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The antimicrobial peptide cathelicidin-BF could be a potential therapeutic for *Salmonella typhimurium* infection

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ABSTRACT

Resistance is increasing to several critical antimicrobials used to treat *Salmonella typhimurium* infection, urging people to search for new antimicrobial agents. In this work, we reported the possibility of a potent antimicrobial peptide cathelicidin-BF found in the venom of the snake *Bungarus fasciatus* in treating *Salmonella typhimurium* infection. We tested its activity in biological fluids and *in vivo* using a mouse model of *Salmonella typhimurium* infection, and examined the effect of cathelicidin-BF on *Salmonella* invasion to epithelial cells. In addition, the biodistribution of cathelicidin-BF was evaluated by using *in vivo* optical imaging. The results revealed that cathelicidin-BF was unstable in gastrointestinal tract, but retained substantially active in murine serum. Cathelicidin-BF attenuated the clinical symptoms of *Salmonella* infected-mice, significantly reduced the number of internalized *Salmonella* and attenuated *Salmonella*-induced decreases in TER in epithelial cells. Our results provide a first indication for the potential of cathelicidin-BF as a novel therapeutic option for salmonellosis.

Introduction

Salmonella infection is a primary enteric pathogenic disease affecting both human and animals and therefore a major public health concern (Dougan et al. 2011; Maiti et al. 2014). Until recently the most common cause of food poisoning by *Salmonella* species was due to *Salmonella typhimurium* (*S. typhimurium*) (Gordon et al. 2008; Kupz et al. 2014). *S. typhimurium* infection manifest within 48 h after ingestion of contaminated food and include nausea, vomiting, mostly self-limiting, diarrhea. This pathogen is able to colonize the intestinal tract and modulate epithelial tight junction integrity enough to allow the physical movement of PMN across the intestinal monolayer, and penetrate the gut epithelium to ultimately gain access to systemic sites (Galan and Collmer 1999; Gruenheid and Finlay 2003; Kohler et al. 2007).

Antibiotics are critical to the treatment of invasive *Salmonella* infections. However, resistance to the older antimicrobials, chloramphenicol and ampicillin, termed multidrug resistant isolates has been present for many years (Harish and Menezes 2015). In this respect, it is therefore urgent to search for new antimicrobial agents.

Cathelicidins are a family of structurally diverse antimicrobial peptides (AMPs) that exert potent antibacterial activity and acting as multifunctional effector molecules of innate immunity (Bals and Wilson 2003; Hing et al. 2013). Cathelicidin-BF (C-BF) was the first cathelicidin family peptide found in reptiles and has been found to exerting potent antibacterial activity against gram-negative bacteria, especially to *Salmonella.* Minimal inhibitory concentration (MIC) of C-BF to *S. typhimurium* is 4 μg/mL, far more effective than the human cathelicidin peptide LL-37 (MIC = 128 μg/mL) (Liu et al. 2011). C-BF is clearly among the most potent cathelicidins discovered to date. In contrast to most AMPs, C-BF is not toxic to mammalian cells at concentrations well above the MIC against microbes (Wang et al. 2008). These features suggest that C-BF might be used *in vivo* to be effective against *Salmonella* infection. But until now, there is little research on whether C-BF is functional against *Salmonella in vivo*.

The aim of this study was to investigate the activity of C-BF in a more physiological context, such as in simulated gastrointestinal fluids and serum, and examined the effects of C-BF on modulating the *Salmonella* infection *in vivo* mouse models and *in vitro* epithelial cells.

Materials and methods

Preparation of peptides

C-BF (KFFRKLKKSVKKRAKEFFKKPRVIGVSIPF) and Fluorescein isothiocyanate-labeled C-BF (FITC-C-BF) was synthesized from GL Biochem (Shanghai, China). Both of them are purified by RP-HPLC

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and analyzed by HPLC and mass spectrometry to confirm their purity higher than 95%.

Preparation of biological fluids

Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared as described in the United States Pharmacopoeia (Anonymous 1995). SGF consists of 3.2 mg/mL pepsin (Sigma P7012) in 0.03 M NaCl at pH 1.2. SIF consists of 10 mg/mL of pancreatin (Sigma P7545) in 0.05 M KH_2PO_4, pH 7.5. They are prepared when needed. Mouse serum was prepared by centrifuging the coagulated healthy mouse blood at 3000 rpm for 15 min to get supernatant, and stored at −20 °C.

Peptide stability in biological fluids

To test the peptide stability in biological fluids, RP-HPLC analysis was performed with Water-600 series HPLC system using a Zorbax SB C18 column (flow = 1 mL/min, detection = 214 nm) as previously described with slightly modifications (Nguyen et al. 2010; Liu et al. 2013). Briefly, C-BF (45 μg) was incubated in 450 μL SGF or SIF or serum at 37 °C, At different time intervals (0, 5, 30, 60, 120, 240 and 360 min), 60 μL aliquots were mixed with 12 μL of 15% trichloroacetic acid (TCA) and incubated at 4 °C for at least 15 min and centrifuged at 13,000 rpm for 5 min to precipitate proteins, The supernatant was submitted to a determination of the relative concentrations of the remaining peptides by RP-HPLC.

Antibacterial activity in biological fluids

The bactericidal activity of C-BF against *Salmonella* was determined by a killing kinetics assay (Benincasa et al. 2010). *Salmonella enterica* serovar Typhimurium CMCC 50013 was purchased from China General Microbiological Culture Collection Center (Beijing, China). It was grown in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich, USA) to exponential growth phase, and diluted in SGF or SIF or serum to a final density of 2×10^5 colony-forming units (CFUs)/mL, and incubated with 10 μg/mL C-BF in a shaking water bath at 37 °C for 1, 2, 3, 4, 5 and 6 h. Samples were serially diluted and plated in duplicate on MH agar to allow colony counts. Data are the mean of three independent determinations with comparable results.

Animals

Male c57/BL6 mice of approximately 19 g and 6 weeks of age were used. Mice maintained under constant temperature (22 °C) and humidity with 12 h light/dark cycle, and received pelleted chow and tap water ad libitum. The study was approved by the Committee on the Ethics of Animal Experiments of Zhejiang University.

In vivo optical imaging

Nu/nu mice of 5 weeks age were used to observe the biodistribution of C-BF. 100 μL of 200 μg/mL FITC-C-BF or 50 μg/mL Free-FITC diluted in PBS were injected i.p. to nu/nu mice. At designated time points (30 min, 2 h, 4 h and 24 h), mice were anesthetized with thiopental, and then placed in the imaging system (MAESTRO, CRI, USA). Filter set "blue" was used for excitation. The tunable filter was automatically stepped in 10-nm increments from 490 to 550 nm. Fluorescence signals were collected and unmixed using commercial software (Maestro software, CRi). After photon collection, a pseudocolor representation of light intensity (red, most intense; blue, least intense) was superimposed over the grayscale body surface.

Mouse model of Salmonella-mediated disease

Mice were randomly divided into the following groups ($n = 10$ per group): control group; *Salmonella* group; C-BF + *Salmonella* group; C-BF group, respectively. All groups were treated with streptomycin sulfate (5 g/L) in drinking water from day-2 to day-1. On day 0, water and food were withdrawn for 4 h before the mice were infected with 10^8 CFU of spontaneous streptomycin-resistant derivatives of *Salmonella enterica* serovar Typhimurium CMCC 50013 (Calderon Toledo et al. 2008) intragastrically or treated with sterile PBS (control and C-BF groups). C-BF + *Salmonella* group and C-BF group were injected with C-BF (2 mg/kg in saline) intraperitoneally from day 0 (30 min after the inoculation) to day 4, the other two groups were injected with saline.

The clinical status of mice was assessed by visual examination for motility, ruffled fur, ataxia and tremor (Ozkaya et al. 2012). Weight was monitored daily and feces were collected from day 0 to day 4, CFU/g shedding were determined by plating series dilutions on Bismuth Sulfite Agar plates (Hopebio, China). At day 4, mice were sacrificed, spleens and livers were removed aseptically from five mice of *Salmonella* group and C-BF + *Salmonella* group, homogenized in 4 °C cold PBS. The numbers of CFU were determined by plating series dilutions on MH Agar plates.

Distal ileum from at least four mice were excised and cut into approximately 3 mm-long pieces, immediately fixed in neutral-buffered formalin for histological evaluation by hematoxylin and eosin staining (H&E staining). Proximate colon sections were gently cut and immediately fixed with 2.5% glutaraldehyde (Polyscience, USA) in 0.1 M cacodylate buffer (pH 7.2–7.4, Agar scientific, UK) for 24 h at 4 °C in dark place. The samples were observed by a transmission electron microscopy (JEOL, Japan) after a series of procedures (Veldhuizen et al. 2009).

Salmonella invasion assay

The gentamicin-protection assay (Darling et al. 2004) was used to assess *Salmonella* invasion. The intestinal porcine epithelial cell line J2 (IPEC-J2), maintained in culture as described previously (Schmidt et al. 2008), were cultured in 12-well cell culture plates (Costar, Corning, NY) and grown to confluency. Before infection, cells were incubated with medium alone or with medium containing C-BF (2 or 10 μg/mL) for 1 h. *S. typhimurium* were grown to mid-logarithmic phase, washed, resuspended in medium, and added to the cells. To evaluate whether the bacterial-killing ability of C-BF interfered with *Salmonella* invasion, C-BF treatment was conducted in two settings. In the first, cells were treated with C-BF for 1 h, and C-BF was washed out before the addition of *Salmonella*. In the second, *Salmonella* were added without washing out the C-BF. After incubation for 1 h, cells were washed three times with PBS to removed non-adherent bacteria. Then a gentamicin solution in medium (200 mg/mL) was added and incubated for 60 min to kill extracellular bacteria. The antibiotic was removed by washing three times with PBS, and the cells were subjected to a Triton X-100 (0.5%; 200 μL/well) solution, followed by a 5-min incubation and subsequent addition of 800 μL PBS. 10 μL aliquots of serial 10-fold dilutions of cell lysates were plated on Bismuth Sulfite Agar to quantify bacteria. The number of CFU was determined after overnight incubation at 37 °C.

Measurement of TER

IPEC-J2 cells were seeded onto transwell filter supports (10^6 cells/well, surface area 1.12 cm^2; Costar Inc., New York, NY) and grown 21 days until cells showed features of polarization as judged by a significant increase in trans-epithelial electrical resistance (TER) monitored by a voltmeter and chopstick electrodes

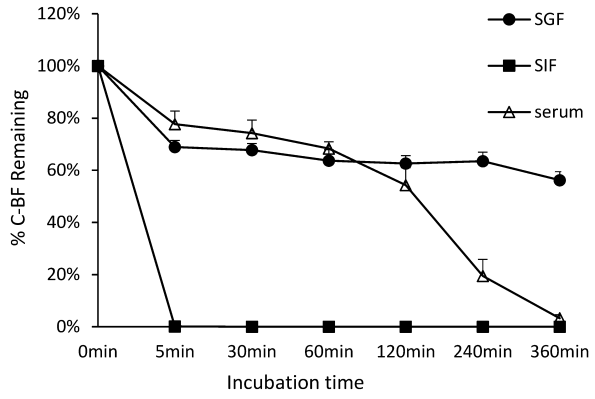

Fig. 1. Degradation of C-BF in biological fluids. The peptides were incubated with the SGF or SIF or serum for different time periods and the remaining peptide amounts were determined by RP-HPLC.

(Millicell-ERS; Millipore, Bedford, MA)(Schierack et al. 2006). The cells were randomly divided into four groups for the measurement of TER. The groups consisted of the following: the control group (treated with sterile saline), the *Salmonella* group (treated with 2×10^6 CFU/mL *Salmonella* for 3 h), and the C-BF + *Salmonella* group (treated with 2 μg/mL C-BF for 1 h, washed out before exposure to *Salmonella*), and the C-BF group (treated with 2 μg/mL C-BF for 1 h). TER was recorded at 30, 60, 90, 120, 150 and 180 min after treatment.

Statistical analysis

Values are given as mean ± SEM. All results were analyzed by Mann–Whitney Rank Sum Test or one-way ANOVA followed by Duncan's multiple range test using SPSS software. A *P*-value of <0.05 was considered as significant.

Results

Stability and antibacterial activity of C-BF in biological fluids

Previous results showed that C-BF has a strong activity against Gram-negative bacteria in LB (Luria-Bertani) broth, and even stronger in 150 mM phosphate buffer solution or NaCl solution (Wang et al. 2008). But whether it remained integrated and active *in vivo* is still unknown. To evaluate the therapeutic potential of C-BF, We first assayed its stability in the presence of simulated gastrointestinal fluids and serum (Fig. 1). RP-HPLC analysis indicated that intact C-BF reduced slowly in SGF, while it degraded quickly within 5 min in SIF. The amount of intact C-BF decreased with a half-life of approximately 1 h, and disappeared after 4 h-incubation in murine serum. Consistent with this, when incubated with SGF or serum, C-BF (10 μg/mL) could still killed *Salmonella* but slower than C-BF alone (Fig. 2A and C), indicating that C-BF could partially preserve its antimicrobial activity in SGF or serum. But SIF completely abolished the antimicrobial activity of C-BF against *Salmonella* (Fig. 2B). These results suggest that C-BF is unstable in gastrointestinal tract, but remains substantially active in serum.

In vivo biodistribution of C-BF

To investigate whether C-BF can be absorbed into the circulation system after injected into peritoneal cavity, we detected it by using fluorescently labeled C-BF (FITC-C-BF). FITC-C-BF exhibiting an antimicrobial activity comparable to that of the unlabeled C-BF. As shown in Fig. 3, the fluorescence intensity signal could be observed in the whole body within 30 min, and then slowly moved to the

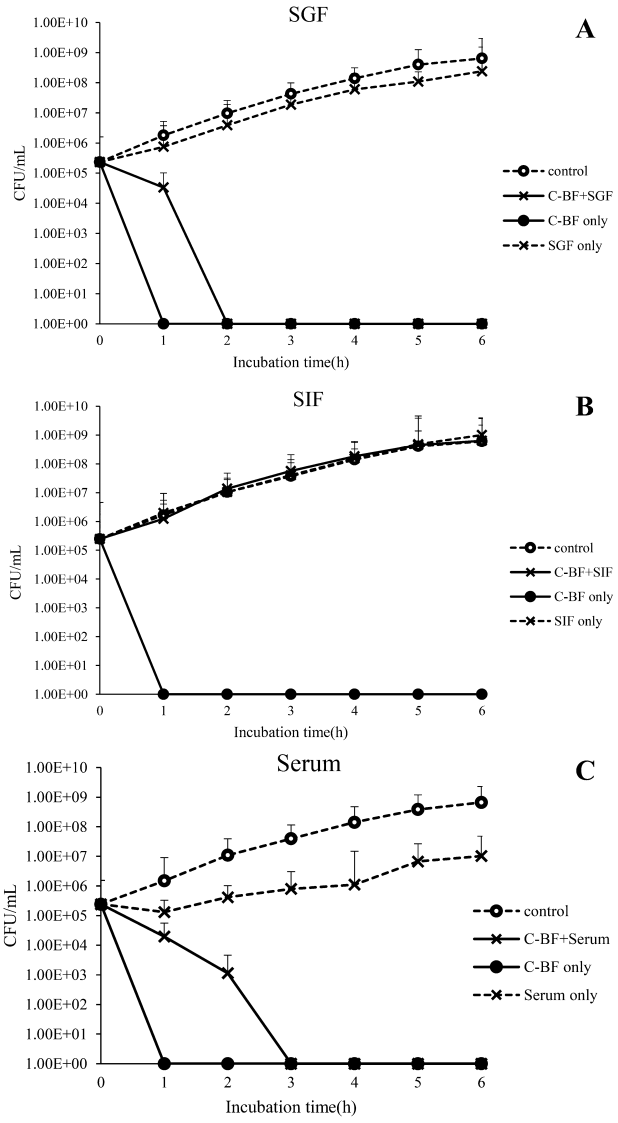

Fig. 2. Antimicrobial activity of the peptides against *Salmonella* in the presence of SGF (A), SIF (B) and serum (C) assessed using the killing kinetics assay.

abdomen (kidney and the bladder). The *in vivo* analyses performed after 24 h confirm that the FITC-C-BF has been totally excreted, while considerable amount of Free-FITC accumulated in mice.

C-BF attenuated the symptoms of Salmonella infection

To assess the potential role of C-BF in treating bacterial infection, we infected C57/BL6 mice with *Salmonella* by oral gavage to induce *salmonellosis*. Two mice of *Salmonella* group were died at day 3 and day 4, and most of the mice in *Salmonella* group lose vigor and vitality and started losing weight on day 1 post-inoculation. Mice of C-BF + *Salmonella* group were less ruffled and more active than the *Salmonella* group. *Salmonella*-infected mice had a sustained body weight loss in comparison with control group. C-BF-treated mice slightly lost weight one day after inoculation, but recovered to gain weight thereafter (Fig. 4).

C-BF reduced the bacterial loads in the spleen and liver

Infection with *Salmonella* may result in bacterial translocation across intestinal barrier. This can be followed by bacterial migration to the spleen and liver, herein we examined the effect of the C-BF

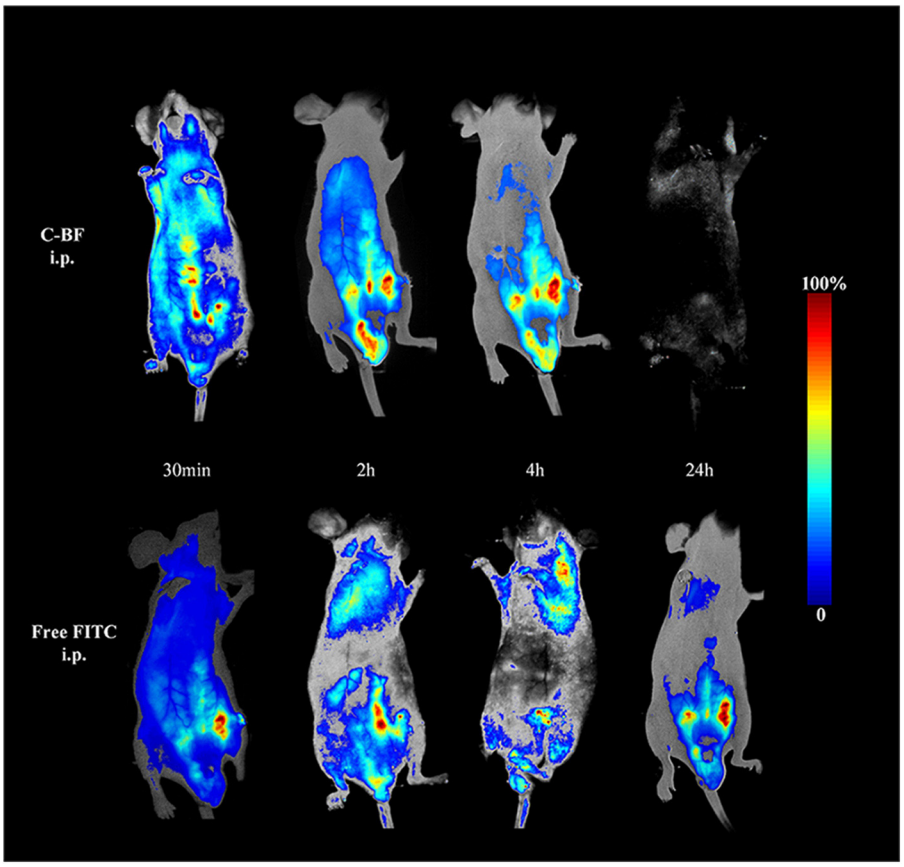

Fig. 3. *In vivo* spectral fluorescence imaging in nu/nu mice. The animal was placed in lying position.

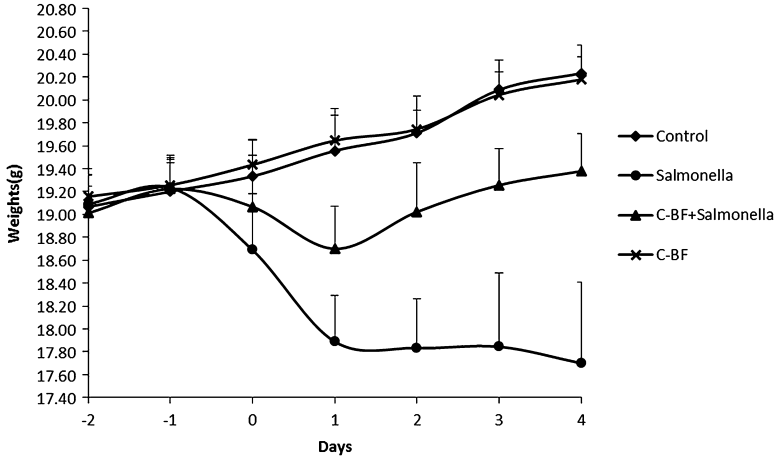

Fig. 4. Body weight of mice in each group ($n = 10$). Data show means and SEM.

treatment on the colonization of spleen and liver by *Salmonella*. Bacteria translocation to both spleen and liver was significantly reduced ($P < 0.05$) by C-BF + *Salmonella* group compared with the *Salmonella* group (Fig. 5). The amount of *Salmonella* shed in the feces was also monitored daily. But there was no significant difference between the *Salmonella* group and C-BF + *Salmonella* group (Fig. 6).

C-BF ameliorates Salmonella-induced alterations to the architecture of intestine

To analyze *S. typhimurium*-induced intestinal pathology, we observed the alterations of gut structure by H&E staining and TEM. As shown by H&E staining (Fig. 7), mice inoculated with *S. typhimurium* developed intestinal epithelial barrier dysfunction with altered mucosal architecture, a significant reduction in villus height were observed in the *Salmonella* group. However, C-BF treatment significantly decreased the *Salmonella*-induced mucosal damage. Electron micrograph images showed that the control group and C-BF group displayed normal microvillus architecture, C-BF administration attenuated the obvious pathological changes in the intestinal mucosa caused by *Salmonella* (Fig. 8). These pathological changes included localized effacement of the microvilli and disrupted TJs due to swollen or shrunken epithelial cells.

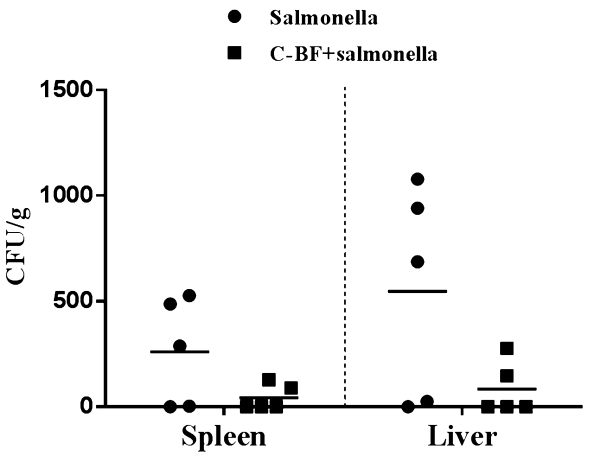

Fig. 5. Bacterial counts evaluated 4 days post-infection in the liver and spleen. The bars indicate the median bacterial load.

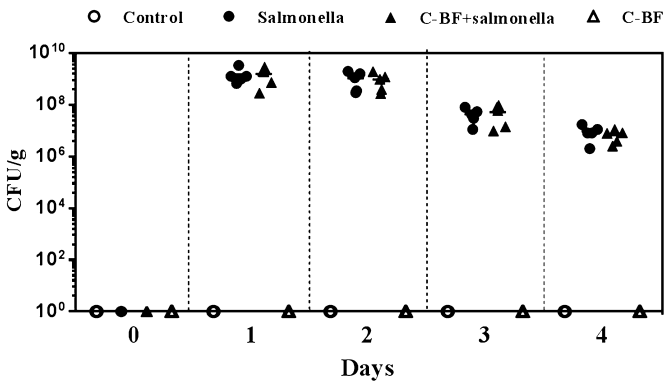

Fig. 6. Fecal shedding of *S. typhimurium* in mice after inoculation.

C-BF prevented Salmonella invasion to epithelial cells

Following the results of mice infection that C-BF ameliorates the architecture of intestine and reduced the bacteria load in liver and spleen, we speculated that C-BF can somehow enhance the gut barrier function to prevent bacteria invasion, so we next examined the effect of C-BF in preventing *Salmonella* invasion using an epithelial cell line IPEC-J2. Because reduced *Salmonella invasion* in the presence of C-BF might be due to antibacterial effects of C-BF, We performed the experiment in two settings in which cells were treated with C-BF for 1 h and then washed out before bacteria addition or kept in the medium during the infection period. The data in Fig. 9 showed that both washed out or consistent C-BF, at concentrations of 2 μg/mL, could significantly reduce the number of internalized *Salmonella*. When concentrations of C-BF up to 10 μg/mL, constant C-BF had strong killing activity against *Salmonella*, while washed out C-BF exhibited equivalent effect as concentrations of 2 μg/mL.

C-BF attenuated Salmonella-induced decreases in TER in epithelial cells

IPEC-J2 cells were used to assess barrier function in response to *Salmonella* infection in the absence or presence of C-BF. As shown in Fig. 10, administered with C-BF alone did not alter the TER of polarized IPEC-J2 cells. *Salmonella* infection resulted in a rapid decrease in TER in the IPEC-J2 cell monolayer 90 min post-infection, but

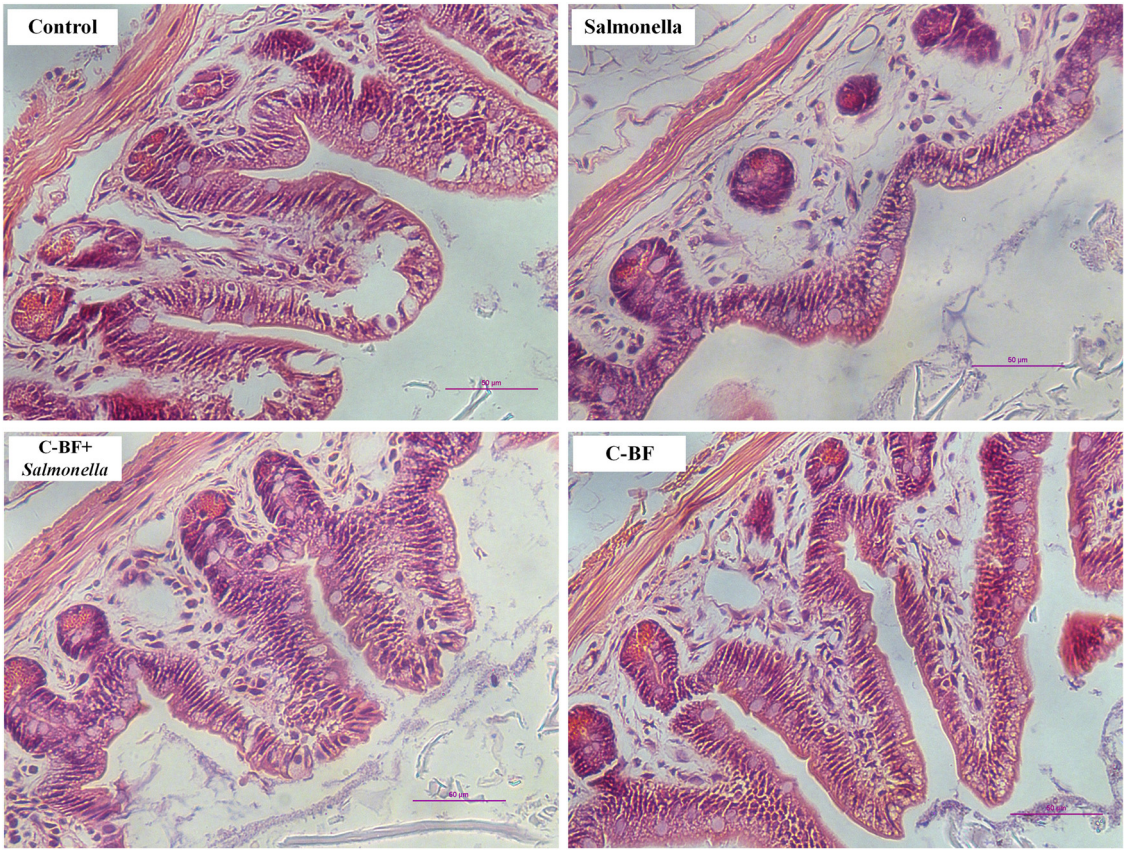

Fig. 7. Hematoxylin and eosin (H&E) staining of distal ileum tissues.

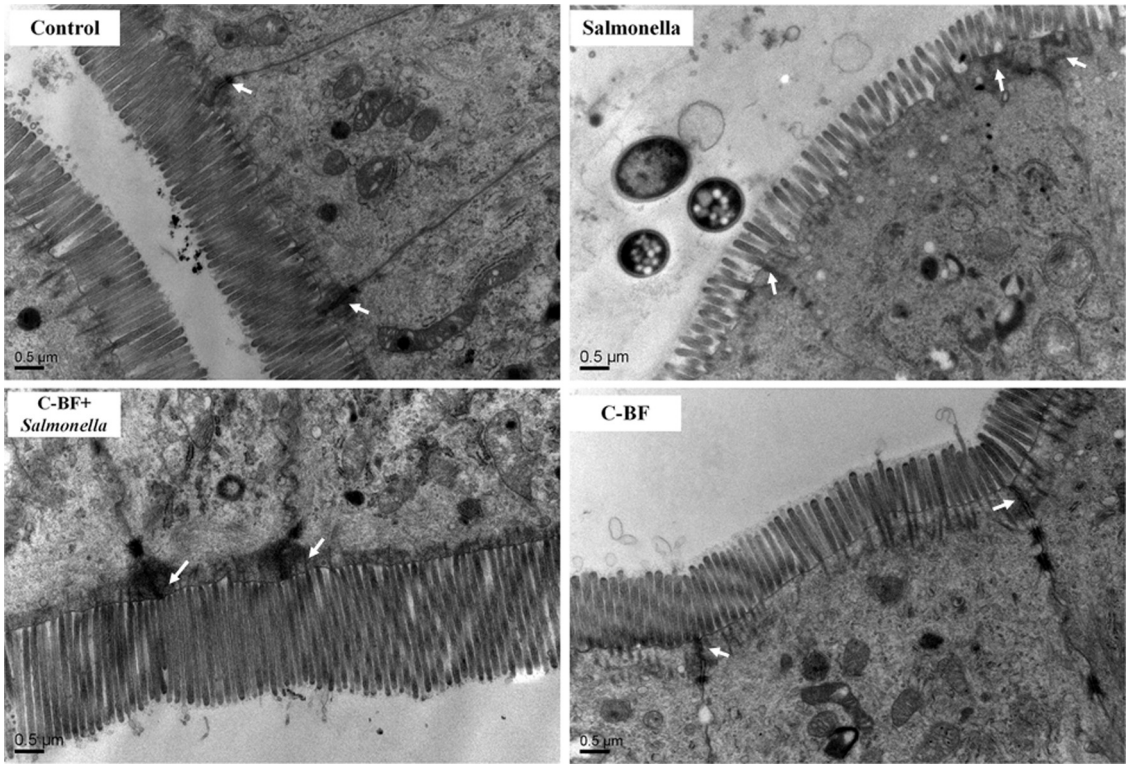

Fig. 8. Electron micrograph images of the colonic tissues. Arrows point to the tight junctions of intestine epithelium.

TER recovered in cells treated with C-BF which had much slower decreases.

Discussion

A major limitation of antimicrobial peptides is their low stability toward proteases, this severely limits the practical application of many peptides as drugs or feed additives (Andres and Dimarcq 2004; Marr et al. 2006). Our study reveals that antimicrobial peptide C-BF exerts antibacterial activity in murine serum, but not stable in simulated intestinal fluids. The small intestine is the most degradative environment for the widest range of peptides and proteins, it contains luminal secreted proteases and membrane-bound peptidases. It has been reported that small peptides (less than 6-aa) have high stability in gastrointestinal fluids. This may be due to their lack of specific cleavage motifs (Smart et al. 2014). C-BF is a 30-aa peptide with amphipathic α-helical conformation (Wang et al. 2008), there is plenty of hydrolysis site for peptidase. The reason why C-BF remains active in serum needs to be further explored. These results implicate C-BF is better administrated by intraperitoneal injection other than gavage. Next we observed the biodistribution of FITC-C-BF using *in vivo* imaging system. FITC-C-BF's MIC against *Salmonella* is equivalent to C-BF, and remains active in serum within 4 h. Results show that FITC-C-BF can be absorbed into circulation system by i.p. within 30 min, and has been totally excreted after 24 h, while considerable amount of Free-FITC accumulated in body. So we speculate that C-BF can function in the circulation system and does not accumulate in body one day after injection. This provides encouraging evidence for a future of C-BF drug.

Salmonella infection continues to be a major world-wide health problem. *Salmonella* can survive exposure to the low pH of the stomach and arrive in the intestine where it can penetrate the epithelial layer (Hughes and Galan 2002). But mice were generally resistant to *Salmonella* pathogenesis. Normally, just 5% of all

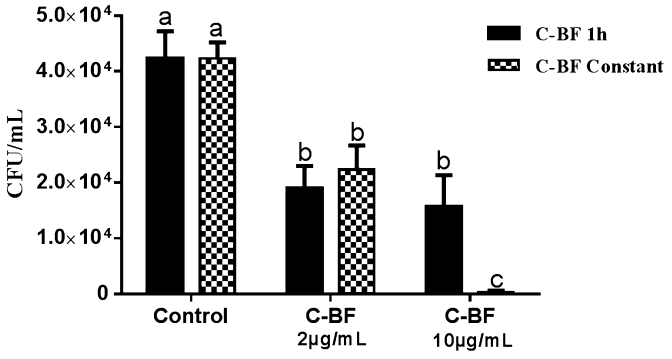

Fig. 9. Invasion of *S. typhimurium* in IPEC-J2 Cells.

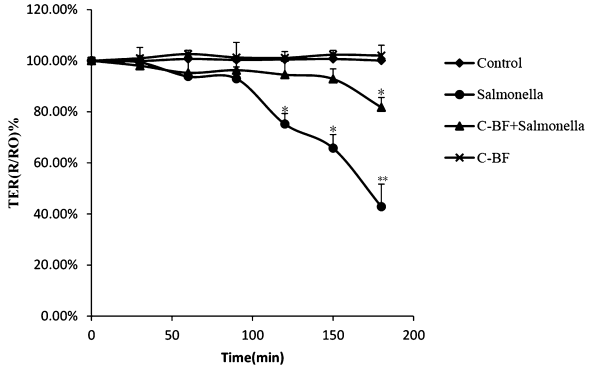

Fig. 10. TER measurements of IPEC-J2 cell monolayer infected with *Salmonella*. Reported as a percentage of the initial TER value and expressed as means and SEM of three different experiments, each performed in duplicate. * $P < 0.05$, ** $P < 0.01$ for a comparison to control group.

mice orally infected with *S. typhimurium* and allowed pathogen growth in the gut lumen and developed mucosal inflammation in colon and cecum (Stecher et al. 2010). So we use the streptomycin mouse model for *Salmonella*, in this model, infection with *S. typhimurium* leads to massive pathogen growth in large intestine and pronounced acute mucosal inflammation within 6–8 h (Barthel et al. 2003; Suar et al. 2006; Kaiser et al. 2012). The current study has shown that C-BF attenuated the symptoms of *Salmonella* infection and reduced the bacterial loads in the spleen and liver, this may due to the antibacterial activity of C-BF against *Salmonella.* At the same time, C-BF also ameliorates *Salmonella*-induced detrimental alterations to the architecture of intestine, indicate C-BF may have multiple roles *in vivo*.

The results of *in vitro* epithelial cells model reveal that C-BF can reduce *Salmonella* invasion and attenuate *Salmonella*-induced decreases in TER in epithelial cells even without the contact with the *Salmonella*. Therefore, we speculated that C-BF can protect host from *Salmonella* infection by affecting certain functions of epithelial cells. Our study contradicts a prior finding that infection with *S. typhimurium* did not influence TER (McCormick et al. 1993). There are key differences between our report and this earlier work that could account for such differences. First, the cell lines and *Salmonella* species used in these two studies are different. Second, they only measure the TER within 120 min, whereas we record it up to 180 min, and the TER of *Salmonella*-infected cells begins decreasing markedly in 120 min.

Conclusion

Our results indicating that C-BF has potentials to play a role in modulating *Salmonella* infection, as well as ameliorating intestinal barrier functions, suggesting that C-BF is an excellent therapeutic candidate for *S. typhimurium* infection in human and farm animals.

Acknowledgments

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