Antibacterial and cell penetrating-effects of LFcin17-30, LFampin265-284 and LFchimera on enteroaggregative *Escherichia coli*

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Abstract

Lactoferrin (LF) is a protein with antimicrobial activity, which is conferred in part by two regions contained in its N-terminal lobe. These regions have been used to develop the following synthetic peptides: Lactoferricin17-30, Lactoferrampin265-284 and LFchimera (a fusion of Lactoferricin17-30 and Lactoferrampin265-284). We have reported that these LFpeptides have antibacterial activity against several pathogenic bacteria; however, the exact mechanism of action has not been established. Here, we report the effects of LFpeptides on the viability of enteroaggregative *Escherichia coli* (EAEC) and the ability of these peptides to penetrate into the bacteria cytoplasm. Materials and Methods: The viability of EAEC treated with LFpeptides was determined via enumeration of colony-forming units, and the binding and internalization of the LFpeptides was followed via immunogold labeling and electron microscopy. Results: Treatment of EAEC with 20 and 40 μM LFpeptides reduced bacterial growth compared to untreated bacteria. Initially the peptides associated with the plasma membrane, but after 5 to 30 min of incubation, the peptides were found in the cytoplasm. Remarkably, bacteria treated with LFchimera developed cytosolic electron-dense structures that contained the antimicrobial peptide. Our results suggest that the antibacterial mechanism of LFpeptides on EAEC involves their interaction with and penetration into the bacteria.

Keywords: Lactoferrin; LFpeptides; LFchimera; EAEC; antibacterial

Introduction

Escherichia coli is a species of the genus *Escherichia* that contains mostly motile gram-negative bacilli that fall within the family Enterobacteriaceae (Nataro and Kaper 1998). The organism typically colonizes the infant gastrointestinal tract within the first hours of life, and thereafter, *E. coli* and the host derive mutual benefit for decades (Kaper 2005; Kaper et al. 2004). Among the *E. coli* that cause intestinal diseases, there are six well-described categories: enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC) and enterotoxigenic *E. coli* (ETEC) (Kaper 2005; Kaper et al. 2004).

EAEC is recognized as an emerging cause of diarrhea and has been implicated in acute and chronic diarrhea among travelers, children, adults, and HIV-infected persons in both industrialized and developing countries (Ahmed et al. 2014; Hebbelstrup Jensen et al. 2014; Weintraub 2007). To cause diarrheal disease, EAEC adheres to the intestinal mucosa, forms a mucoid biofilm and induces toxic effects on the intestinal mucosa that result in diarrhea. The exact pathogenic mechanism is not fully understood; however, adhesins, toxins and several other factors have been implicated (Flores and Okhuysen 2009). The clinical presentation of EAEC infection is characterized by watery diarrhea, usually unaccompanied by blood or mucus. Travelers to endemic countries, such as Jamaica, India, Mexico, etc., are at risk for EAEC infection (Hebbelstrup Jensen et al. 2014; Weintraub 2007). Many EAEC infections are self-limited, but symptomatic infections are usually treated empirically because laboratory diagnosis is not routinely available (Adachi et al. 2001). EAEC susceptibility varies by region. In most regions, EAEC strains are susceptible to fluoroquinolones, azithromycin, rifaximin, amoxicillin/clavulanic acid, and nalidixic acid; however, there have been reports of EAEC resistance to ampicillin, tetracycline,

trimethoprim, sulfamethoxazole, and chloramphenicol (Canizalez-Roman et al. 2013; Kaur et al. 2010; Kong et al. 2015). In addition to EAEC resistant strains, host susceptibility factors to EAEC diarrheal infection has been reported. There are factors that are important in determining susceptibility to EAEC infection, for example IL-8 and LF. IL-8 is a proinflammatory chemokine that functions as a neutrophil chemoattractant, and LF is an important element of the immune system. A single-nucleotide polymorphisms (SNPs) in the IL-8 gene are associated with EAEC-related symptoms (Jiang et al. 2003). Furthermore, a single nucleotide polymorphism with a T/C substitution in exon 15 (LTFEx15 codon 632 [T/C]) of the lactoferrin gene was associated with susceptibility to inflammatory diarrhea caused by EAEC in North Americans who were traveling in Mexico (Adachi et al. 2001; Mohamed et al. 2007). The spread of antibiotic-resistant pathogens, such as EAEC, requires new treatments. As the rate of development of new antibiotics has severely declined, alternatives to antibiotics must be considered in both animal agriculture and human medicine (Gomez de Leon et al. 2014). In this regard, the use of human or bovine lactoferrin (hLF and bLF), as well as their natural or synthetic peptides, must be considered an option for the treatment of pathogens due their multiple functions and health benefits (Brock 2012).

We have previously reported that bLF and its synthetic derived peptides, LFcin17-30, LFampin265-284 and LFchimera (a fusion of LFcin17-30 and LFampin265-284 with a lysine), have antimicrobial activity against *Vibrio parahaemolyticus, Staphylococcus aureus, enterohemorrhagic Escherichia coli, Streptococcus pneumoniae, Entamoeba histolytica* and *Giardia intestinalis* (Flores-Villasenor et al. 2012a; Flores-Villasenor et al. 2012b; Leon-Sicairos et al. 2014; Leon-Sicairos et al. 2009). Their mechanism of action involves causing damage at the ultrastructural level and disrupting bacterial membranes,

resulting in microbial death. In bacteria, we have found that in addition to interacting with the membrane and entering the cytoplasm, LF derived peptides may possibly interact with internal organelles. In this work, we evaluated the ability of bLF and peptides to penetrate and kill EAEC.

Materials and Methods

Peptides, chemotherapeutic agents and antibodies

The synthetic peptides LFcin17-30, LFampin265-284 and LFchimera were obtained by solid phase peptide synthesis using Fmoc chemistry, as described previously (Bolscher et al. 2012; Bolscher et al. 2009). Bovine LF (bLF, 20% iron saturated, was kindly donated by Morinaga Milk Industries Co (Tokyo, Japan). The purity of bLF (>98%) was checked by SDS-PAGE stained with silver nitrate. bLF concentration was assessed by UV spectroscopy on the basis of an extinction coefficient of 15.1 (280 nm, 1% solution). The bLF iron saturation was about 20% as detected by optical spectroscopy at 468 nm on the basis of an extinction coefficient of 0.54 (100% iron saturation). LPS contamination of bLf, estimated by Limulus Amebocyte assay (LAL Pyrochrome kit, ThermoFicherScientific, Waltham, MA, USA), was equal to 0.7±0.06 ng/mg of bLF. Cecropin (C7927), magainin II (M7402), rabbit-anti-LF (3262), ciprofloxacin (17850) and amikacin (1019508) were purchased from Sigma Chemical Co. (ST., MO, USA). The goat-anti rabbit gold-coupled antibodies were purchased to BB International (Cardiff WA, UK). Antibiotics Stock solutions were prepared in phosphate-buffered saline.7.4.

Strains and cultures

Enteroaggregative *Escherichia coli* clinical and reference strains were maintained in MacConkey agar (Difco, NJ, USA.) at 37 °C for 24 h, as previously reported (Canizalez-Roman et al. 2013). For the experiments, EAEC strains were grown in LB broth at 37 ºC for 18 h with shaking (200 rpm). Then, the bacteria were transferred to LB broth without iron for 2 h at 37 ºC and washed twice in iron-free PBS (bacteria were pelleted by centrifugation at 4,500 x g for 5 min). Iron was removed from the media and PBS as previously was reported (Leon-Sicairos et al. 2009).

153 **Antibacterial assay**

154 To test the bactericidal activity of the synthetic LFpeptides and bovine LF, approximately
155 $1x10^7$ CFU/ml of each strain was incubated in 96-well microplates (Corning NY, USA)
156 containing LB broth with 40 μM LFcin17-30, 40 μM LFampin265-284, 40 μM LFchimera,
157 or 20 μM bLF. The effects of 50 μg/ml cecropin 1 or 50 μM magainin II were also tested.
158 Untreated bacteria in LB medium were used as a control of growth (viability), and bacteria
159 treated with 100 μg/ml amikacin or ciprofloxacin were used as a positive control of growth
160 inhibition. MICs and CFU/ml were determined using the broth microdilution method
161 according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) or by
162 using the agar dilution method on Mueller-Hinton agar supplemented with 25 μg/ml
163 glucose-6-phosphate. Bacterial viability was expressed as a percentage relative to the
164 untreated control cells, and the mean and standard deviation are indicated. Statistical
165 analysis was conducted using the two-tailed Student's t test using SigmaPlot software
166 (version 11.0; Systat Software Inc., USA). A P value < 0.05 was considered statistically
167 significant.

168

169 **Electron microscopy**

170 Bacterial cells ($1x10^8$ UFC/ml) were treated with 20 μM LFcin17-30, LFampin265-284 or
171 LFchimera for 1 h at 37 ºC. Processing was done according to Gomez de León et al (2014).
172 For identification of the ultrastructural changes in the bacteria produced by exposure to the
173 peptides, exposed bacteria were washed with PBS and then fixed with 2.5% glutaraldehyde
174 for 60 min at room temperature (RT): Bacterial were washed three times with PBS (bacteria
175 were pelleted by centrifugation at 1000 × g) and then post-fixed with 1% osmium tetroxide
176 for 30 min at RT followed by 30 min at 4 °C. The pellet was washed three times with PBS

177 and then gradually dehydrated by incubation of the samples in increasing concentrations of
178 ethanol (from 50 to 100%) for 20 min at each step at RT with constant shaking. Bacterial
179 pellets were gradually embedded at RT in Spurr´s resin (EMS, Electron Microscopy
180 Sciences, Washington, DC): Spurr´s resin mixed with LR White Embedding Medium at a
181 ratio of 1:1, 3:1, and lastly in 100% resin. Pellets were transferred to molds and
182 polymerized at 60ºC for 48 h. Thin sections were obtained with an Ultracut R
183 ultramicrotome (Leica EM UC7, Mannheim, Germany), collected on copper grids, and
184 stained with uranyl acetate and lead citrate. Grids were observed and micrographed with a
185 transmission electron microscope (TEM) JEOL 1400X, (JEOL LTD, Japan) (Gomez de
186 Leon et al. 2014).

187 For immunolocalization of the peptides in the bacteria by IEM, pellets were washed
188 with PBS and fixed in a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde in PBS
189 for 1 h at RT. Bacteria were postfixed with 0.01% OsO_4 for 5 min at 4ºC. Samples were
190 gradually dehydrated in ethanol and embedded in LR White resin (London Resin,
191 Polysciences, Inc. USA). Samples were polymerized overnight under ultraviolet light at
192 4ºC in gelatin capsules. Thin sections were obtained with an Ultracut R ultramicrotome and
193 mounted on formvar-covered nickel grids. Immunolabeling was carried out at RT: to
194 minimize nonspecific labeling, grids were incubated with PBS containing 1% skim milk
195 and 0.05% Tween-20 (PBS-MT) for 30 min; sections were then incubated with anti-LF
196 polyclonal antibodies at a dilution 1:50 in PBS-MT for 1 h at RT or overnight at 4ºC. Grids
197 were thoroughly washed with PBST (PBS 1 0.05% Tween-20) and then incubated for 2 h at
198 RT with a goat anti-rabbit polyclonal antibody coupled to 20 nm gold particles (Cardiff
199 WA, UK) (diluted 1:40 in PBS-T). Incubation with each antibody solution was performed
200 in a humidified chamber with intervening washes. After thorough washings in PBS and

distilled water, sections were contrasted with 2% uranyl acetate and then examined by TEM. As negative controls, sections were incubated with the pre-immune rabbit serum diluted in PBS-MT and then with the secondary antibody coupled to gold particles.

Results and Discussion

Lactoferricin17-30, lactoferrampin265-284 and LFchimera have antibacterial activity against enteroaggregative *Escherichia coli*

The capacity of LFcin17-30, LFampin265-284 and LFchimera to inhibit the growth of EAEC strains was tested in LB medium. EAEC showed different susceptibilities to the LFpeptides (Fig. 1). EAEC cells were most susceptible to the antibacterial effect of LFchimera: at 2 h of exposure, 0 CFU/ml were detectable (Figure 1 panel C, arrowhead). LFcin17-30 and LFampin265-284 had relatively little antibacterial effect on EAEC after 2 h of exposure, with both peptides demonstrating approximately 20% reduction in CFU/ml (Figure 1, Panel A and B; arrowheads) compared to untreated EAEC. The chemotherapeutic drug ciprofloxacin and the peptide cecropin, which were used as controls of growth inhibition, had antibacterial activity as expected. In addition, native bLF at 20 μM had significant antibacterial activity (data not shown). The inhibition of bacterial growth by LFchimera was similar to that of ciprofloxacin (Fig. 1, panel C). The peptides magainin II and amikacin had no effect on EAEC viability at the concentrations and conditions used here (data not shown).

The greater antimicrobial efficacy exhibited by LFchimera compared to its peptides of origin, LFcin17-30 and LFampin265-284 has been observed in other studies, including studies testing the antibacterial effects of these peptides against pathogenic microorganisms, such as Candida, the halophiles *V. parahaemolyticus* and *V. cholera, S. aureus, E. histolytica and E. coli* (Bolscher et al. 2012; Flores-Villasenor et al. 2012a; Flores-Villasenor et al. 2010; Leon-Sicairos et al. 2014; Leon-Sicairos et al. 2009; Lopez-

Soto et al. 2010; Puknun et al. 2016). LFchimera has also been reported to exert antimicrobial activity against *Burkholderia pseudomallei*, *Burkholderia thailandensis* and *Leishmania donovani* (Puknun et al. 2013; Puknun et al. 2016; Silva et al. 2012; Silva et al. 2013). The high antimicrobial activity of LFchimera may be related to its structure, which adopts a secondary conformation (three-dimensional) that resembles that of native bLF (Bolscher et al. 2009). In addition, LFchimera contains two active domains of native bLF (LFcin17-30 and LFampin265-284). It also has a higher net cationic charge (12+) at neutral pH than LFcin17-30 (6+) or LFampin265-284 (4+) (Bolscher et al. 2012; Bolscher et al. 2009), and the highly cationic nature of LFchimera may confer multifunctional activity that is higher than that of bLF, LFcin17-30 or LFampin265-284 (Bolscher et al. 2009; Flores-Villasenor et al. 2012b; Haney et al. 2012; Silva et al. 2012; Silva et al. 2013).

EAEC is an increasingly recognized cause of acute diarrhea among both children and adults. Contamination of food and water plays a central role in its transmission. It has been reported that human milk components may contribute to the defense of infants against enteropathogens, such as EAEC (Nascimento de Araujo and Giugliano 2000; Nataro and Kaper 1998). Similarly, it has been reported that hLF (purified from human milk), recombinant hLF (rhLF), and bLF inhibit the aggregative adherence "stacked-brick pattern" of EAEC in cultured cells (Ochoa et al. 2006). bLF also affects surface adhesins and inhibits EAEC biofilm formation (Ochoa et al. 2006). However, clinical trials are needed to determine if LF and its variants are able to cure or prevent EAEC infections. The results presented here suggest that the antibacterial activities of LFpeptides, specifically LFchimera, may provide an effective alternative for the treatment of EAEC infections.

Internalization of LF17-30, LFampin265-284 and LFchimera peptides within enteroaggregative *Escherichia coli* bacteria

In order to know if the antibacterial activity of LFpeptides depends on their interaction with and penetration into bacteria, LFpeptides were immunolocalized by electron microscopy (IEM). After 30 min of incubation LFcin17-30 (Panel B), LFampin265-284 (Panel C) and LFchimera (Panel D) can be found in the EAEC cytoplasm, and only a low proportion of the peptides were visualized on the membrane. Interestingly, LFchimera showed a different pattern of distribution (Panel D) in EAEC. It was localized in molecular aggregates or conglomerates, whereas LFcin17-30 (Panel B) and LFampin265-284 (Panel C) showed a diffuse pattern of distribution in the cytoplasm. In conclusion, the three peptides are able to translocate across the bacterial plasma membrane.

Antimicrobial peptides (AMPs) exist in a wide range of the multicellular organisms including animals and plants, which suggests that the AMPs play very important roles in basic protection against invading microorganisms. Natural AMPs have antibacterial properties and exhibit anticancer activities (Lopez Cascales et al. 2014; Madani et al. 2011; Wallbrecher et al. 2014). Cationic antimicrobial peptides have been isolated from many different organisms during the last decade. They vary in primary and secondary structure, but have some common properties: they are short, most of them consisting of less than 45 amino acids, amphipathic, and carry a net positive charge (Last and Miranker 2013; Lopez Cascales et al. 2014; Madani et al. 2011). A number of these peptides have been extensively studied in order to elucidate their antimicrobial mode of action. Interaction with the bacterial cytoplasmic membrane is thought to be responsible for the bactericidal effect of many cationic antimicrobial peptides. Different models for the bactericidal effect have been proposed: it has been reported that peptides might form pores or act by thinning the membrane or by destabilizing the membrane bilayer. For example, Cecropin P1 (Cec P1) is most active against Gram-negative bacteria, killing these bacteria by increasing the

297 permeability of the membrane and lysis of the bacterial cel1 (Huang 2006; Last and
298 Miranker 2013; Lopez Cascales et al. 2014). Magainins are antimicrobial peptides isolated
299 from the skin of the African clawed frog Xenopus laevis that also kill bacteria by
300 permeabilizing their membranes. D-Analogues of cecropins and magainins retain their
301 antibacterial effect, and hence their mode of action is not thought to be receptor-mediated
302 (Ho et al. 2012; Last and Miranker 2013). The acting mechanisms of Mag 2 and Cec P1 are
303 limited exclusively to the cytoplasmic membrane. However, antimicrobial peptides have
304 also been reported to have intracellular targets such as DNA and RNA. Buforin II binds to
305 DNA and RNA, indolicidin inhibits DNA synthesis and to a lesser extent RNA synthesis,
306 and cecropin PR39 inhibits DNA and protein synthesis (Hartmann et al. 2010; Ho et al.
307 2012). These peptides also have effects on the outer and inner membranes of bacteria. It is
308 not known whether their bactericidal mode of action is due to their membrane effects, their
309 effects on intracellular targets, or a combination of these effects (Hartmann et al. 2010; Ho
310 et al. 2012).

311 It has been reported that the peptides Lfcin B 17-41 and Lfcin B 17-31 penetrate
312 through the membrane and into the cytoplasm of *E. coli* and *S. aureus* (Haukland et al.
313 2001). Notably, the identification and characterization of a 22-amino acid cell penetrating
314 peptide (CPP) corresponding to amino acids 19-40 of human lactoferricin has been reported
315 (Duchardt et al. 2009). This peptide exhibits conformation-dependent uptake efficiency that
316 is correlated with efficient binding to heparan sulfate and lipid-induced conformational
317 changes. The peptide contains a disulfide bridge formed by terminal cysteine residues
318 (Duchardt et al. 2009). The precise mode by which this peptide penetrates into the
319 cytoplasm is not fully elucidated, but human lactoferricin has been shown to cause
320 depolarization and loss of integrity of the cytoplasmic membrane and loss of the pH

gradient and to exert a bactericidal effect on *E. coli* (Haukland et al. 2001). LPS and TA have been reported as the initial binding sites for Lfcin B, but Lfcin B neither lyses bacteria nor causes major leakage from liposomes. However, Lfcin B does depolarize the membranes of susceptible bacteria and induces fusion of negatively charged liposomes. Kinetic studies have shown that the lactoferricins have a slow bactericidal action, which might indicate an intracellular target (Haukland et al. 2001). Ho et al (2012) demonstrated that LFcin B inhibits the growth of bacteria by binding to and inhibiting the phosphorylation of the two-component system response regulators BasR and CreB. Therefore, reported data indicates that Lfcin B penetrates through the cell membrane and has intracellular activities, and supports the idea that the bactericidal actions of at least some cationic antimicrobial peptides includes intracellular targets in addition to effects on the cell membrane.

Regarding the results reported here (Fig 2), the cytoplasmic localization of the synthetic LFpeptides in EAEC agrees with the premise that these peptides, like Lfcin B, have intracellular targets. Importantly, the different distribution pattern of LFchimera compared to LFcin17-30 and LFampin265-284 could partially explain the greater antibacterial activity of LFchimera in EAEC and in other bacteria such as Vibrio spp, *S. aureus*, *EHEC and S. pneumoniae* (Flores-Villasenor et al. 2010; Leon-Sicairos et al. 2014; Leon-Sicairos et al. 2009). Perhaps, due to the potent and efficient killing activity of LFchimera, bacteria are quickly damaged and destroyed, allowing DNA release from the cell, and peptides are bound to the clustered DNA as a secondary event. This could explain the external molecular aggregates seen in the cultures exposed to LFchimera (see Fig 2 panel D). Notably, these extracellular clusters are not seen in bacteria exposed to LFcin17-30 or LFampin265-284 (see Fig 2 panels B and C), suggesting the possibility that

LFchimera is more efficient at disrupting the integrity of the cell membrane than LFcin17-30 or LFampin265-284. Additionally, once the peptides enter into bacteria it is likely, given the different cytoplasmic distributions of the peptides, that the intracellular targets and effects of LF chimera, LFcin17-30 and LFampin265-284 are different.

Based on the present data, while we do not know the mechanism by which LFchimera enters into the bacteria, we can speculate that the antibacterial properties of these synthetic peptides are caused by their ability to disrupt the integrity of bacterial cell membranes and to penetrate into the bacteria and reach intracellular targets. Our results raise the possibility that LFchimera has potential as an alternative candidate in the treatment or prevention of infections caused by EAEC and other pathogenic bacteria.

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515 **Figure legends**

516

517 **Figure 1. Antibacterial effects of LFchimera, LFcin17-30 and LFampin254-284 on**
518 **enteroaggregative *Escherichia coli*.**

519 EAEC (10^7 CFU/ml) were treated with 40 μM LFcin17-30 (Panel A), LFampin265-284
520 (Panel B) or LFchimera (Panel C) for 2 h at 37ºC. The samples were then washed and
521 processed to determine the CFU/ml. Untreated bacteria in LB medium were used as a
522 control of growth (viability), and bacteria treated with 100 μg/ml ciprofloxacin were used
523 as control of growth inhibition. Bacterial viability was expressed as a percentage relative to
524 the untreated control cells, and the mean and standard deviation are shown.

525

526 **Figure 2. Lactoferricin17-30, Lactoferrampin265-284 and Lactoferrin chimera**
527 **penetrate into the cytoplasm of enteroaggregative *Escherichia coli*.**

528 Micrograph (A) corresponds to EAEC bacteria that were untreated and processed to
529 preserve their ultrastructure. Panels B-D correspond to EAEC bacteria exposed to 20 μM of
530 LFcin17-30 (Panel B), LFampin265-284 (Panel C), or LFchimera (Panel D) for 1 h at 23 ºC
531 and then immunolabeled with anti-lactoferrin polyclonal antibodies coupled to 20 nm gold
532 particles. Arrows indicate labeling with the gold particles. A diffuse distribution pattern (in
533 the cytoplasm and membranes) is observed in EAEC treated with LFcin17-30 (B) or
534 LFampin265-284 (C). In contrast, molecular aggregates (mostly in the cytoplasm) are
535 observed in EAEC treated with LFchimera (D). Scale bars= 100 nm.

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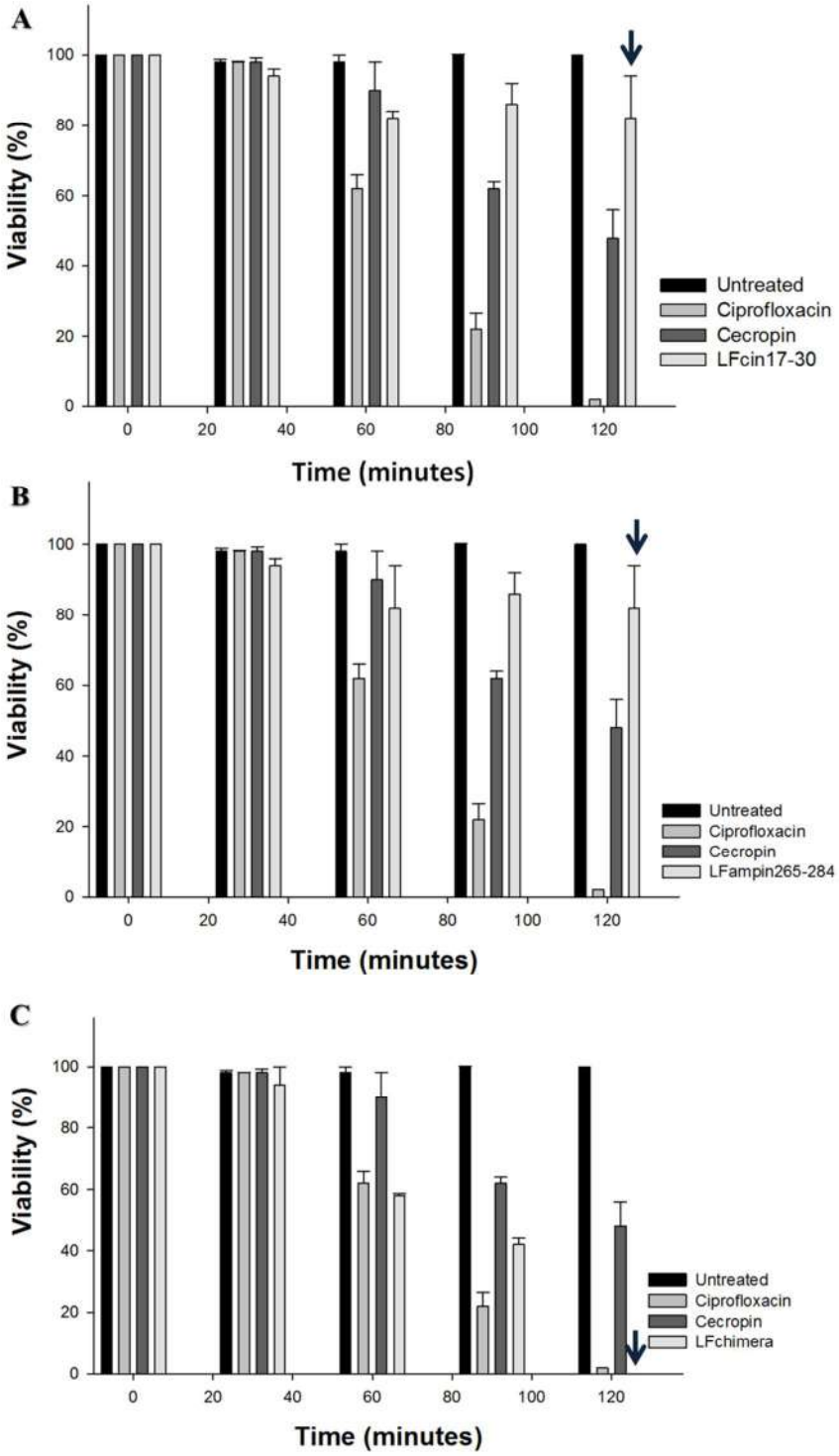
540 Figure 1



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544 Figure 2

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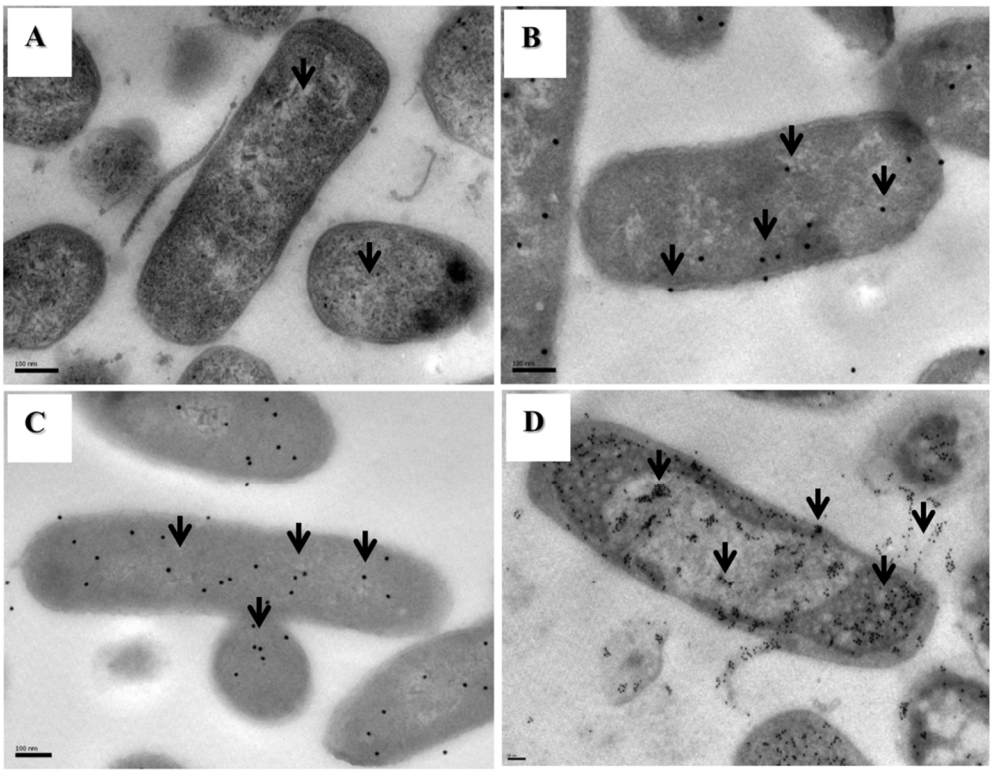

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