Synergistic effects of antimicrobial peptides and antibiotics against *Clostridium difficile*

Running title: Antimicrobial peptides and antibiotics against *C. difficile*

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Abstract

Accelerating rates of healthcare-associated infections caused by *Clostridium difficile* with increasing recurrence rates and rising antibiotic resistance have become a serious problem in the recent years. This study was conducted to explore whether the combination of antibiotics with human antimicrobial peptides may lead to an increase of the antibacterial activity. The *in vitro* activities of the antimicrobial peptides HBD1-3, HNP1, HD5 and LL-37, the antibiotics tigecycline, moxifloxacin, piperacillin/tazobactam or meropenem alone or in combination against 10 toxinogenic and 10 non-toxinogenic *C. difficile* strains were investigated. Bacterial viability was determined by flow cytometry and toxin production by ELISA. When combined at subinhibitory concentrations antimicrobial peptides and antibiotics generally led to an additive killing effect against toxinogenic and non-toxinogenic *C. difficile* strains. However, LL-37 and HBD3 acted in synergism with all antibiotics tested. Electron microscopy demonstrated membrane pertubation in the bacterial cell wall by HBD3. In 3 out of 10 toxinogenic strains, HBD3 and LL-37, as well as piperacillin/tazobactam or meropenem led to an increased toxin release which could not be prevented by addition of HNP1. Antimicrobial peptides increase the bacterial killing of antibiotics against *C. difficile* regardless of the mode of action of the antibiotics. Membrane perturbation or pore formation of the bacterial cell wall may enhance the uptake of antibiotics and increase their antibacterial effect. Therefore, the combination of antibiotics with antimicrobial peptides may represent a promising novel approach to treat *C. difficile* infections.
Introduction

Clostridium difficile infections (CID) have become an increasing health care problem, occurring often during or after antibiotic therapy. The transmission of *C. difficile* in the nosocomial environment is primarily caused by the ingestion of spores, which may have been acquired from other patients directly, via the hands of health care workers or indirectly from inanimate objects. The severity of the infections, increasing recurrence rates and the development of resistance against antibiotics has limited effective antibiotic therapy of *C. difficile* infections in the last years (1). While most antibiotics act on the vegetative bacterial cell of *C. difficile*, only a few antibiotics such as vancomycin or fidaxomicin may have an inhibitory effect on the sporulation. However, there are still recurrence rates of 15 % with fidaxomicin and 25 % with vancomycin (2).

*C. difficile* infections can lead to symptoms from diarrhea to severe pseudomembranous colitis and toxic megacolon. In these gastrointestinal infections the proper balance between the gut flora and host defense is disturbed. Normally, although the colonic mucosa is constantly exposed to a high number of bacteria, bacterial infections are rare. One important mechanism to prevent microbial invasion of the epithelium is the production and secretion of antimicrobial peptides such as defensins or the cathelicidin LL-37. The family of defensins comprises small cationic peptides with a low molecular weight of 3-6 kDa. Due to a different position of three intramolecular disulphide bonds human defensins are divided in α- and β-defensins (3). The α-defensins derive mainly from intestinal Paneth cells (HD5 and -6) or neutrophils (HNP1-4), whereas the beta defensins (HBD1-4) are predominantly of epithelial origin (4-7).

The only human cathelicidin LL-37 is released by proteolytic cleavage from the precursor hCAP-18, which is synthesized from epithelial cells, but also from a variety of immune cells (8).
The main mode of action of antimicrobial peptides is the formation of pores in the bacterial cell membrane but they can also act on intracellular targets such as nucleic acid and protein biosynthesis (9). It is conceivable that antimicrobial peptides may augment the activity of antibiotics which share some of the targets with them.

Despite numerous studies investigating the effects of antimicrobial peptides on a broad range of bacterial species, information on their impact on the viability of *C. difficile* is limited. Mc Quade *et al.* recently examined the effect of the cathelicidin LL-37 on *C. difficile* strains and found strain specific susceptibility (10). In addition, it was shown by Giesemann *et al.* (11), that human alpha defensins inhibit toxin B production of *C. difficile*. In this study, synergism between selected antibiotics and the human beta defensins HBD1-3, the alpha defensin HD5 and the cathelicidin LL-37 against 10 toxin positive and 10 toxin negative clinical strains of *C. difficile* was assessed employing a flow cytometric test. This assay was previously developed for viability assessment of other anaerobic bacteria based on membrane polarization (12). Bacterial strains were first preincubated with the various antimicrobial peptides and then exposed to subinhibitory concentrations of the antibiotics tigecycline, moxifloxacin, piperacillin/tazobactam or meropenem. Subsequently, bacterial membrane depolarization was measured by flow cytometry and toxin release was controlled by ELISA.

**Material and methods**

**Bacterial strains**

10 non-toxinogenic and 9 toxinogenic clinical isolates were obtained from the routine laboratory testing of the Institute of Laboratory Medicine of the Hospital ALB FILS KLINIKEN. As 10th toxinogenic strain served *C. difficile* DSM 1296. All strains were recovered on Columbia-agar with 5% defibrinated sheep blood (BD, Sparks, USA). Toxin production of the toxinogenic strains was verified by toxin A/B ELISA (Ridascreen...
Clostridium difficile®, R-biopharm, Darmstadt, Germany). The strains were characterized molecular genetically with Genotype CDiff (Hain Lifescience, GmbH Nehren, Germany).

**MIC-Determination**

The minimal inhibitory concentrations (MIC) of the *C. difficile* isolates against tigecycline, moxifloxacin, piperacillin/tazobactam and imipenem were determined by Etest® (BioMerieux Nürtingen, Germany) according to the manufacturer’s instructions. As control strains served *S. aureus* ATCC 25923, *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741.

Briefly, an inoculum of about $1.5 \times 10^7$ colony forming units was plated on Mueller Hinton Agar supplemented with 5% sheep blood (BD, Sparks, USA). The Etest® strips were placed on the agar and the plates incubated for 48 h under anaerobic conditions (Anaero Gen, Oxoid Limited, Hampshire, UK). The MIC was recorded as concentration at which the elliptical inhibition zone met the Etest strip. To determine subinhibitory concentrations with the flow cytometric test, $1.5 \times 10^6$ CFU/ml of mid-logarithmic phase *C. difficile* were incubated with antibiotics in the following concentrations: Moxifloxacin 64, 32, 16, 8 and 4 µg/ml, tigecycline 2, 1, 0.512, 0.256, 0.128 and 0.0064 µg/ml, piperacillin 6, 3, 1.5, 0.75, 0.375 µg/ml combined with 4 µg tazobactam, and meropenem 512, 256, 128, 64, 16 and 4 µg/ml for 8 hours at 37 °C under anaerobic conditions (AnaeroGen, Oxoid Limited, Hampshire, UK).

Subsequently, the suspensions were incubated for 10 minutes with 1 µg/ml of the membrane potential sensitive dye DiBAC₄(3) [bis-(1,3-dibutylbarbituric acid) trimethine oxonol] (Invitrogen, Carlsbad, CA). Cell damage leads to the breakdown of the membrane potential followed by the uptake of the dye in the bacterial cells and to an increasing fluorescence. The suspensions were centrifuged for 10 minutes at $4,500 \times g$, the bacterial pellets were resuspended in 300 µl PBS and analyzed by flow cytometry as described below. For several strains the flow cytometric results were confirmed by plating.
Effect of defensins and LL-37 on C. difficile

For a total of 10 toxinogenic or non-toxinogenic C. difficile strains the subinhibitory concentrations of the antimicrobial peptides were determined. C. difficile suspensions with a concentration of 1.5 x 10^6 CFU/ml were incubated with the antimicrobial peptides HBD1, HBD2, HBD3, HD5 (all peptides from Peptide Institute, Osaka, Japan) and LL-37 (Innovagen, Lund, Sweden) at varying concentrations ranging from 0.5 to 15 µg/ml for 90 min at 37 °C under anaerobic conditions (AnaeroGen, Oxoid Limited, Hampshire, UK). Then 1 µg/ml DiBAC₄(3) (Invitrogen, Carlsbad, CA) was added and after 10 minutes the suspensions were centrifuged for 10 minutes at 4,500 x g, the pellets were resuspended in 300 µl PBS and analyzed by flow cytometry.

Activity of reduced antimicrobial peptides

To reduce the antimicrobial peptides these were preincubated with 10 mM DTT at 37 °C for 2 hours (13). The peptides were incubated in the native/oxidized or reduced form with 5 C. difficile strains in a concentration of 15 µg/ml as described above and analyzed by flow cytometry.

Combined effect of antibiotics and antimicrobial peptides

Synergism studies were performed using a concentration of 1 µg/100 µl of the various antimicrobial peptides, since at this peptide concentration alone no significant depolarization of bacteria was observed. Based on pilot experiments we used tigecycline at 0.512 µg/ml, moxifloxacin at 32 µg/ml, piperacillin/tazobactam at 6 µg/ml/4 µg/ml, and meropenem at 64 µg/ml for these studies because this concentrations don’t lead to a significant depolarization. Suspensions with 1.5 x 10^6 CFU/ml were incubated with the antimicrobial peptides for 30 minutes at 37 °C. Subsequently, the antimicrobials were added. After further 8 hours incubation at 37 °C under anaerobic conditions (AnaeroGen, Oxoid Limited, Hampshire,
UK), the suspensions were incubated for 10 minutes with 1 μg/ml DiBAC₄(3). The suspensions were centrifuged for 10 minutes at 4,500 × g and the bacterial pellets were resuspended in 300 μl PBS each. The percentage of depolarized fluorescent bacteria in the suspension was determined by flow cytometry as described below.

All experiments were performed at a minimum in duplicates and repeated.

Flow cytometry

In each sample 10,000 events were analyzed on a FACSCalibur™ flow cytometer (BD, Sparks, MD), using Cell Quest software (BD). With the parameters Forward Scatter and Side Scatter, referring to relative cell size and granularity, the bacterial population was gated for evaluation of the fluorescence 1 (DiBAC₄(3)) in a corresponding histogram. Antibacterial activity was determined as percentage of fluorescent bacteria with respect to the untreated bacterial control.

Electron microscopy

To visualize the effect of antimicrobial peptides on C. difficile cells, 1.5 × 10⁷ bacteria/ml were exposed to HBD3 in a concentration of 200 μg/ml and incubated for 4 hours at 37 °C. (Due to a 10-fold bacteria concentration for microscopy, the concentration of antimicrobial peptides was also increased 10-fold.) Untreated bacteria served as control. After centrifugation, the bacterial pellets were fixed with Karnovsky’s fixative. The pellets were embedded in 3.5 % agarose at 37 °C, coagulated at room temperature, and fixed again in Karnovsky’s fixative. Postfixation was carried out with 1 % osmium tetroxide containing 1.5 % K-ferrocyanide in 0.1 M cacodylate buffer for 2 h. Subsequently the specimens were embedded in glycid ether. Ultra-thin sections, 20-30 nm thick, were mounted on uncoated copper grids and imaged by electron microscopy with a Zeiss LIBRA 120 microscope (Carl Zeiss Oberkochen, Germany).
For the antimicrobial peptides that exhibited synergism with antibiotics, HBD3 and LL-37, as well as for HNP1, we investigated the influence on toxin release by *C. difficile*.

The 10 toxinogenic strains were grown overnight in Schaedler broth and adjusted to to 4.5 x 10^6 cells/ml in Schaedler broth (BD, Sparks, USA), diluted 1:3 in dH2O). The dilution was performed because the salt content in undiluted Schaedler broth has an inhibitory effect on the activity of some antimicrobial peptides. The suspensions were incubated with the respective antimicrobial peptide at a concentration of 10 µg/ml for 30 minutes at 37 °C. The antimicrobials were added in the following concentrations: tigecycline 0.512 µg/ml; moxifloxacin 32 µg/ml; piperacillin/tazobactam at 4 and 6 µg/ml respectively and meropenem 64 µg/ml. The bacteria suspensions were incubated for 8 h under anaerobic conditions (AnaeroGen, Oxoid Limited, Hampshire, UK). 100 µl of the suspension were used in a toxin A and B-ELISA, which was performed according to the manufacturer’s instructions (R-biopharm, Darmstadt, Germany).

**Statistics**

Graphing and statistical analyses were carried out using Prism 5.0 software. Data are presented as means with standard deviation (SD). For comparison of bacterial killing with or without preincubation with antimicrobial peptides, the Mann-Whitney test was used. Values of p< 0.05 were considered statistically significant.

A depolarization rate of the combination of antimicrobial peptide and an antibiotic equivalent to the sum of their individual killing rates was defined as additive, whereas a higher percentage than the sum of their individual rates was considered synergistic.
Results

MIC-Determination

For the control strains *S. aureus* ATCC 25923, *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741 the MIC determined by Etest was for all antibiotics tested (tigecycline, piperacillin, moxifloxacin and imipenem) within the range provided by the manufacturer. For the *C. difficile* strains the MIC for tigecycline was equal to or less than 0.016 mg/l. The MIC for piperacillin ranged from 0.75 mg/l to > 32 mg/l, with the MIC of most strains (n=13) between 3 and 6 mg/l. The MIC for moxifloxacin was for 14 strains over 32 mg/l and only 6 strains were sensitive with a MIC of 0.125 or 0.75 mg/l. For imipenem also 17 strains had a MIC above 32 mg/l and only three strains were sensitive with a MIC of 2, 6 or 12 mg/l (Supplementary data table 1).

Effect of defensins and LL-37 on *C. difficile*

With concentrations of 0.5 or 1 µg/ml no antibacterial effect was seen with all peptides tested. Using higher concentrations of 2.5-15 µg/ml no effect on *C. difficile* integrity was observed when incubating the strains with the constitutive defensin HBD1. Some strains were marginally affected by the inducible HBD2 or by HD5. In contrast, with concentrations > 5 µg/ml, both, LL-37 and HBD3 effectively depolarized vegetative cells (Figure 1A) and reduced significantly colony forming units in cultures of all *C. difficile* strains examined (data not shown).

Electron microscopy performed of *C. difficile* with the defensin HBD3 showed disruptions of the cell wall and less dense cytoplasmic regions or even dissolution of the cytoplasm (Figure 1B). After reduction of the antimicrobial peptides the activity of HBD3 was lower than the activity of the native (oxidized) form. This effect was not observed reducing LL-37. No
significant differences occurred in the activity of reduced HBD1, HBD2 and HD5 against *C. difficile* compared to the native form of these peptides (Figure 2).

**Synergism between antibiotics and antimicrobial peptides**

With an average of 3.83 % (range 0.89 - 9.73 %) no significant depolarization of bacterial cells was found in the untreated controls. Incubated with subinhibitory concentrations of the various antibiotics, the percentage of depolarized cells was on average 9.02 % (range tigecycline 3.69-29.6 %, moxifloxacin 1.03–12.69 %; piperacillin/tazobactam 1.07-12.69 % and meropenem 0.29–26.5 %) without any statistically significant difference among the different antibiotics. The subinhibitory concentrations of HBD1-3, HD5 and LL-37 revealed on average depolarization rates of 11.16 % (range 0.02- 39.12 %).

When combined the antibiotics exerted an additive effect with nearly all antimicrobial peptides. Only HD5 showed in non-toxinogenic strains an additive effect with tigecycline, but not with moxifloxacin, piperacillin/tazobactam or meropenem. Furthermore in combination with HBD3 or LL-37 all antibiotics exhibited significant synergistic effects (Table 1, supplementary figure 1). In 15 out of 20 strains tested a synergistic effect could be observed after adding HBD3 with an average increase of depolarized cells from 9.02 % with the various antibiotics alone to 41.42 % in the combination with HBD3. With LL-37 this effect was seen in 16 strains, but the average increase of depolarized cells was generally lower with 29.12 % after addition of LL-37. Therefore the degree of additive or synergistic effects of antibiotics combined with antimicrobial peptides was mainly strain specific.

There were no significant differences, when the averages of depolarized bacteria were compared between the various antibiotics/LL37 or antibiotics/HBD3-combinations.
In contrast to LL-37 and HBD3, the peptides HBD1 and HBD2 showed no synergistic effect in toxigenic and non-toxigenic strains. HD5 demonstrated statistically significant synergism in toxin-positive strains with meropenem only (P < 0.01).

**Influence of antibiotics and antimicrobial peptides on toxin production**

When tested in subinhibitory concentration no antibiotic inhibited toxin production of toxinogenic strains, nor was a conversion seen in toxin-negative strains. An increase in detectable toxin was seen in three toxinogenic strains in response to subinhibitory concentrations of piperacillin/tazobactam and meropenem. The same strains reacted similarly, when adding HBD3 or LL-37 alone. The addition of HBD3 or LL-37 to piperacillin/tazobactam or meropenem did not further increase the toxin release. In other strains no significant increased toxin amounts could be measured after addition of antibiotics or antimicrobial peptides. **C. difficile**-toxins were not detectable by toxin ELISA in all strains, when incubated with the alpha-defensin HNP1 alone (Table 2). This was also observed for all strains tested with moxifloxacin or tigecycline in combination with HNP1. However, when treated with piperacillin/tazobactam or meropenem, the toxin release of those strains which showed an induction could not be neutralized by the applied HNP-1 concentration.

**Discussion**

Investigating the bacterial killing effect of antimicrobial peptides against **C. difficile**, HBD3 has the strongest activity, followed by LL-37 and HBD2, whereas HBD1 had no or little antimicrobial effect. To our knowledge, this is the first report, which describes antimicrobial activity of defensins on vital **C. difficile**-cells, whereas activity of LL-37 against **C. difficile** was recently reported (10).
In a preceding study, the reduced form of HBD1 showed higher antimicrobial activity against Bifidobacterium sp. and Candida albicans (13). In this study reduction of the disulfide bonds led not to a pronounced effect of HBD1 but to a decreased activity of the defensin HBD3 against C. difficile-strains. The activity of LL-37 was not influenced by the pretreatment with DTT, because in contrast to the defensins the cathelicidin LL-37 is a linear helical peptide without disulfide bonds in the secondary structure.

We found that the flow cytometric test required often higher concentrations of the different antibiotics for depolarization of bacteria than MICs causing growth inhibition found using the Etest. This is in concordance with prior findings (14) with other bacterial species, because the Etest measures growth inhibition, but the flow cytometric test measures killing reflected by depolarization of the bacterial membrane. Nevertheless, subinhibitory antibiotic concentrations were adapted to the concentrations showing no or little antibacterial effect in the flow cytometric assay. Combined with antibiotics, defensins or the cathelicidin LL-37 led to an additive or synergistic effect, with meropenem and moxifloxacin even against the C. difficile strains, which were mostly resistant to these two antibiotics. The increased bacterial killing was most evident for the combination of antibiotics with the defensin HBD3 or the cathelicidin LL-37. As electron microscopic investigations with HBD3 showed (Figure 1B), it may be assumed, that access of antibiotics into the bacterial cell may be supported by the membrane perturbation by antimicrobial peptides. The observed effect was independent from the mode of action of the different antibiotics and whether the antibiotics act bactericidal or bacteriostatic such as tigecycline.

Concerning the toxin release of C. difficile strains incubated with antibiotics or antimicrobial peptides, none of the antibiotics tested in subinhibitory concentration had an inhibiting effect on the toxin production of toxigenic C. difficile strains. Tigecycline and moxifloxacin provided antimicrobial efficacy without induction of toxin release. This is in concordance with the finding of Baines et al. (15), who showed, that tigecycline leads not to an increased
cytotoxin production in a human gut model. Therefore the combination of tigecycline with antimicrobial peptides is assumed to be a therapy option in *C. difficile* colitis, whereas the synergistic effect of defensins with piperacillin/tazobactam and meropenem may contribute in an adverse outcome, due to increased release of toxin from *C. difficile* cells in some strains. Onderdonk *et al.* demonstrated that toxin production was affected by external stress factors (16). They measured increased amounts of toxin in response of alterations of the oxidation-reduction potential and subinhibitory concentrations of vancomycin and penicillin in a *C. difficile*-strain, whereas clindamycin had no increasing effect. Comparative analysis from the supernatant and sonicated cells lead to the hypothesis, that *C. difficile* rather released more toxin in the medium than produced more toxin. Inhibition of cell wall synthesis with subsequent membrane perturbation or formation of pores of the cell wall may contribute to an increased release of *C. difficile*-toxin, which then was not neutralized by the added amount of HNP-1. The *C. difficile*-toxin release from the bacterial cell is also supported by the fact, that a similar amount of toxin increase was detected using the antimicrobial peptides HBD3 and LL-37 alone, which pass through pore formation or inhibition of protein biosynthesis for cell wall synthesis to kill the bacterial cell. Many studies have shown the effects of antibiotics on toxin production of *C. difficile* and these were partly strain specific and therefore partially contradictory.

Using Ciprofloxacin in subinhibitory concentrations, Aldape *et al.* demonstrated significant and dose-dependent increase of Toxin A gene expression and a shift of its expression to the earlier growth cycle in a highly ciprofloxacin-resistant isolate (17). TcdB gene expression was also increased but was less sensitive to low-dose ciprofloxacin. Nevertheless Moxifloxacin in subinhibitory concentrations did not lead to any alterations in toxin-release in our strains. Prophage carriage seems to be common in clinical relevant strains of *C. difficile* (18). Goh *et al.* suggested, that lysogens carrying temperate phages could modify toxin production in *C. difficile* and furthermore Sekulovic *et al.* were able to detect 1.6-2.1 fold more TcdA and
TedB in a NAP1/O27 strain, infected with ΦCD38-2, compared to the wild type strain (19, 20). Therefore, prophage carriage may contribute to the strain specific behavior, when affected to various antimicrobials and defensins, but in particular cases this have to be further verified.

It is expected that only few new antimicrobials are in developmental stages to be feasible on the market in the coming years. The cationic peptides, like defensins or the cathelicidin LL-37 are substances that have found little application in practice. But as combination partners that show synergistic effects in sub-inhibitory concentrations, they provide the opportunity to increase bacterial killing of antibiotics even at low dosages.

Acknowledgements and Funding

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The authors thank Birgit Fehrenbacher (Department of Dermatology, University of Tübingen, Germany) for excellent technical assistance and Edith Porter (Department of Biological Sciences, California State University, Los Angeles) for critical revision of the manuscript.

Transparency declarations

None to declare.
**Figure legends**

**Figure 1:**

A) Flow cytometric analysis of the antimicrobial activity of the defensins HBD2 and HBD3 and the cathelicidin LL-37 against vegetative cells of *C. difficile* 109. The grey filled histogram shows the green fluorescence of untreated bacteria, the lines the fluorescence of bacteria incubated with antimicrobial peptides in a concentration of 15 µg/ml (Green line HBD-1, blue line HBD2, red line HBD3, brown line HD5 and black line incubated with LL-37). Cell damage leads to an uptake of the membrane potential sensitive dye DiBAC4(3) in the bacteria and therefore to an increasing fluorescence compared to the untreated control.

B) Electron microscopy of *C. difficile* cells incubated with 200 µg HBD3/ml. Untreated control (left panels) and pore formation after treatment with HBD3 (right panels, metering bars 0.2 µm). HBD3 led to disruptions in the cell membrane (arrows) and to a decrease in the density of the cytoplasm.

**Figure 2:**

Activity antimicrobial peptides in native (oxidized) or reduced form in a concentration of 15 µg/ml against 5 *C. difficile* strains (Means + SD). The reduction of HBD3 led to a diminished antimicrobial activity (*p<0.05), whereas the antibacterial activity of LL-37 was not significant different with both forms.
Reference list


Table 1: Bacterial killing of toxinogenic (n=10) and non toxinogenic (n=10) *C. difficile* strains after incubation with antimicrobial peptides, antibiotics or the combination of both.

<table>
<thead>
<tr>
<th></th>
<th>without antibiotic</th>
<th>+Tigecyclin</th>
<th>+Moxifloxacin</th>
<th>+Piperac./Tazob.</th>
<th>+Meropenem</th>
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<td><strong>control</strong> (No AMP)</td>
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<tr>
<td>toxinogenic strains</td>
<td>3.8 (SD 2.67)*</td>
<td>10.8 (SD 7.90)</td>
<td>5.5 (SD 3.70)</td>
<td>14.3 (SD 11.16)</td>
<td>9.6 (SD 8.41)</td>
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<tr>
<td>non toxinogenic</td>
<td>6.9 (SD 1.93)</td>
<td>13.8 (SD 13.76)</td>
<td>8.2 (SD 11.77)</td>
<td>13.2 (SD 16.37)</td>
<td>14.4 (SD 12.38)</td>
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<td><strong>LL37</strong></td>
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<tr>
<td>toxinogenic strains</td>
<td>11.7 (SD 8.60)</td>
<td>27.2 (SD 20.21) *</td>
<td>31.9 (SD 17.62)</td>
<td>37.7 (SD 21.61) **</td>
<td>31.6 (SD 19.93) **</td>
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<tr>
<td>non toxinogenic</td>
<td>13.6 (SD 19.91)</td>
<td>39.3 (SD 22.25) **</td>
<td>33.9 (SD 24.31) **</td>
<td>37.9 (SD 23.52) **</td>
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<td><strong>HBD1</strong></td>
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<tr>
<td>toxinogenic strains</td>
<td>12.7 (SD 6.99)</td>
<td>21.8 (SD 19.32)</td>
<td>17.3 (SD 20.21)</td>
<td>22.5 (SD 17.14)</td>
<td>19.5 (SD 19.37)</td>
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<td>19.2 (SD 19.45)</td>
<td>12.0 (SD 18.69)</td>
<td>22.3 (SD 18.44) *</td>
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<td>8.1 (SD 5.63)</td>
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<td>27.0 (SD 19.58) *</td>
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<td>12.7 (SD 10.87)</td>
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<td>21.6 (SD 19.55)</td>
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<td>49.9 (SD 30.49) **</td>
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<td>52.8 (SD 26.41) ***</td>
<td>38.0 (SD 28.74) ***</td>
<td>49.3 (SD 25.71) **</td>
<td>44.0 (SD 27.69) **</td>
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<td><strong>HD5</strong></td>
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<td>23.1 (SD 21.68)</td>
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<td>20.0 (SD 19.71)</td>
<td>38.9 (SD 24.26) **</td>
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<td>13.7 (SD 16.45)</td>
<td>21.4 (SD 23.65)</td>
<td>13.8 (SD 21.71)</td>
<td>17.4 (SD 19.30)</td>
<td>17.4 (SD 20.29)</td>
</tr>
</tbody>
</table>

* Mean percentage of depolarized bacteria

** Number of strains with synergistic antimicrobial killing

(*: p< 0.05; **: p<0.005; ***: p<0.0005; ****: p<0.0001; ***: p<0.0005; **: p<0.005; *: p< 0.05)
Table 2: Levels of toxin A and B of 10 toxinogenic *C. difficile* strains after incubation with antimicrobial peptides, antibiotics or the combination of both.

<table>
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<th>strains with increased toxin levels (n=3)</th>
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<td>control</td>
<td>0.425</td>
<td>0.282 (SD 0.23)</td>
<td>0.761 (SD 0.63)</td>
</tr>
<tr>
<td>LL-37</td>
<td>1.264</td>
<td>0.390 (SD 0.25)</td>
<td>3.302 (SD 0.21)</td>
</tr>
<tr>
<td>HBD3</td>
<td>1.113</td>
<td>0.300 (SD 0.22)</td>
<td>3.011 (SD 0.39)</td>
</tr>
<tr>
<td>HNP1</td>
<td>0.180</td>
<td>0.005 (SD 0.02)</td>
<td>0.587 (SD 0.49)</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>0.365</td>
<td>0.251 (SD 0.22)</td>
<td>0.628 (SD 0.46)</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>0.413</td>
<td>0.236 (SD 0.20)</td>
<td>0.824 (SD 0.57)</td>
</tr>
<tr>
<td>Piperac. Tazob.</td>
<td>0.954</td>
<td>0.293 (SD 0.24)</td>
<td>2.495 (SD 0.54)</td>
</tr>
<tr>
<td>Meropenem</td>
<td>0.892</td>
<td>0.261 (SD 0.20)</td>
<td>2.365 (SD 0.28)</td>
</tr>
<tr>
<td>LL-37+Moxi</td>
<td>1.158</td>
<td>0.342 (SD 0.21)</td>
<td>3.061 (SD 0.54)</td>
</tr>
<tr>
<td>LL-37+Tige</td>
<td>1.177</td>
<td>0.338 (SD 0.22)</td>
<td>3.138 (SD 0.32)</td>
</tr>
<tr>
<td>LL-37+Pip</td>
<td>1.190</td>
<td>0.349 (SD 0.21)</td>
<td>3.152 (SD 0.34)</td>
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<tr>
<td>LL-37+Mero</td>
<td>1.212</td>
<td>0.354 (SD 0.23)</td>
<td>3.215 (SD 0.36)</td>
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<tr>
<td>HBD3+Moxi</td>
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<td>0.347 (SD 0.21)</td>
<td>3.117 (SD 0.33)</td>
</tr>
<tr>
<td>HBD3+Tige</td>
<td>1.135</td>
<td>0.327 (SD 0.21)</td>
<td>3.021 (SD 0.38)</td>
</tr>
<tr>
<td>HBD3+Pip</td>
<td>1.178</td>
<td>0.337 (SD 0.25)</td>
<td>3.141 (SD 0.40)</td>
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<tr>
<td>HBD3+Mero</td>
<td>1.077</td>
<td>0.285 (SD 0.17)</td>
<td>2.295 (SD 0.56)</td>
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<tr>
<td>HNP1+Moxi</td>
<td>0.042</td>
<td>0.000 (SD 0.00)</td>
<td>0.140 (SD 0.13)</td>
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<tr>
<td>HNP1+Tige</td>
<td>0.713</td>
<td>0.005 (SD 0.03)</td>
<td>0.566 (SD 0.29)</td>
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<tr>
<td>HNP1+Pip</td>
<td>0.471</td>
<td>0.008 (SD 0.01)</td>
<td>1.553 (SD 0.23)</td>
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<tr>
<td>HNP1+Mero</td>
<td>0.564</td>
<td>0.004 (SD 0.01)</td>
<td>1.869 (SD 0.49)</td>
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</table>