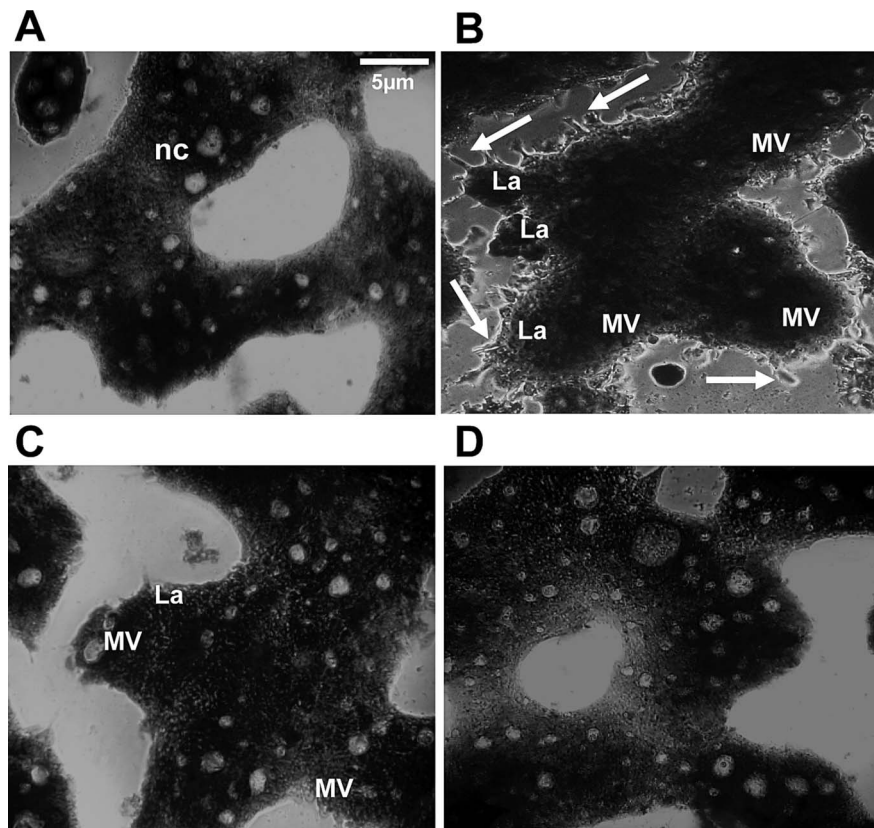


FIGURE 4. Colicin killing of intestinal epithelial cell-associated AIEC bacteria reduced appearance of abnormal cell morphology induced by infection. T84 cells were grown on glass coverslips in 6-well plates to form epithelial monolayers and infected with AIEC LF82 (MOI of 10 for 3 h). For colicin-treated samples, colicin E9 (100 μ g/mL) was added 15 minutes after infection. Cells were Giemsa stained and visualized with Zeiss Axioskop ($\times 40$ magnification). Light micrographs show: (A) untreated control, (B) LF82-infected T84 cells, (C) LF82-infected T84 cells treated with colicin E9 (100 μ g/mL for 3 h), (D) uninfected T84 monolayer treated with colicin E9 (100 μ g/mL for 3 h). Scale bar shown is 5 μ m. Images represent 2 independent experiments. La, lamellipodia; MV, membrane vesiculation; nc, nuclei; white arrow, adhered bacteria.

E9 treatment of infected T84 monolayers significantly reduced the production of cell protrusions, and a smoother cell monolayer was observed (Fig. 4C). LF82-infected T84 monolayers treated with colicin E9 also showed reduced cell detachment and reduced levels of debris in the apical supernatant. Addition of colicin E9 only to uninfected T84 cells did not induce changes in the cell morphology (Fig. 4D).

Colicins Reduce the Persistence of Intracellular AIEC Within RAW264.7 Macrophages

Previous studies have shown the ability of LF82 and other AIEC strains isolated from patients with CD to survive and replicate within macrophages.²⁵ AIEC infection of these cells stimulates the production of proinflammatory cytokines, most notably TNF- α .²⁵ We therefore sought to determine whether intramacrophagic AIEC could be killed through colicin treatment. Although previous studies have reported extensive replication of AIEC strain LF82 within macrophages in vitro, we were unable to reproduce this result.^{26,44} However, in our hands, LF82 was able to infect macrophages and persist intracellularly for extended periods with viable bacteria being detected after 48 hours (see Fig., Supplemental Digital Content 2, <http://links.lww.com/IBD/A972>). Bacterial survival in RAW264.7 macrophages was determined using the gentamicin protection assay.⁴¹ RAW264.7 macrophages were infected with LF82 at an MOI of 50 for 2 hours, treated with gentamicin for 1 hour, and then exposed to antimicrobial treatment in the presence of gentamicin for 4 or 24 hours. Antimicrobial treatments were colicin E1, colicin E9, and colicin E9-H575A, a variant protein that lacks antibacterial activity because of a mutation in the active site of the cytotoxic DNase domain, or ciprofloxacin, which has previously been shown to be highly active against intramacrophagic AIEC.⁴⁵ For antibiotic-free controls, the number of bacteria present in RAW264.7 macrophages at 4 hours and 24 hours was approximately 1.6×10^4 and 800 CFUs per well, respectively (see Fig., Supplemental Digital Content 2, <http://links.lww.com/IBD/A972>). Both colicin E9- and colicin E1-treated macrophages showed reduced intracellular survival of LF82 within macrophages at both 4 and 24 hours, but to a lesser extent than ciprofloxacin (Fig. 5A). In contrast, treatment with inactive colicin E9-H575A after gentamicin treatment did not reduce survival of intramacrophagic LF82, which indicated that the cytotoxic function of the protein was directly responsible for bacterial killing (Fig. 5A).

Colicins Enter AIEC Containing Compartments Within Infected RAW264.7 Macrophages

To demonstrate colicin entry into RAW264.7 macrophages, we constructed a GFP-expressing LF82 strain (LF82-GFP) and also an RFP-tagged fluorescent colicin E9 (colicin E9-RFP) in which the DNase domain is replaced with RFP leading to a cytotoxically inactive protein. RAW264.7 macrophages grown on glass coverslips in 6-well plates were infected with LF82-GFP (green) as described above and treated with colicin E9-RFP (red)

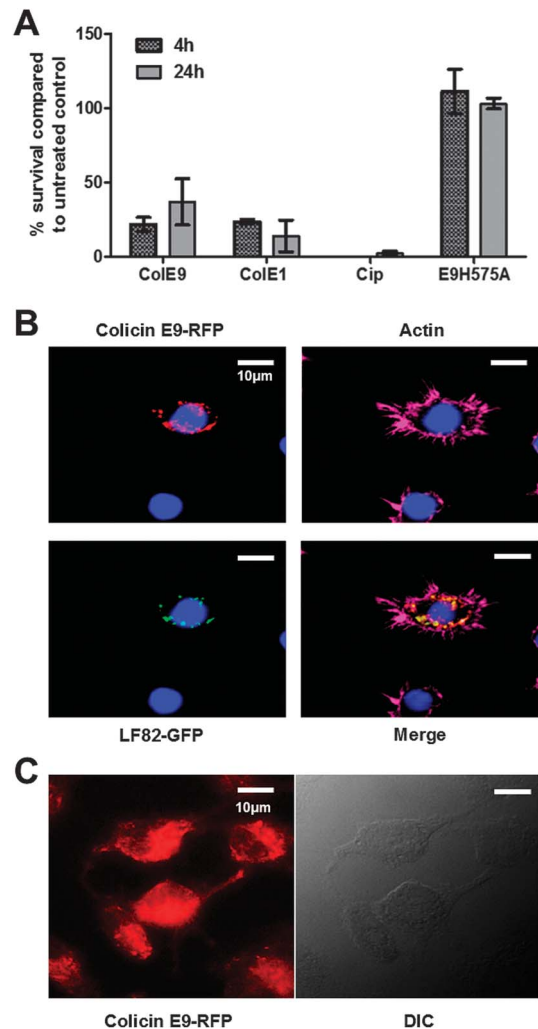


FIGURE 5. The cytotoxic function of colicin caused reduced persistence of intramacrophagic AIEC LF82 bacteria. A, Colicins show activity against LF82 residing within RAW264.7 macrophages. RAW264.7 macrophages were infected with LF82 (MOI of 50; 2 h) and treated with gentamicin (100 μ g/mL for 1 h) to kill extracellular bacteria. Antimicrobial treatments were performed for 24 hours at 100 μ g/mL for all tested colicins. At 4 and 24 hours, macrophages were lysed and viable intracellular bacteria determined by CFU counts. E9H575A is an active site-mutant protein of colicin E9. Data shown represents mean and SE of 3 independent experiments. One-way ANOVA followed by post hoc analysis with the Bonferroni correction showed significant differences between the inactive colicin H575A group and colicin E9-, colicin E1-, and ciprofloxacin-treated groups at 4 and 24 hours ($P < 0.05$). B, Colicin enters LF82-containing compartments in RAW264.7 macrophages. Figure shows confocal micrographs of RAW264.7 macrophages infected with LF82-GFP (green) and treated with colicin E9-RFP (red) for 24 hours. Actin is stained with phalloidin-Alexa 647 conjugate (purple), and nuclear DNA is stained with DAPI (blue). Scale bars shown are 10 μ m. A total number of 50–200 macrophages were assessed per experiment. Confocal micrographs represent 3 independent experiments. C, Colicin adheres to RAW264.7 macrophages. The figure shows the fluorescent micrograph of RAW264.7 macrophages treated with colicin E9-RFP for 4 hours. The second panel shows the DIC image. Scale bar shown is 10 μ m.

and incubated for 24 hours. To selectively visualize intracellular colicin protein, macrophages were treated with trypsin to inactivate extracellular and cell-associated colicin E9-RFP protein. Macrophage cells were extensively washed with PBS to remove macrophage-bound protein. Using confocal microscopy, we showed the presence of both colicin protein and LF82 bacteria within infected macrophages (Fig. 5B). Confocal micrographs showed that colicin E9-RFP was present within infected RAW264.7 macrophages, and in some cases, colocalized together with LF82-GFP within specific compartments (Fig. 5B). Although colocalization of colicin with bacteria was detected, we also detected colicin-RFP protein within macrophages that was not colocalized with bacteria (see Fig., Supplemental Digital Content 3, <http://links.lww.com/IBD/A973>). Interestingly, similar experiments in which colicin E9-RFP was added to RAW264.7 macrophages that were not subsequently treated with trypsin showed that the colicin adheres to the surface of the macrophage, which suggests that colicin could be nonspecifically taken up by endocytosis (Fig. 5C). To determine whether this is the case, macrophages were pretreated with inhibitors of multiple endocytic pathways, treated with colicin E9-RFP for 4 hours, were trypsin

treated, and washed as described. Confocal micrographs showed that pretreatment of macrophage cells with cytochalasin B, an inhibitor of actin polymerization, significantly reduced the uptake of colicin E9-RFP (Fig. 6). The processes of clathrin-mediated endocytosis, pinocytosis, mannose receptor-mediated endocytosis, and scavenger receptor-mediated endocytosis inhibited by dynasore, DMA, mannan, and polyinosinic acid, respectively, were not shown to influence colicin uptake. These data indicate that an actin-regulated endocytic pathway regulates colicin uptake into AIEC-infected macrophage compartments (Fig. 6).

Effect of Colicin Treatment on Macrophage TNF- α Production

In previous *in vitro* studies, AIEC-infected macrophages have been shown to produce increased amounts of proinflammatory cytokine TNF- α .²⁵ We therefore sought to investigate whether colicin killing of intramacrophagic AIEC would cause a decrease in the production of proinflammatory cytokines. To achieve this, RAW264.7 macrophages were infected with AIEC strain LF82 and treated with colicin E9. For this assay, colicin protein was further purified using endotoxin removal columns to

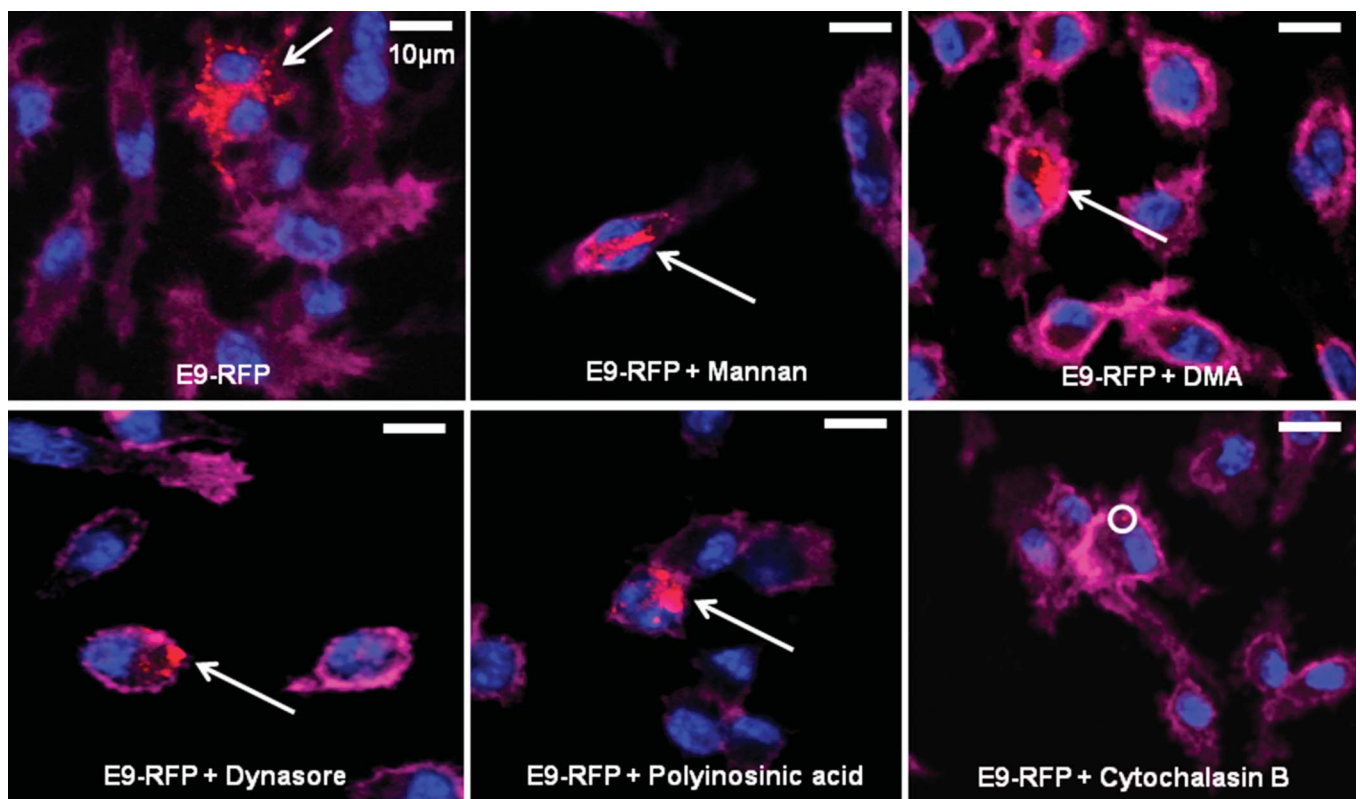


FIGURE 6. Pretreatment of RAW264.7 macrophages with chemical inhibitor of actin polymerization significantly reduced uptake of colicin E9-RFP. Confocal micrographs of RAW264.7 macrophages pretreated with inhibitors of endocytosis (30 min) and then treated with colicin E9-RFP (red) for 4 hours. Inhibitors used were mannan (mannose receptor-mediated endocytosis), dynasore (clathrin-mediated endocytosis), dimethylamiloride (pinocytosis), polyinosinic acid (scavenger receptor-mediated endocytosis), and cytochalasin B (actin polymerization). Actin was stained with phalloidin-Alexa 647 conjugate (purple), and nuclear DNA was stained with DAPI (blue). Arrows and circle highlight colicin E9-RFP inside macrophage cells. Scale bars shown are 10 μ m. Confocal micrographs represent 2 independent experiments.

ensure that LPS was extracted from protein fractions. LPS (1 $\mu\text{g/mL}$) was used as a control for cytokine release. For cytokine detection, macrophage cell supernatants were removed at 24, 30, and 40 hours, and enzyme-linked immunosorbent assays were performed for TNF- α . Although colicin E9 did not reduce the production of TNF- α from LF82-infected macrophages, increased production of TNF- α was not detected after colicin treatment (Fig. 7A). A similar result was obtained for ciprofloxacin-treated LF82-infected macrophages indicating that at least in this assay, AIEC killing is not associated with reduced levels of TNF- α . Colicin E9 treatment of uninfected macrophages was shown to produce similar levels of TNF- α as the untreated mock controls at all time points tested (Fig. 7A). LDH assays showed that colicin E9 treatment was not cytotoxic to macrophages. Untreated macrophages were shown to produce increased amounts of LDH compared with colicin-treated and LF82-infected cells at 24 hours (Fig. 7B).

DISCUSSION

A range of chronic immune-related diseases such as inflammatory bowel disease, diabetes, and rheumatoid arthritis are associated with intestinal dysbiosis, and in a subset of patients, associations with specific bacterial species have been determined. For CD, AIEC have been implicated in playing a role in disease pathogenesis; and for rheumatoid arthritis, a specific expansion in the population of *Prevotella copri* has been observed.⁴⁶⁻⁴⁸ For the most part, the use of broad-spectrum antibiotics for the treatment of chronic immune-related disorders, such as CD, generally show poor efficacy.⁴⁹ This can itself lead to the development of severe and life-threatening secondary infections resulting in extended hospital care and requirement for further treatments.^{50,51} Moreover, more frequent antibiotic use in early childhood is also linked to the development of chronic inflammatory diseases, most notably CD and asthma.^{52,53} This is believed to be due to the altered (T cell) immune responses caused by antibiotic-induced changes in the gut flora composition.^{54,55}

A potential alternative treatment for the correction of dysbiosis is the use of narrow-spectrum antibiotics such as the colicin-like bacteriocins, which demonstrate several highly attractive attributes. These include highly targeted killing on the species level and potent activity. In this work, colicins were shown to (1) effectively kill drug-resistant AIEC biofilms, (2) kill AIEC bacteria associated with intestinal epithelial cells, and (3) kill AIEC bacteria growing intracellularly within macrophages. For the latter, colicins entered AIEC-containing compartments within RAW264.7 macrophages by exploiting actin-mediated endocytosis.

The use of colicin-like bacteriocins for the treatment of biofilm-associated bacteria was previously investigated in a study by Smith et al.,⁵⁶ which showed that pyocins are able to effectively kill *P. aeruginosa* in the biofilm state. In this study, colicins were shown to effectively kill *E. coli* biofilms and showed superior killing compared with the commonly used antibiotics metronidazole and ciprofloxacin. Indeed, the ability

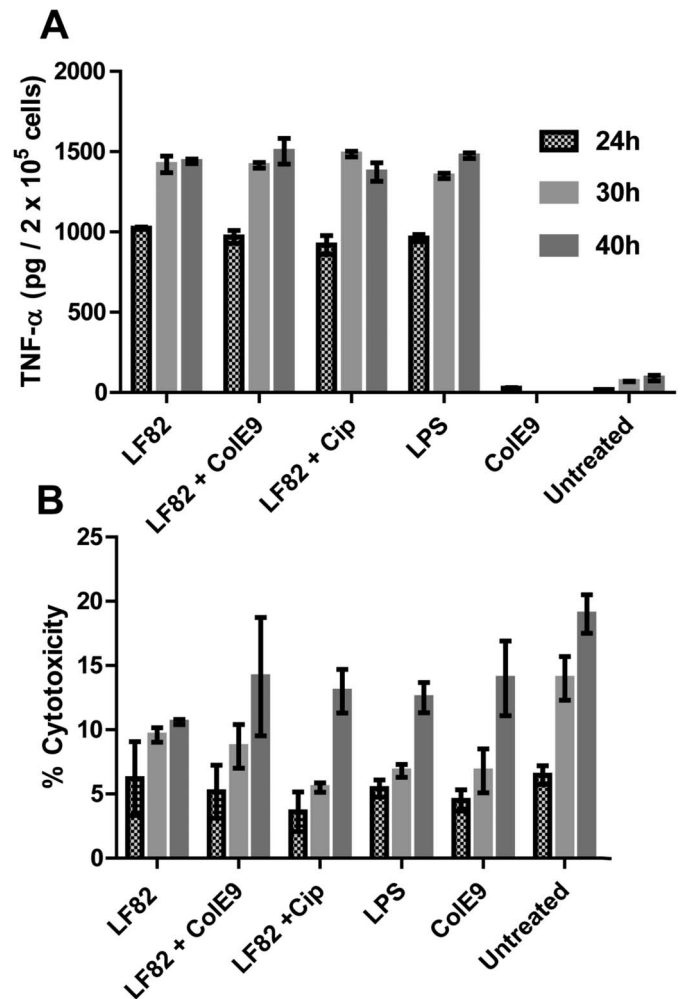


FIGURE 7. Colicin is noncytotoxic to macrophage cells and fails to increase the production of TNF- α . TNF- α (A) and LDH (B) production by RAW264.7 macrophages infected with LF82 (MOI of 50; 3 h) and treated with E9; ciprofloxacin (100 $\mu\text{g/mL}$) for 24, 30, and 40 hours after gentamicin treatment. Untreated represents mock infected control. LPS (1 $\mu\text{g/mL}$) stimulation of macrophages was the control for cytokine production. % Cytotoxicity was determined by measurement of LDH release relative to uninfected controls. Data are expressed as mean \pm SE of 2 independent experiments. A, ANOVA followed by post hoc analysis with the Bonferroni correction indicated differences ($P < 0.05$) for both untreated and ColE9-treated versus LF82-, LF82 + ColE9-, LF82 + Cip- and LPS-treated cells at 24, 30, and 48 hours and no difference ($P > 0.05$) between untreated and ColE9 treated cells. B, ANOVA followed by post hoc analysis with the Bonferroni correction indicated differences ($P < 0.05$) at 40 hours for untreated versus LF82, LF82 + ColE9, LF82 + Cip, LPS, and ColE9 but no other significant differences between groups on pairwise comparison.

of colicins to inhibit biofilms grown by a range of clinical AIEC isolates (HM95, 419, 615) highlights their potential as a therapeutic against diverse infections. For treatment of intestinal infections of this kind, we envisage that colicin delivery would take the form of a colicin-producing probiotic. Indeed, we

demonstrated the ability of a colicin-producing commensal *E. coli* to kill the majority of biofilm-associated cells in established AIEC biofilms. This result indicates that colicin-producing bacteria, which can be readily isolated from the healthy gut microbiome, may be effective in the treatment of drug-resistant biofilm-associated infections.^{57,58} To fully determine the potential of colicins as a targeted antimicrobial treatment, future work must investigate the in vivo efficacy of this antibiotic. Specific targeting of AIEC bacteria within the gastrointestinal tract by colicins or colicin-producing bacteria would be highly supportive of this. Indeed, recent mouse models showing AIEC persistence in the intestine may represent a suitable model for this work.^{27,59}

The use of narrow-spectrum therapies has been previously investigated for acute intestinal infections through use of species-specific vaccines for EHEC, UPEC, and recurrent *Clostridium difficile* infection.^{60,61} Furthermore, recent studies suggest the use of “personalized medicine,” which considers variations in individual gut profiles.⁶² The manipulation of the intestinal microbiota is being recognized as potential mechanism for the modulation of immune responses in the protection against allergic disease.⁶³ It also represents a highly desirable method to combat disorders associated with intestinal dysbiosis.⁶² Current therapies of this kind include fecal microbiota transplant, bacteriophage therapy, and probiotic or prebiotic therapies. For treatment of inflammatory bowel disease, however, the long-term efficacy and general applicability of such therapies remain to be proven.^{64,65}

Overall, the selectivity and potent activity of colicins suggest that they could be used to effectively eradicate a specific bacterial pathogen, such as AIEC, while preserving the bacterial population that constitutes the normal healthy microbiome. In light of current concerns over antibiotic resistance, they also represent a novel class of antimicrobials that have the potential to treat a wide range of resistant gram-negative infections. Furthermore, because of the availability of diverse colicins, which target a number of different uptake pathways, the use of “colicin cocktails” and combination therapies could be used to limit the emergence of resistance.

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