

Bacteria Inhibitory Action of Peptides P753 and P359 in 2D and 3D Cell Cultures

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ABSTRACT

2 METHODS

The current study investigated two proprietary peptides, p359 and p753, for their antibacterial activity against *Escherichia coli* (*E.Coli*) and *Staphylococcus aureus* (*S. aureus*). Minimum Inhibitory Concentration (MIC) of bacteria for p359 and p753 was 50µg/mL and 25µg/mL respectively. MTT assay found p359 and p753 was not toxic to HEp-2 cells up to 100µg/mL. HEp-2 cells were infected with bacteria at Multiplicity of Infection (MOI) of 1:0.1 for 2D studies and 3D studies on 3D printed PLA scaffolds. Peptides p359 and p753 were added 24 hours after bacterial infection. MTT assay of the 2D cell culture found cells with peptide showed 80% more cell viability compared to the control. Live/dead assay and plate count of bacteria treated with peptides showed a 95% decrease in bacteria of 2D cell culture and a 70% decrease in bacteria of 3D cell culture versus without peptide. qPCR was performed on bacteria treated with peptide. There was a down regulation in gene expression of 16s, KatA, RecA, and *grpE* in *E.coli* and of SPY1258 in *S.aureus* with peptides compared to the control.

Keywords: Bacteria inhibition, antimicrobial peptide, 2D cell culture, 3D cell culture, scaffold

1 INTRODUCTION

Worldwide there are foodborne disease outbreaks caused by *E. coli* every year, most recently in 2018 by contaminated romaine lettuce. *E.coli* is a gram-negative bacteria that can cause nausea, vomiting, and fever symptoms. Another bacteria is *S. aureus*, a gram-positive bacteria that is highly contagious and causes skin infections. The symptoms are often boils and oozing blisters of the skin. Due to the increasing rate of antibiotic resistant bacteria, new antibiotics need to be developed. A promising type of antibiotic is antimicrobial peptides. Antimicrobial peptides are a part of the host defense system for many organisms including plants, insects, fish, and mammals. 2-D cell cultures are used because of their ease and cost efficiency however, they do not necessarily provide an accurate representation of what will happen in later clinical trails. Therefore, 3D cell cultures are used to better replicate the environment *in vivo*. In the present study, peptides p359 and p753 were investigated under 2D and 3D cultures for their antibacterial properties against *E. coli* and *S. aureus*.

2.1 Kirby-Bauer Disc Diffusion Assay

E. coli (1×10^8 cfu/mL) was spread (50 µl) onto a nutrient agar plate. Filter paper disks were saturated with 20µl of 1mg/ml peptide p359 and peptide p753. The paper disks were placed onto the nutrient agar plate that contained the *E.coli*. AMC antibiotic disk was added as a positive control. This was repeated with *S. aureus* (1×10^8 cfu/mL) on a separate plate. The plates were incubated at 37°C for 24 hours. The zone of inhibition was measured after 24 hrs.

2.2 Minimum Inhibitory Concentration

The Minimum Inhibitory Concentration (MIC) values of peptide p359 and p753 were determined in quadruplicates. LB broth was added to a sterile non-tissue culture treated 96-well plate. Serial dilution was used at 2 fold dilution to achieve concentrations of 100 µg/ml, 50 µg/ml, 25 µg/ml, 12 µg/ml, and 0 µg/ml of peptide p359 or peptide p753. *E. Coli* or *S. aureus* was added to each well at (1×10^5 cfu/mL). The MIC plate was incubated at 37°C for 24 hours. The absorbance was measured at 600 nm. Resazurin dye was added and the plate was incubated for 1 hour. The change in color from blue to red indicated the presence of live bacteria showing the MIC for the peptide.

2.3 Peptide Toxicity to HEp-2 cells through MTT Assay

The viability of HEp-2 cells in the presence of peptide p359 and peptide p753 was determined by MTT assay, The HEp-2 cells were plated in a 96 well tissue culture plate with 20,000 cells per well and 100µl of MEM10 media. The plate was incubated for 24 hours in 37°C with 5% CO₂. The plate was then washed with HBSS. Peptide p359 or peptide p753 was added at the concentrations 100 µg/ml, 50 µg/ml, 25 µg/ml, 10 µg/ml, 5 µg/ml, or 0 µg/ml to 100µl of MEM10 media, vortexed, and added to the plate. The plate was incubated for 24 hours in 37°C with 5% CO₂. At the end of incubation 15µl of MTT dye was added to each well. The plate was incubated in the dark for 4 hours at 37°C with 5% CO₂. After the 4 hours, 100µl of stop solution was added to each well. The plate was incubated for 1 hour in 37°C with 5% CO₂. The plate was then read using TECAN Sunrise at 570nm and reference 690 nm.

2.4 Multiplicity of Infection

HEp-2 cells were plated on a 24-well tissue culture plate with 70,000 cells in each well and incubated in 37°C with 5% CO₂ for 24 hours. Assuming the growth of 60% Multiplicity of Infection (MOI) 11,000 bacteria was added for a MOI of 1:0.1 and incubated in 37°C with 5% CO₂ for 24 hours. The cells were observed under a light microscope for viability.

2.5 Two-Dimensional Cell Culture

HEp-2 cells were plated on a 24-well tissue culture treated plate with 70,000 cells and 500µl of MEM10 media per well. The plate was incubated for 24 hours in 37°C with 5% CO₂. The plate was washed with HBSS. Additionally, 100µl of MEM10 media without antibiotics was combined with the peptide, vortexed, and added to the plate. Peptide p359 was added in the concentration 25µg/ml or 50µg/ml. Peptide p753 was added in the concentrations 12µg/ml and 25µg/ml. Peptide concentrations were dependant on the MIC results. Bacteria *E. coli* and *S. aureus* were added to the plate at 11,000 cfus a 1:0.1 ratio. The plate was incubated for 24 hours in 37°C with 5% CO₂. The plate was viewed under a light microscope. The plate was scrapped with a cell scrapper and the media was pipetted into an eppendorf tube. The cells were pelleted in a centrifuge at 10,000rpm for 5 minutes. The supernatant was removed. The contents of the wells were stained using live/dead staining of bacteria. A sample was prepared for plate count, gene expression analysis, and live/dead bacterial staining using Invitrogen LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen).

2.6 Three-Dimensional Cell Culture

PLA scaffolds were synthesised at the University of Alabama Birmingham. PCL scaffolds were cut into pieces approximately 1cm x 0.5 cm. The scaffolds were placed in media for 24 hours in 24 or 48-well tissue culture treated plate. Cells (60,000/well) were placed onto each scaffold with 500µl or 200µl of MEM10 media respectively. The plate was incubated for 24 hours in 37°C with 5% CO₂. The plate was washed with HBSS. Additionally, 100µl of MEM10 media without antibiotics was combined with the peptide, vortexed, and added to each well. Peptide p359 was added in the concentration 25 µg/ml or 50µg/ml. Peptide p753 was added in the concentrations 12µg/ml and 25µg/ml. Peptide concentrations were based on MIC results. Bacteria *E. coli* and *S. aureus* were added to the plate at 11,000 cfus a 1:0.1 ratio. The plate was incubated for 24 hours in 37°C with 5% CO₂. The plate was viewed under a light microscope. The scaffolds underwent live/dead staining of bacteria and DAPI staining to be viewed by immunofluorescence microscope. The sample was also prepared for plate count, live/dead bacteria staining, and gene expression analysis.

2.7 Bacteria Survival Determined by Plate Count Analysis

Cells were lysed using TritonX-100 (25µl) with 20 minutes incubation to lyse the cells. After the cells were lysed, 100µl of LB broth was added to the bacteria. The bacteria was diluted by a 10 fold serial dilution to 10⁵ dilution and 25µl was spread onto a nutrient agar plate. The plate was incubated for 24 hours at 37°C. The plate was then viewed and counted using QCount.

2.8 Evaluating Gene Expression with qRT-PCR

Bacteria culture samples were centrifuged into a pellet and RNA was extracted using mini RNA easy kit (Qiagen). Extracted RNA was stored at -80°C. cDNA was synthesized using superscript first strand synthesis system (invitrogen). Gene expression analysis was done through qPCR. qPCR was performed on cells infected with bacteria and bacteria along with peptide. Genes were downregulated in 16s, KatA, RecA, and grpE in *E. coli* and of SPY1258 in *S. aureus* in samples treated with peptide compared with bacteria control samples.

3. RESULTS AND DISCUSSION

3.1 Kirby-Bauer Disc Diffusion Assay

The Kirby-Bauer disc diffusion assay showed that both p359 and p753 were effective at inhibiting bacteria growth of *E. coli* and *S. aureus*.

Table 1. Kirby Disc Zone of Inhibition for *E. coli* and *S. aureus* with p753 and p359

	Zone of Inhibition	
	<i>E. coli</i>	<i>S. aureus</i>
AMC 30µg	15mm	15mm
P359 200µg	12mm	25mm
P753 200µg	12mm	15mm

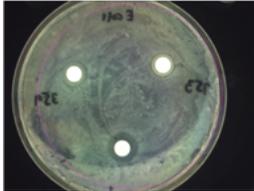
A.  B. 

Figure 1. Kirby Disc Diffusion assay with p753, p359, and Amoxicillin/Clavulanic acid as control (a) *E. coli* (b) *S. aureus*

3.2 Minimum Inhibitory Concentration

The MIC of *E. coli* and *S. aureus* by peptide p359 and p753 were determined. Resazurin dye was added to the plate to visually see the inhibitory effects; blue showing concentrations that have been inhibited. Both *E. coli* and *S. aureus* were inhibited at 50µg/µL with peptide p359 or with 12µg/µL of p753 .

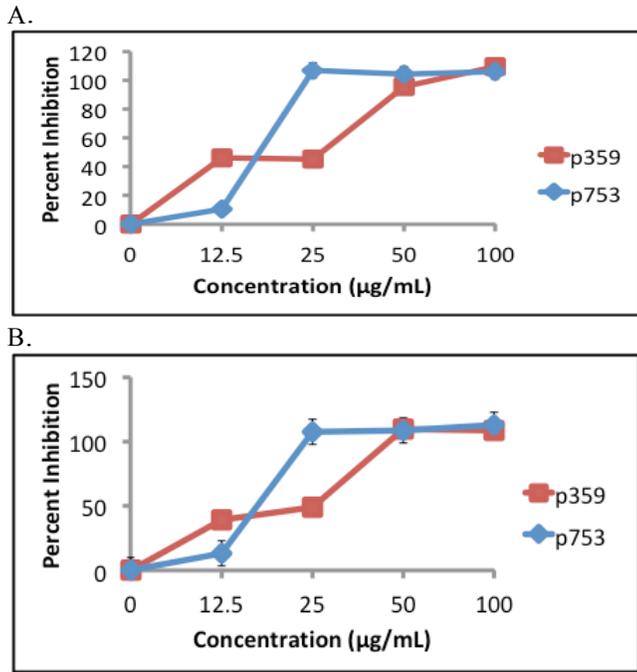


Figure 2. MIC of bacteria incubated with p753 and p359 (a) *E.coli* (b) *S. aureus*

3.3 HEp-2 Cell Viability using MTT Assay

HEp-2 cells were viable at concentrations up to 100 µg/mL of p753 and p359 showing no toxicity to cells. The HEp-2 cells were incubated for 24 hours, bacteria and peptide were added. HEp-2 cells with bacteria and p359 or p753 were 80% more viable than cells with bacteria alone.

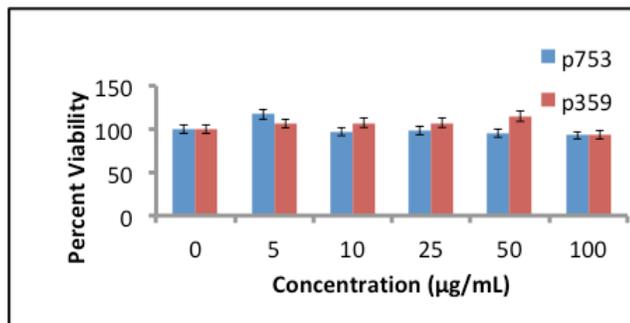


Figure 4. HEp-2 cells are viable up to concentrations of 100 µg/mL of p753 and p359, showing no toxicity to HEp-2 cells

3.4 Bacteria Plate Count Analysis

Plate counts showed up to 70% decrease in bacteria in cell cultures that were treated with p359 or p753 compared to control

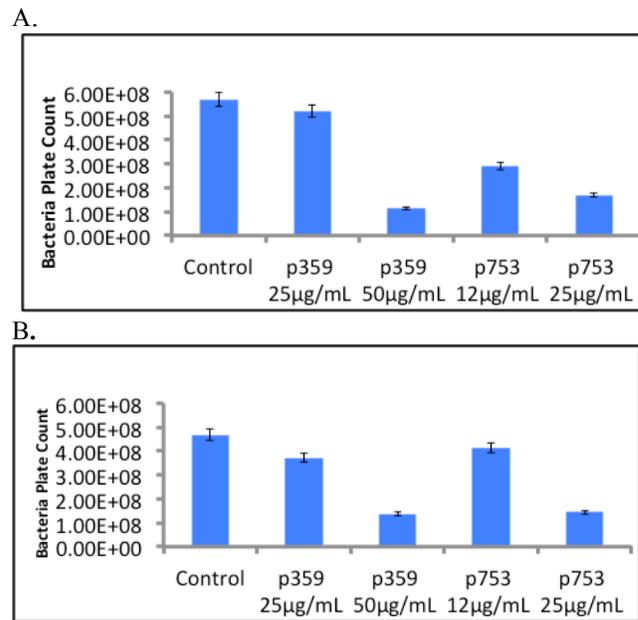


Figure 5. (a) 2D HEp-2 cell culture with *E.coli* and peptide p359 and p753 (b) 2D HEp-2 cell culture with *S. aureus* and peptide p359 and p753

3.5 Live/dead Bacteria Viability Assay

Live bacteria were stained green with SYTO9 and dead bacteria were stained red with propidium iodine and imaged with fluorescent microscope. There was a higher concentration of live bacteria (green) when the bacteria was not treated with peptides. When treated with peptides, there was a higher concentration of dead bacteria (red).

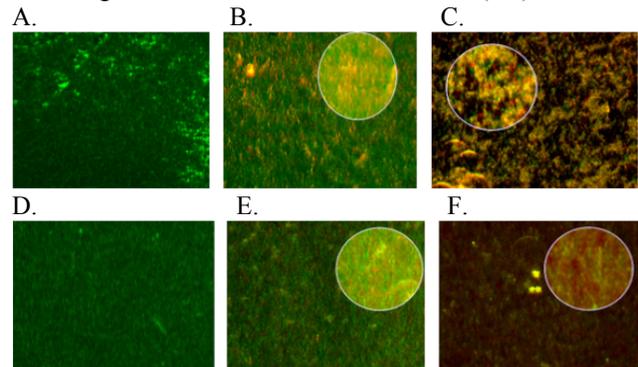


Figure 6. 2D HEp-2 cell culture (a) *E. coli* (b) with *E. coli* and 50µg/µL of p359 (c) with *E. coli* and 25µg/µL of p753 (d) with *S. aureus* (e) with *S. aureus* and 50µg/µL of p359 (f) with *S. aureus* and 25µg/µL of p753

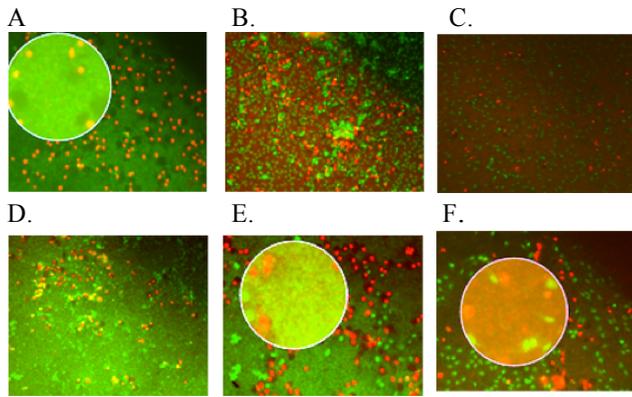


Figure 7. 3D HEp-2 cell culture (a) with *E. coli* (b) with *E. coli* and 50µg/µL of p359 (c) with *E. coli* and 25µg/µL of p753 (d) with *S. aureus* (e) with *S. aureus* and 50µg/µL of p359 (f) with *S. aureus* and 25µg/µL of p753

3.6 HEp-2 Imaging in 3D Cell Cultures Using Dapi Staining

HEp-2 cells infected with bacteria alongwith peptide showed more viability as shown by DAPI stained nucleus in 3D culture compared to HEp-2 cells infected only with bacteria.

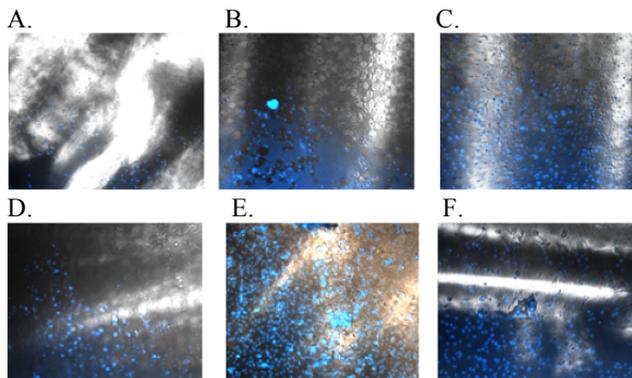


Figure 8. 3D HEp-2 cell culture (a) with *E. coli* (b) with *E. coli* and 50µg/µL of p359 (c) with *E. coli* and 25µg/µL of p753 (d) with *S. aureus* (e) with *S. aureus* and 50µg/µL of p359 (f) with *S. aureus* and 25µg/µL of p753

3.7 Gene Expression Analysis

Changes in gene expression of bacteria after treatment with p359 and p753 were quantified using qPCR. In *E. coli*, *Kat A* (catalase) was down regulated, *Rec A* (SOS response) was down regulated, and *grpE* (heat shock) was down regulated. In *S.aureus*, *SPY 1258* (transcription regulator) was down regulated.

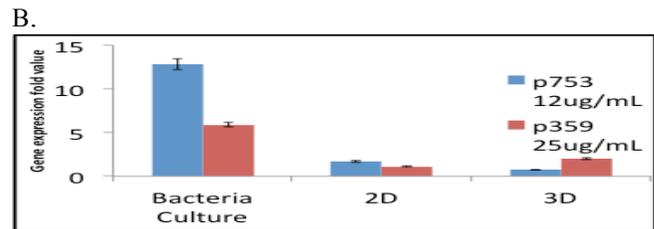
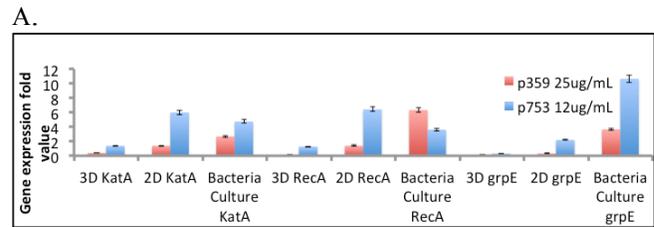


Figure 9. (a) Gene expression of Kat A, Rec A, grpE in *E. coli* treated with p359 and p753. (b) Gene expression of SPY 1258 in *S. aureus*

4. CONCLUSION

In the present study, the peptides p359 and p753 were effective as bacterial inhibitors against gram-negative *E. coli* and gram-positive *S. aureus*. Inhibited by peptides p359 and p753. p753 was effective at 25µg/µL and p359 was effective at 50µg/µL. These levels of peptide were not toxic to the HEp-2 cells. The cells were 80% more viable with the peptide and bacteria compared to bacteria alone. Future studies will include testing the peptides bacteria inhibition properties at different time periods.

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