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CHARACTERIZATION OF A NARROW-SPECTRUM ANTIMICROBIAL THAT EXHIBITS SPECIFIC ACTIVITY AGAINST UROPATHOGENIC BACTERIA

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14. ABSTRACT

Narrow-spectrum antimicrobials offer an alternative to widely used broad-spectrum antimicrobials which have been implicated as a critical cause of the rise of drug resistant bacteria. Additionally, the presence of females in the field has precipitated the need to address female-specific events that may compromise mission performance such as urinary tract infections (UTIs), reported to affect more than half the population of female Soldiers. Such frequency clearly warrants a technology, such as a novel textile with narrow-spectrum antimicrobial treatments, to prevent this infection state to enable the Warfighter to maintain performance to execute mission objectives. The objective of the work described in this report is to identify a narrow-spectrum antimicrobial that exhibits targeted activity against uropathogenic bacteria and characterize its production and purification to yield preliminary data toward the development of a narrow-spectrum technology that prevents the development of UTIs. The narrow-spectrum antimicrobial of focus for this work was colicins, a family of bacteriocins, generated by *Escherichia coli* that demonstrate specific activity. A collection of colicins was screened for activity against representative uropathogenic bacteria and commensal microorganisms. Analysis of the activity results lead to down-selection of one colicin for further characterization. Conditions to promote the growth and synthesis of the colicin by the host microorganism were studied. Further, the stability of the colicin over time was evaluated in a stabilizing solution. The efforts detailed in this technical report offer preliminary data toward the development of an antimicrobial technology to enable female Soldiers to achieve mission objectives in the field.

15. SUBJECT TERMS

BACTERIA, BACTERIOCINS, ESCHERICHIA COLI, ANTIMICROBIAL AGENTS, WOMEN, RESISTANT, PURIFICATION, MICROORGANISMS, ANTIMICROBIAL PEPTIDES, COLICIN, INFECTIONS, ANTIMICROBIALS, NARROW-SPECTRUM, TARGETED ANTIMICROBIALS, ANTIBIOTICS, URINARY SYSTEM, CULTURES(BIOLOGY), UTI(URINARY TRACT INFECTION), COMMENSAL, UROPATHOGENIC, RESISTANCE(BIOLOGY), NARROW-SPECTRUM ANTIMICROBIALS

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Preface

This report documents work performed by the Biological Sciences and Technology Team (BSTT) of the Warfighter Directorate at the U.S. Army Natick Soldier Research, Development and Engineering Center (NSRDEC) during the period of October 2015 to September 2016. This work was comprised of selecting and characterizing an antimicrobial agent that exhibits narrow-spectrum activity against pathogenic bacteria that cause urinary tract infections (UTIs). The goal is eventual incorporation of this antimicrobial agent into the development of a hygiene wipe to be used by the Warfighter in the field to prevent the incidence of UTIs.

The objectives of this project were (1) to identify a narrow-spectrum antimicrobial peptide from a well-characterized collection of colicins that demonstrates targeted activity against a representative collection of uropathogenic pathogens; (2) to develop procedures to acquire and purify sufficient yields of the selected colicin; and (3) to investigate the effects of a stabilizing solution on the activity of the colicin over time.

Previous work related to the investigation of narrow-spectrum antimicrobials was executed from October 2011 to September 2015 and is documented in "Bactericidal Coatings on Textiles for Remediation of Intermicrobe Activity (BaCTeRIA) Summary Report" TR-17-017.

CHARACTERIZATION OF A NARROW-SPECTRUM ANTIMICROBIAL THAT EXHIBITS SPECIFIC ACTIVITY AGAINST UROPATHOGENIC BACTERIA

1 Introduction

The Biological Sciences and Technology Team (BSTT), Warfighter Directorate, of the U.S. Army Natick Soldier Research, Development and Engineering Center (NSRDEC) has been investigating the use of narrow-spectrum antimicrobials in non-traditional textiles to protect the Warfighter from pathogenic bacteria. This investigation comprised several parts, including identification and characterization of bacteriocins, a class of narrow-spectrum antimicrobials, against specific pathogenic targets. This project, which started in October 2015 and ran through September 2016, shifted the focus from developing the protocols for identifying and characterizing bacteriocins to a specific application; namely, developing a wipe that contains bacteriocins for the purpose of preventing urinary tract infections (UTIs).

The objective of this technical report is to describe the selection and production of a bacteriocin that exhibits specific activity against uropathogenic bacteria and the effects of a stabilizing solution on the long-term stability of the selected bacteriocin.

The investigation of narrow-spectrum antimicrobials was motivated by the need to develop antimicrobial technologies that can be employed as alternatives to broad-spectrum antimicrobials. The widespread use of broad-spectrum antimicrobials is a critical contributing factor toward the development of bacteria strains that are resistant to treatment by antimicrobials in a polymicrobial community (Levy *et al*.). As a result, infections that are currently treatable may not be in the near future. Broad-spectrum antimicrobials function by targeting the entire bacterial population to be killed, though some bacteria may be genetically pre-disposed to be resistant to the fatal effects of the antimicrobial. These bacteria will survive exposure to the antimicrobial while benign microorganisms that contribute to keeping drug-resistant pathogenic strains under control are killed. Because of this, the resistant strains of bacteria are able to grow unrestrained (Levy *et al*., Gulberg *et al*.). Multiple exposures of the polymicrobial community to the same broad-spectrum antimicrobial will promote the growth of the resistant strains so that they dominate the microbial community.

Narrow-spectrum antimicrobials function through specific activity against a bacterial target or targets while having little or no effect on other bacteria in a polymicrobial community.
One such group currently being investigated is bacteriocins, a class of narrow-spectrum antimicrobial peptides. Bacteriocins are peptides produced by one bacterial species to kill related bacterial species competing for the same resources. Many bacteriocins have very specific activity spectrums, as they only target one or two species. This selectivity offers a paradigm shift of applying antimicrobials where instead of broadly killing everything, specific pathogens could be targeted, leaving beneficial bacteria unaffected and capable of thriving (Birkemo *et al*.). Another attractive feature is that few bacteriocins have demonstrated adverse effects on eukaryote cells (Cotter *et al*., Cox *et al*., Galvz *et al*.). This beneficial effect is not always true for antimicrobials. These and other characteristics make bacteriocins an appealing option to supersede broad-spectrum antimicrobials.

The BSTT has been engaged in studies of bacteriocins to develop antimicrobial treatments for textiles that are exposed to the skin. The objective of those studies has been to limit the growth of pathogenic species on the skin to prevent infection while keeping the beneficial commensal bacteria intact. The development of novel textiles with narrow-spectrum antimicrobial treatments could prevent the inception of infection and enable the Warfighter to maintain performance to execute mission objectives. Previous work by the BSTT ("Bactericidal Coatings on Textiles for Remediation of Intermicrobe Activity Summary Report" 2017) focused on establishing protocols to identify bacteriocins with desired activity against a specific pathogenic strain of bacteria; exploration of growth conditions to optimize yield of bacteriocin production by the host microorganism; and developing purification procedures to deliver high yields of purified antimicrobial peptide for further development. Other work by the BSTT (Functional Oxides as Reactive Coatings for Enhanced Protection on Textiles [FORCE ProTex]) investigated the encapsulation of a narrow-spectrum antimicrobial peptide for application on a textile. The results of that effort suggested that the encapsulation method evaluated (encapsulation by titania precipitation) did not successfully stabilize the antimicrobial peptide for long-term storage, eluding the challenge of applying a biologically based antimicrobial treatment to a textile.

Due to the increased presence of women on the battlefield, technologies that target female-centric infections, such as UTIs, are of great interest. The prevalence of UTIs in women is higher than in men with about 81% of UTIs occurring in women, peaking between the ages of 16 and 35. Estimates suggest that one out of three women will experience a UTI episode during their lifetime (Salvatore *et al*.). Further, 50% of women who are deployed report acquiring a UTI. This percentage is only an estimate, as it is speculated that not all women who contract a UTI when deployed report the event (Doherty *et al*.). Gram-negative and Gram-positive bacteria, as well as some fungi, cause UTIs. The most common cause of UTIs is uropathogenic *Escherichia coli* (*E. coli*) (Flores-Mireles *et al.*). While in the field, there are several risk factors that increase a woman's susceptibility to contracting a UTI including infrequent urination, dehydration, and poor hygiene. Deployed females frequently practice voluntary dehydration to reduce the frequency of urination, and 77% of female Warfighters have reported holding their urine for an extended amount of time. Treatment of UTIs has been estimated to cost $81 million per year and frequently results in time out of the theater. In the military, current approaches for UTI prevention focus primarily on training, but this approach has had minimal impact on preventing the occurrence of UTIs (Doherty *et al*.). Symptomatic UTIs are commonly treated with untargeted antibiotics. However, the application of these treatments may lead to long-term effects on the normal microbiota of the vagina and gastrointestinal tract and promote the development of multi-drug resistant microorganisms (Flores-Mireles *et al.*).

The increase of females in the field, the high incidence of UTIs encountered by women, the detrimental effects of UTIs on mission performance, and the resulting effects of using broad-spectrum antibiotics as treatments for UTIs render this infection a critical occurrence that should be addressed to improve the performance of the Warfighter. The objective of the work, detailed in this report, was to identify a colicin, a type of bacteriocin produced by and active against species of *E. coli,* that exhibits specific activity against uropathogenic bacteria to target the prevention of UTIs. Additionally, optimization of the colicin's production and purification was also investigated. Uropathogenic *E. coli* were specifically chosen as the target of this work due to their high incidence of causing UTIs. The specific class of bacteriocins chosen to investigate was

colicins. Previous work has demonstrated that colicins provide a novel alternative to prevent UTIs. Results of an *in vitro* study found that catheter colonization by a susceptible clinical isolate was completely prevented by coating the catheter with a colicin-producing strain of *E. coli* (Trautner *et al*). Additionally, colicins have been shown to exhibit highly specific antimicrobial activity with a decreased incidence of pathogen resistance (Dorit *et al.*).

The identification and production of a narrow-spectrum antimicrobial, such as a colicin that targets uropathogenic bacteria, may provide the basis for the development of a novel textile that prevents the contraction of UTIs. However, previous work (FORCE ProTex) demonstrated that application of a narrow-spectrum antimicrobial peptide to a textile is not trivial. Antimicrobial peptides are sensitive to environmental conditions and arrangement/orientation on the textile. Further, the application of the antimicrobial peptide to the textile must be robust and durable to ensure that the antimicrobial peptide retains activity over the lifecycle of the textile. To circumvent the challenges associated with applying a narrow-spectrum antimicrobial to a textile, a hygiene wipe was propositioned as a novel textile for narrow-spectrum antimicrobial peptide application. A hygiene wipe can be associated with an aqueous solution that could be tailored to retain the activity of the biological agent. Wipes containing narrow-spectrum antimicrobials are currently being used to combat infections in farm and domestic animals. The application of these hygiene wipes with narrow-spectrum antimicrobials have yielded positive results leading to significant reductions in the incidence of infection compared with more traditional approaches, such as broad-spectrum antibiotics or untargeted antimicrobials (Cotter *et al*., Immucell *et al.*, Field *et al.*). Many of the side effects associated with broad-spectrum antibiotics—irritation, chapping, lesions, and increases in antibiotic resistance—were reduced or eliminated when narrow-spectrum antimicrobials were employed (Mueller *et al*., Nickerson *et al*.). This precedence suggests that application of narrow-spectrum antimicrobials in a hygiene wipe could provide a method for preventing infection.

The efforts described in this report focus on the selection and production of a colicin from a host microorganism. These preliminary studies evaluated the antimicrobial activity of the colicin against representative uropathogenic and commensal microorganisms. Exploratory investigations were conducted to assess growth conditions of the host microorganism and production conditions for the production of the colicin as well. The preliminary results from this investigation may provide the basis for the development of technology based on this colicin, such as a hygiene wipe to prevent the inception of UTIs in the field. Engagement with interested and relevant parties, such as the Army Medical Department and industry, would be necessary to exploit the efforts of this investigation to develop a viable hygiene wipe to ensure performance of the female Warfighter is maintained in the field.

2 Materials and Methods

2.1 Bacterial Strains

An established collection of colicin-producing *E. coli* was used as the source of colicins to evaluate activity against the chosen target microorganisms. Table 1 describes the collection of colicin-producing microorganisms including the host strain, molecular weight, and extinction coefficient (Cursino *et al*., Pugsley, Riley *et al.* 2000.). This collection of colicin-producing bacterial strains was a generous gift from Professor Margaret Riley at UMass Amherst.

Information for the colicins used in the study is provided, including extinction coefficient, molecular weight, and host strain. The extinction coefficients and molecular weights were obtained from Bactibase (http://bactibase.pfba-lab-tun.org/main.php), an international database for bacteriocins and/or from calculations based on the peptide's sequence using ExPASy ProtParam tool (http://web.expasy.org/protparam/), an online bioinformatics resource. The extinction coefficient for colicin L could not be determined.

Table 1. Collection of Colicins Used for Screening.

Colicin	Extinction Coefficient ($M^{-1}cm^{-1}$)	Molecular Weight (kDa)	Host Strain
A	51350	62.989	BZB2101
B	63370	54.732	BZB2102
D	73340	74.688	BZB2103
E1	41370	52.279	BZB2104
E2	51450	61.561	BZB2125
E3	57410	57.960	BZB2106
E4	23950	56.000	BZB2107
E5	26470	19.874	BZB2108
E6	58900	58.011	BZB2109
E7	51450	61.349	BZB2110
E8	17990	23.197	PAP247
E9	47440	61.587	PAP1407
G	66015	46.574	CA46
H	48025	40.656	CA58
IA	59360	69.406	BZB2114
IB	52370	69.963	ColIb-P9
Js	70820	54.085	ColJs
K	46870	59.611	BZB2116
L	-	64.000	L-JF246
M	33457	29.453	PAP1
N	50880	41.696	BZB2123
S4	70820	54.085	PAP2
V	29575	17.907	PAP222
Y	60850	67.162	K339
10	47900	53.342	BZBlOl1-pCol10
5	35410	53.137	ECOR5

A representative collection of *E. coli* uropathogenic bacteria was selected to evaluate the activity of colicins against bacterial strains that cause UTIs. The uropathogenic bacterial strains were purchased from ATCC (Manassas, VA). The collection of strains evaluated were ATCC 53498, ATCC 700417, ATCC 700928, ATCC 700415, ATCC 25922, ATCC 700414, and ATCC 700336.

To determine the effect of colicins on commensal microorganisms, a selection of commensals representative of the bacteria community in the vaginal area was evaluated. These commensals were *Atopobium vaginae* ATCC BAA-55 (ATCC, Manassas, VA), *Lactobacillus vaginalis* ATCC 49540 (ATCC, Manassas, VA), *Staphylococcus epidermidis* (collected by lab), and *Staphylococcus aureus* ATCC 27515 (ATCC, Manassas, VA).

2.2 Colicin Production

Preparation of Crude Lysate for Screening Assays

A crude, unpurified cell lysate of colicin-producing *E. coli* was used to test for activity against uropathogenic and commensal microorganisms (Herschman *et al.*). 10 mL of Luria Broth (VWR, Radnor, PA) was inoculated with a colony of colicin-producing microorganisms. The culture was grown overnight with shaking (220 rpm) at 37 °C. The next day (~16 h), 1 mL of colicin-producing *E. coli* was transferred to 9 mL of Luria Broth and incubated for 1 h at 37 °C with shaking (220 rpm) until an optical density (OD) of 0.3-0.5 was achieved. The culture was then induced with 3 µL of 0.5 mg/mL (1.5 µg) mitomycin C (Alfa Aesar, Ward Hill, MA) and incubated for 3 h with shaking (220 rpm) at 37 °C. Following induction, 2 mL of the cell culture was removed and placed in a 2 mL Eppendorf tube. To each culture, 50 µL of chloroform (Sigma Aldrich, St. Louis, MO) was added and the sample was vortexed to mix thoroughly. The sample was then centrifuged for 5 min at 15,000 rpm. The supernatant was removed from the centrifuged samples (1.5 mL) and placed into a new Eppendorf tube. Samples were stored at 4 °C until needed.

Larger Scale Preparation for Characterization Assays

To produce greater quantities of colE7, two 100 mL aliquots of Luria Broth were each inoculated with the colicin-producing *E. coli*. The cultures were incubated overnight with shaking (220 rpm) at 37 °C. The next day, the overnight cultures were used to inoculate two 900 mL flasks of Luria Broth. These cultures were incubated at 37 °C with shaking for 1-2 h until an OD of 0.3 was achieved. Each culture was induced with the addition of 500 µL of 0.5 mg/mL (250 µg) of mytomycin C and then incubated for an additional 3 h. Centrifugation at 10,000 rpm at 4 °C for 15 min was used to remove the cells from the culture. The resulting supernatant was removed and stored at 4 °C until needed.

2.3 Evaluation of Colicins for Antimicrobial Activity Against Uropathogenic Bacteria

Lysates of colicin-producing microorganisms were screened for antimicrobial activity using an activity drop test assay against several strains of uropathogenic bacteria. The uropathogenic microorganisms were prepared by inoculating a 10 mL aliquot of Luria Broth. The culture was incubated at 37 °C with shaking (220 rpm) until an OD of 1.0 was achieved. A 7 mL aliquot of Luria Broth soft agar was inoculated with 20 µL of the target culture. This mixture was poured over a fresh Luria Broth agar plate, ensuring even coating of the plate, and allowed to dry thoroughly. For the activity drop test, 6 µL of the colicin-producing cell lysate was dropped in

triplicate onto the field of target organism. Plates were incubated at 37 °C overnight and assessed the following day for activity. Activity was determined by the presence of a zone of clearing around the lysate drop.

2.4 Evaluation of Colicins for Antimicrobial Activity Against Commensal Bacteria

Cultures of commensal bacteria were prepared as follows: *Atopobium* was inoculated in 10 mL of Tryptic Soy Broth. *Lactobacillus* was inoculated in 10 mL of De Man, Rogosa and Sharpe broth (MRS). *S. epidermidis* and *S. aureus* 27515 were inoculated in 10 mL nutrient broth. Each were incubated at 37 °C with agitation (220 rpm), with the inclusion of a CO_2-rich environment for *Atopobium* and *Lactobacillus*, for 4.5-5 h until an OD of 0.7-1 was achieved.

Colicin-producing microorganisms and crude lysates of colicins were prepared as previously described. A soft agar overlay was prepared with each commensal culture to evaluate activity of the colicin-producing microorganisms by activity drop test. A 7 mL aliquot of Luria Broth soft agar was inoculated with 20 µL of the commensal target culture. This mixture was poured over a fresh Luria Broth agar plate, ensuring even coating of the plate, and allowed to dry thoroughly. For the activity drop test, 6 µL of the colicin-producing microorganism was dropped in triplicate onto the field of target organism. Plates were incubated at 37 °C overnight and assessed the following day for activity. Activity was determined by the presence of a zone of clearing around the lysate drop.

2.5 Determination of Arbitrary Activity Units

To quantify the activity of colicin in cellular lysates, a critical dilution assay was conducted (Parente *et al*. 1995). Crude lysates containing colicins were serially diluted two-fold. A 6 µL aliquot of each dilution was dropped on a lawn of target *E. coli* ATCC 53498 in soft agar and incubated overnight at 37 °C. The next day, the plates were evaluated for activity as determined by zones of clearing. The critical dilution was determined as the highest dilution to yield an explicit zone of clearing. When a definite zone of clearing was followed by an indistinct one, the critical dilution was calculated as the average of the two dilutions. The arbitrary activity units (AU/mL) were calculated as the reciprocal of the volume of the drop used for the activity assay in mL (1/0.006 mL) multiplied by the dilution factor for the critical dilution.

2.6 Optimization of Colicin E7 Production

Evaluation of Colicin Production in Varying Growth Media

Cultures of *E coli* producing colA, colE7, colN and col10 were produced by inoculating 10 mL of Luria Broth with a single colony and incubated overnight at 37 °C with shaking (220 rpm). The next day (~16 h), a 1 mL aliquot of each culture of microorganism that produces colicin was used to inoculate 9 mL of Luria Broth, Nutrient Broth, Brain Heart Infusion Broth, and Tryptic Soy Broth, respectively. The cultures were incubated at 37 °C with shaking (220 rpm) for 1 h. Each culture was induced with the addition of 3 µL of 0.5 mg/mL mitomycin C and incubated at 37 °C with shaking (220 rpm). A 2 mL aliquot of the induced cultures was transferred to a fresh Eppendorf tube and 50 µL of chloroform was added. Each tube was vortexed and then centrifuged for 5 min at 14260 xg. A 1.5 mL aliquot of each supernatant was transferred to a fresh Eppendorf tube and stored on ice. Activity was assessed by activity drop test against a field of *E. coli* ATCC 53498.

Evaluation of Alternative Induction Methods

A single colony of *E. coli* ATCC strains 25922, 700414, and 700417 were each inoculated in 10 mL of Luria Broth and incubated at 37 °C with shaking (220 rpm) until an OD of 1.0 was achieved. A 2 mL aliquot of each culture was transferred to a clean Eppendorf tube and centrifuged at maximum speed for 10 min. A 1.5 mL aliquot of each supernatant was filtered through a 0.2 µm syringe filter and stored at 4 °C overnight. A single colony of colE7-producing microorganism was inoculated in 10 mL of Luria Broth and incubated at 37 °C with shaking overnight. Eleven cultures of colE7-producing microorganism were prepared by combining a 1 mL aliquot of the colE7 overnight culture with 9 mL of Luria Broth and incubating at 37 °C with shaking until an OD of 0.3-0.5 (~1 h) was achieved. To each colE7-producing microorganism culture the following aliquots were added to assess alternative induction methods to enhance colicin production: 1) 3 µL (0.5 mg/mL) mitomycin C; 2) 500 µL ATCC 700417 supernatant; 3) 250 µL of ATCC 700417 supernatant; 4) 125 µL of ATCC 700417 supernatant; 5) 500 µL ATCC 25922 supernatant; 6) 250 µL of ATCC 25922 supernatant; 7) 125 µL of ATCC 25922 supernatant; 8) 500 µL of ATCC 700414 supernatant; 9) 250 µL of ATCC 700414 supernatant; 10) 125 µL of ATCC 700414 supernatant; and 11) no addition of induction agent. The induced cultures were incubated at 37 °C with shaking for 3 h and then a 2 mL aliquot of each was transferred to an Eppendorf tube. A 50 µL aliquot of chloroform was added to each tube, then vortexed and centrifuged for 5 min at 14260 x g. A 1.5 mL aliquot of each supernatant was removed and transferred to a clean Eppendorf tube. Samples were evaluated for activity using an activity drop test against a field of *E. coli* ATCC 53498.

Evaluation of Incubation Time Before Induction

A single colony of *E. coli* producing colE7 was used to inoculate 10 mL of Luria Broth. The culture was incubated overnight at 37 °C with shaking. The next day, six preparations of colE7-producing microorganism were made by combining 1 mL of the overnight culture with 9 mL of Luria Broth. Cultures were incubated at 37 °C before induction with mitomycin C. The pre-induction incubation times evaluated were: 30 min, 60 min, 90 min, 120 min and 150 min. After the respective pre-induction incubation time, the culture was induced by the addition of 3 µL of 0.5 mg/mL mitomycin C and incubated for 3 h at 37 °C with shaking. A 2 mL aliquot was then transferred to a clean Eppendorf tube and 50 µL of chloroform was added. Each tube was vortexed and centrifuged for 5 min at 14260 x g. A 1.5 mL aliquot of each supernatant was transferred to a fresh Eppendorf tube. An activity drop tested was employed to assess the activity of each sample against a field of *E. coli* ATCC 53498.

Evaluation of Mitomycin C Concentration for Induction

Three 10 mL aliquots of Luria Broth were inoculated with a single colony of colE7-producing *E. coli* and incubated overnight at 37 °C with shaking. The next day, six preparations of colE7-producing *E. coli* were prepared by combining 1 mL of the overnight culture with 9 mL of Luria Broth. Cultures were incubated at 37 °C before induction for 1 h or 2 h. Varying volumes of mitomycin C were added to assess the effect of mitomycin C concentration on enhanced production of colE7. The volumes of 0.5 mg/mL of mitomycin C evaluated were 3 µL, 5 µL and 10 µL. After addition of mitomycin C, samples were incubated for 3 h. A 2 mL aliquot was removed and transferred to a fresh Eppendorf tube and 50 µL of chloroform was added. Each tube was vortexed and centrifuged for 5 min at 14260 x g. A 1.5 mL aliquot of each supernatant

was transferred to a fresh Eppendorf tube and stored at 4 °C until needed. Activity was assessed using an activity drop test assay against a field of ATCC 53498.

2.7 Determination of Total Protein Concentration

The total protein concentration was determined by Bicinchoninic Acid (BCA) protein assay using the Pierce® BCA Protein Assay Kit (Thermo Scientific, Waltham, MA). First, diluted bovine serum albumin (BSA) standards were prepared following the manufacturer's instructions. The BCA working reagent was prepared by mixing 50 parts BCA Reagent A with 1 part BCA Reagent B. A 25 µL of each standard or sample replicate was transferred into the wells of a micro-well plate. A 200 µL aliquot of the working reagent was added to each well and the plate was placed on a plate shaker for 30 s to mix. The plate was then covered and incubated at 37 °C for 30 min. The plate was removed from the incubator and allowed to equilibrate to room temperature. Absorbance readings at 562 nm were taken using an Eon™ High Performance Microplate Spectrophotometer (Biotek, Winooski, VT).

2.8 Colicin Purification by FPLC

The lysate collected from the colicin E7 producing *E. coli* bacterial strain was processed by tangential flow filtration (TFF) using a Millipore 30,000 molecular weight cutoff (MWCO) filter. The retentate was washed with 25 mM Tris (American Bioanalytical, Natick, MA) pH 8.5 with 25 mM NaCl (Mallinckrodt, Ireland) two times. The retentate was further purified by Fast Protein Liquid Chromatography (FPLC) using ion exchange chromatography. The retentate was loaded onto a HiPrep Q FF 16/10 column (10 mL bed) on a NGC BioRad FPLC (Hercules, California). The column was washed with 30 mL 25 mM Tris pH 8.5 with 25 mM NaCl. Peptides were eluted using a salt gradient of 25mM Tris with a range of 0 to 250 mM NaCl. Fractions containing colicin E7 were determined by evaluating the fractions for antimicrobial activity using an activity drop test. ColE7 purified by FPLC was characterized by gel electrophoresis using a 4-12% Bis Tris gel. The gel was stained using SimplyBlue Coomassie stain (Life Technologies, Carlsbad, CA) to visualize the protein bands. Active fractions were pooled and stored at 4 °C until needed.

2.9 Colicin Purification by HPLC

High pressure liquid chromatography (HPLC) analysis was conducted using a C18 column (GE Healthcare Life Sciences, Marlborough, MA) with 20% acetonitrile/water as elution buffer A and 100% acetonitrile as elution buffer B. Both elution buffers contained 0.1% trifluoroacetic acid (TFA) (EMD Millipore, Billerica, MA). A 25 µL aliquot of pooled elution fractions of FPLC purified colE7 was injected into the HPLC and analyzed using the wavelengths of 250-280 nm. To calculate the yield and purity, the blank chromatograph was first subtracted from the chromatograph of the sample. Purity was calculated by determining the percentage of the target peak area from the total area of all the peaks. The yield was calculated by dividing the peak area obtained from the HPLC chromatogram by the extinction coefficient of colE7 and the flowcell distance.

2.10 Evaluation of Colicin Stability in Stabilizing Solution

A 32.75 mL aliquot of the pooled antimicrobial active fractions from FPLC purification containing colicin E7, with 25 mM Tris pH 8.5 as necessary to obtain a consistent volume, was combined with the stabilizing solution for a final volume of 50 mL. The composition of the

stabilizing solution is listed in Table 2. The pH of the stabilizing solution was tested using a pH meter before combination with the purified colicin E7. To evaluate the effect of individual components of the stabilizing solution on stability of colicin E7, several unique variations of the stabilizing solution were prepared, each with one component omitted. Deionized (DI) water was substituted for the volume of the omitted component. The unique omission stabilizing solutions were also combined with a 32.75 aliquot of the pooled FPLC-purified colicin E7 fractions. Solutions were stored at 4 °C for a time course to investigate the effects of the stabilizing solution with and without components omitted over time. The subsequent activity of colicin E7 was determined by activity drop test assay.

Table 2. Components of Stabilizing Solution.

Reagent	Volume	Initial Concentration	Final Concentration
Ethanol	6 mL	99%	12%
Tween 20	500 µL	100%	1%
Glycerol	10 mL	50%	10%
Catalase			60 units/mL
EDTA	300 µL	500 mM	3 mM
Sodium citrate	205 µL	1 M	varies
Citric acid	~450 µL	1-2 M	9-18 mM

3 Results and Discussion

3.1 Down-Selection of Colicins for Activity Against Uropathogenic Bacteria

To evaluate the feasibility of developing a colicin-based technology to prevent the inception of UTIs, an established collection of colicins was initially screened for activity against an array of representative uropathogenic bacteria. The collection of colicins chosen for this study is well characterized and readily available in the public domain (Cursino *et al*., Pugsley, Riley *et al.*). Other collections of colicins do exist but are privately held. Due to constrained resources, the number of isolates related to UTIs that could be screened was limited. In this study, several representative uropathogenic microorganisms were specifically chosen as characteristic bacterial strains located on the skin with known association with causing UTIs. The uropathogenic strains of *E. coli* were chosen based on specific selection criteria including phylogenetic information and virulence factor. *E. coli* strains are classified into four phylogenetic groups: A, B1, B2 and D. Most uropathogenic strains of *E. coli* are categorized into phylogenetic groups B2 and D (Hancock *et al*., Rijavec *et al*., Smajs *et al*., Vejborg *et al*.). The B2 phylogenetic group has been found to possess more virulence factors than any other phylogenetic group (Johnson *et al*. 2004). Virulence factors characteristic of UTI-causing *E coli* include the following: P fimbriae, type 1 fimbriae, hemolysin, aerobactin, serum resistance, and the K1 capsule (Johnson 1991, Johnson *et al*. 2004). Based on this information, representative uropathogenic bacterial strains for this study were selected based on the criteria that they were classified as the B2 phylogenetic group and/or that they were characterized by the virulence factors listed above.

The collection of colicins was evaluated for activity against representative uropathogenic bacterial strains. Ten *E. coli* uropathogenic bacterial strains were obtained from ATCC: 1) 53498, 2) 700417, 3) 700928, 4) 700415, 5) 25922, 6) 700414, 7) 700336, 8) 700416, 9) BAA1161, and 10) 29425. Unfortunately, only the first seven strains arrived in time for the screening assay and thus the collection of colicins was only screened for activity against these seven. Initially, an activity drop test using crude lysates acquired from colicin-producing microorganisms was used to evaluate the activity of the collection of colicins. An aliquot of each unique lysate was dropped onto a lawn of uropathogenic target bacteria in soft agar and activity was determined by the observation of a zone of inhibition. Minimal activity of the lysates from the colicin-producing organisms was observed. This result may be due to a low concentration of the colicin present in the lysate. To overcome this challenge and rapidly screen the collection of colicins, the colicin-producing host microorganisms were used for the activity drop test. Using the microorganism for the activity drop test instead of the lysate enables the colicin to be directly secreted on the lawn of target and evaluated for antimicrobial activity.

Figure 1 shows representative activity results of several of the colicin-producing microorganisms against two of the seven strains of the uropathogenic bacteria that were screened. In Figure 1A, results for the drop test antimicrobial activity assay with microorganisms producing colE3, colE4, colE5, colE6 and colE7 against *E. coli* ATCC 53498 are presented. The zone of clearing around each colicin-producing microorganism suggests that the colicins secreted by these microorganisms induce antimicrobial activity against the target strain *E. coli* ATCC 53498. The microorganism that produces colE4 did not grow, which may be because colE4 was outcompeted by colicins produced by *E. coli* ATCC 53498. Figure 1B shows the results of the activity drop test using microorganisms that produce colicins M, N, S4, S4s, and S4l against the target *E. coli*

ATCC 700414. The microorganisms that produce colM and colS4 did not grow. While the microorganisms that produce colN, colS4s and colS4l grew, a zone of clearing was observed only around the microorganism that produces colN. This implies that colN is active against the target *E. coli* ATCC 700414 while colS4s and colS4l are not.

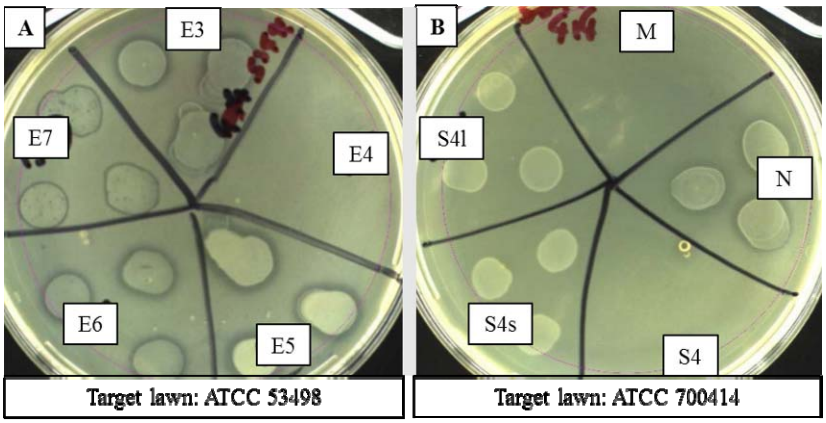

Figure 1. Representative Results of Activity Drop Tests Using Colicin-Producing Microorganisms Against Uropathogenic Bacterial Strains ATCC 53498 (A.) and ATCC 700414 (B.).

Table 3 presents a summary of the antimicrobial activity of colicin-producing microorganisms against the uropathogenic bacterial strains, with the uropathogenic bacterial strains listed in the top row and the collection of colicins screened in the first column. Cells colored in green indicate that antimicrobial activity was observed. For example, colA demonstrated activity against *E. coli* ATCC strains 53498, 700417, 25922, and 700338, while colV demonstrated no antimicrobial activity against any of the uropathogenic bacteria strains tested. Analysis of the data resulting from the activity drop tests using the colicin-producing microorganisms enabled down-selection of the collection of colicins based on several criteria. First, colicins that demonstrated no activity against the seven uropathogenic bacterial strains evaluated were excluded (colicins: Ib, Js, M, and Y). Next, colicins that exhibited activity against only one of the uropathogenic bacterial strains were eliminated (colicins: E1small, E2, E5, E6, E8, E9, H, Ia, K, L3, L, and 5). Finally, colicins that demonstrated activity against less than half of the seven uropathogenic bacterial strains were disregarded unless they uniquely exhibited activity against one uropathogenic bacterial strain that no other colicin displayed activity against. Colicins that demonstrated activity against less than half of the bacterial strains screened include colB, colD, colE1, colE3, colE4, colE7, colG, colL2, colS4small, colS4large, and col10. The results of the activity drop assays for these colicins were further assessed to determine if they uniquely demonstrated antimicrobial activity against uropathogenic strains that the other colicins did not. For example, colE7 and colE4 were the only two colicins to demonstrate activity against *E. coli* ATCC 700938. Because the activity of colE7 was greater than the activity of colE4 against *E. coli* ATCC 700938, colE7 was selected for further testing. Applying the down-selection criteria resulted in selection of four colicins for further evaluation: colA, colE7, colN, and col10. Both colA and colN exhibited activity against more than half of the target uropathogenic bacterial strains.

Table 3. Summary of Antimicrobial Activity of Colicin-Producing Microorganisms Against Uropathogenic Bacteria.

	53498	700417	700938	700414	700415	25922	700338
A							
B							
D							
E1							
E1 small							
E2							
E3							
E4							
E5							
E6							
E7							
E8							
E9							
G							
H							
IA							
IB							
Js							
K							
L2							
L3							
L							
M							
N							
S4sm							
S4 large							
V							
Y							
10							
5							

To further down-select to a single colicin that demonstrates appreciable antimicrobial activity against the representative uropathogenic microorganisms, the cellular lysates of the host micoorganisms for colicins A, E7, N, and 10 were evaluated for activity. The size and strength of the zone of inhibition induced by the lysates for the activity test provides an approximate indication of the quantity of colicin produced by each host microorganism. Figure 2 displays representative activity results of the four colicin cellular lysates (colE7, colA, colN, and col10) against four of the *E. coli* uropathogenic bacterial strains (ATCC 53498, ATCC 25922, ATCC 700928, and ATCC 700414). A zone of clearing where the colicin-containing lysate was dropped among the turbid background of the target microorganism lawn signifies that the colicin-

producing microorganism's lysate exhibits antimicrobial activity against that target strain, suggesting that the colicin present in the lysate is active. The more pronounced the zone of clearing, the greater the activity observed, suggesting a greater yield of the antimicrobial protein. For example, Figure 2 shows that colE7 demonstrates significant activity against target strain ATCC 53498 while colA exhibits less activity against this strain as the zone of clearing for the colE7 lysate is more prominent than that of the colA lysate. Also of note, Figure 2 shows that uropathogenic strain *E. coli* ATCC 53498 was susceptible to antimicrobial activity from each of the four colicin-containing lysates tested, which suggests that this strain of bacteria may be the most sensitive to colicin-induced antimicrobial activity of all the uropathogenic bacterial strains tested.

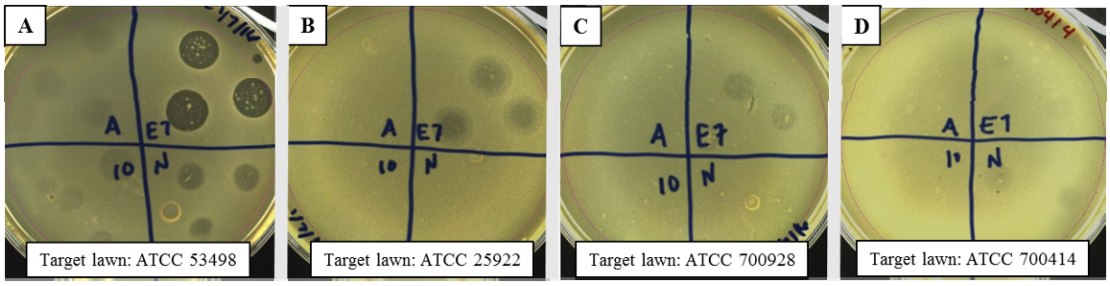

Figure 2. Representative Results of Activity Drop Tests Using Crude Lysates of Microorganisms Against Uropathogenic Bacteria. (A) ATCC 53498, (B) ATCC 25922, (C) ATCC 700928, and (D) ATCC 700414.

Table 4 displays a summary of the activity of colicin-producing microorganism *lysates* (LYS) as well as the colicin-producing *microorganisms* (ORG) against the uropathogenic bacterial strains. The uropathogenic bacterial strains are listed along the top row and the collection of colicins screened are listed in the first column. Boxes colored in green correspond to the observation of antimicrobial activity. For example, the microorganism that produces colA and the cellular lysate of the microorganism that produces colA both demonstrated antimicrobial activity against ATCC 53498, but only the microorganism that produces colA exhibited activity against ATCC 700417. For some of the colicins evaluated, the cellular lysate did not exhibit activity where the colicin-producing microorganism did. This may be due to a diluted, lower concentration of the antimicrobial protein present in the lysate after processing. Whereas when the colicin-producing microorganism is assayed, the more directed and concentrated delivery of colicin secreted by the microorganism toward the lawn of target bacteria enabled activity to be observed. Also of note, the colE7 lysate demonstrated activity against four of the representative uropathogenic bacteria strains, while activity for the colE7-producing microorganism was only observed against two bacterial strains. This observation of activity may be because the microorganism that produces colE7 may be sensitive to colicins produced by the target strain, which inhibited the production of colE7. Further investigation is necessary in order to ascertain the resulting antimicrobial activity of secreted colE7 from the host microorganism.

Table 4. Summary of Antimicrobial Activity of Down-Selected Colicins Against Uropathogenic Bacteria.

	53498		700417		700938		700414		700415		25922		700338	
	LYS	ORG	LYS	ORG	LYS	ORG	LYS	ORG	LYS	ORG	LYS	ORG	LYS	ORG
A														
E7														
N														
10														

The four down-selected colicins were further evaluated by quantifiably characterizing the activity of the colicins in the cellular lysates. This evaluation was achieved by determining the arbitrary activity units for the crude colicin-containing cellular lysates. Crude lysates were serially diluted two-fold and a 6 µL aliquot of each dilution was used in a drop test activity assay against uropathogenic *E. coli* strain ATCC 53498. Uropathogenic *E. coli* strain ATCC 53498 was selected as the lawn of choice as the greatest antimicrobial activity was observed against this strain. The critical dilution was determined as the highest dilution to yield an explicit zone of clearing. When a definite zone of clearing was followed by an indistinct one, the critical dilution was calculated as the average of the two dilutions. The arbitrary activity units (AU/mL) were calculated as the reciprocal of the volume of the drop used for the activity assay in mL (1/0.006mL) multiplied by the dilution factor for the critical dilution. The more dilutions required to observe a decrease in activity, the greater the value of AU/mL, and the greater the activity of the lysate, indicative of a greater quantity of colicin present in the lysate. Using this data, a quantifiable comparison of the crude colicin-producing microorganism lysates for colicins A, E7, N, and 10 was conducted.

Figure 3 shows the results of activity drop tests used to calculate the arbitrary activity units of the crude cellular lysates for colA, colE7, colN and col10 (from left to right). The lysates were serially diluted two-fold and dropped (6 µL) on a lawn of ATCC 53498. The dilution factor for each triplicate is listed in the column on the left and is separated in rows by the dotted lines. Listed in the table in the bottom of Figure 3 are the quantifiable metrics used to assess the activity and yield of each colicin in the crude cellular lysate. The critical dilution factor and the arbitrary activity units were determined as described previously. The arbitrary activity units provide an estimate of activity and quantity of colicin produced and present in the crude cellular lysate. This provides a rapid means to screen the crude, unpurified colicin for antimicrobial activity. Comparison of the arbitrary activity units for the crude colicin lysates suggests that colE7 is the most active while colA is the least active. These results imply that colE7 is produced in a greater quantity by its host microorganism or demonstrates greater activity compared to the other colicins analyzed. To more accurately evaluate the yield of colicin produced by the host microorganism and the subsequent antimicrobial activity of the colicin, additional studies should be carried out to purify the antimicrobial peptide from the cellular lysate to characterize the antimicrobial activity and yield of the individual peptides.

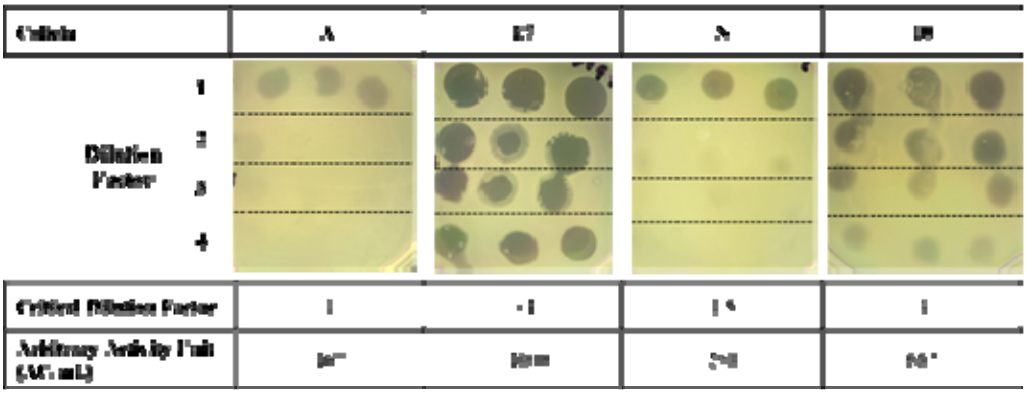

Figure 3. Determination of Arbitrary Activity Units (AU/mL).

To ensure that the colicins do not elicit a threat to commensal microorganisms, colicin-producing micoorganisms were evaluated for activity against several representative vaginal and skin commensals. In order to maintain the health of the commensal microorganism population, a colicin-derived technology to prevent the inception of UTIs should only exhibit specific activity against uropathogenic bacteria that cause UTIs and not trigger a deleterious response by the native microorganism community. Figure 4 displays representative results of the investigation of colicin activity against the commensal microorganisms *S. aureus* (Figure 4A), *Atopobium* (Figure 4B), *S. epidermidis* 106 (Figure 4C), and *Lactobacillus* (Figure 4D). Each panel shows the resulting outcome of an activity drop test, with each row representing a unique colicin-producing microorganism, labeled accordingly. Growth of the colicin-producing microorganisms is observed on each target lawn of commensal microorganisms except for the microorganism that yields colS4. This result suggests that the commensal microorganisms exhibited activity against the microorganism that produces colS4, which prevented its growth. Though more likely, it is possible that the glycerol stock used to initiate the culture of the microorganism that produces colS4 was no longer active as no growth was observed against any microorganism tested. Figure 4 shows that no zones of inhibition are observed around the growth of colicin-producing microorganisms on each commensal target lawn. These results were similar for evaluation of activity against *S. epidermidis* 104 (results not shown). The outcome of this investigation suggests colicin produced and excreted by the colicin-producing microorganisms does not exhibit activity against the commensal microorganisms assessed, which is advantageous for the development of a colicin-based technology targeted to prevent UTIs.

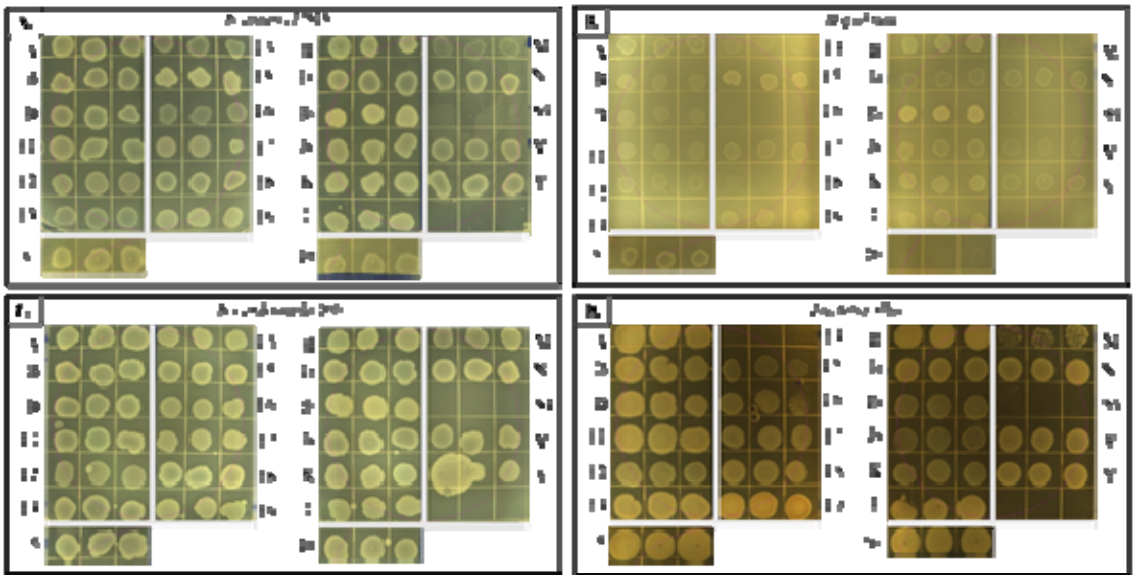

Figure 4. Evaluation of Colicin-Producing Microorganisms Against Commensal Microorganisms. (A.) *S. aureus* 27515, (B.) *Atopobium*, (C.) *S. epidermidis* 106 and (D.) *Lactobacillus*.

While the microorganisms that produce colicins did not elicit a harmful response to the commensals tested, further investigation to assess the effects of the cellular lysate containing the colicins was carried out. Crude lysates acquired from microorganisms that produce colA, colE7, colN, and col10 were evaluated by activity drop test against a target lawn of selected commensal microorganisms. Figure 5A and Figure 5B show the results of the activity drop test against *S. aureus* 27515 and *Atopobium,* respectively. No zones of clearing are observed, suggesting that the crude lysates of microorganisms that produce colA, colE7, colN and col10 do not exhibit activity against these commensal microorganisms. This finding is in agreement with the evaluation of colicin-producing microorganisms for activity against the representative commensal microorganisms. Figure 5C presents the results of the activity drop test against *S. epidermidis* 104. Lysates from microorganisms that produce colA and col10 do not produce any zones of clearing, though very faint zones of clearing are observed for the colN lysates. Figure 5D shows the results of the activity drop test against *S. epidermidis* 106, similar to the activity results against *S. epidermidis* 104. ColA and col10 lysates do not induce activity against *S. epidermidis* 106 while colE7 and colN lysates do. The observed antimicrobial activity against the *S. epidermidis* strains suggest that this commensal microorganism may be susceptible to activity by colE7 and colN, which is contrary to the results observed for evaluating activity of the colicin-producing microorganism (Figure 5C). Activity may have only been observed when evaluating the lysate because the colicin has been liberated from the confines of the cell and is not dependent on excretion, which may hinder the activity of the colicin. A similar result was observed for the evaluation of colE7 against the uropathogenic bacteria. For two uropathogenic strains, antimicrobial activity was observed for the colE7 lysate but no activity was observed for just the colE7-producing microorganism. Finally, Figure 5E displays results of the activity drop test against *Lactobacillus*. Similar to *S. aureus* 27515 and *Atopobium*, no activity is observed for the cellular lysates tested as no zones of clearing are seen.

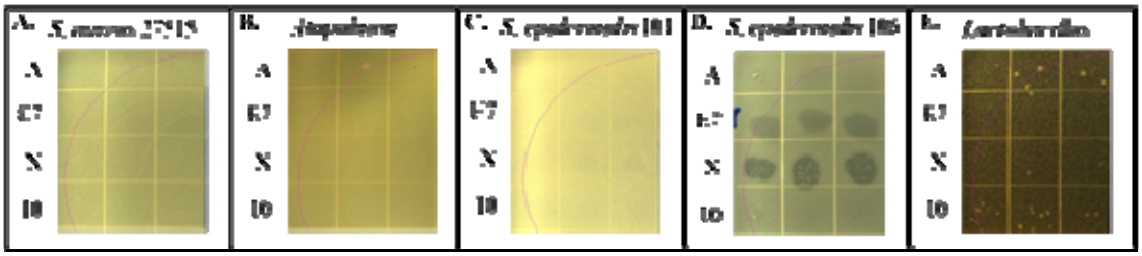

Figure 5. Antimicrobial Activity of Crude Lysates Against Commensal Microorganisms. **(A.),** ***Atopobium*** **(B.),** ***S. epidermidis*** **104 (C.),** ***S. epidermidis*** **106 (D.), and** ***Lactobacillus*** **(E.).**

While the results of this study suggest that commensal microorganisms may be susceptible to antimicrobial activity by colicins, further investigation using purified colicins is necessary in order to fully evaluate the effects of these colicins on the viability of commensal microorganisms. It is possible that the activity observed from the cellular lysates is due to other components present in the lysate and not the colicins, such as cellular debris and other bacteriocins. In addition, a purification of colicins would enable accurate quantification of the antimicrobial peptide so the effects of colicin concentration on antimicrobial activity could be evaluated.

As a result of the analysis previously described in this section, colicin E7 was selected for future efforts. ColE7 demonstrated activity against four out of the seven uropathogenic bacterial strains and against only one out of the five commensal microorganisms evaluated. In addition, the cellular lysate of the colE7-producing microorganism appears to exhibit a significant quantity and activity of colE7.

3.2 Optimization of Colicin E7 Production and Purification

Integration of colE7 into future applications, such as the development of a colicin-based technology to prevent the inception of UTIs, will require industrial scale quantities and purities of the antimicrobial peptide at a reasonable cost. Several experimental conditions were evaluated to assess the effects on the production of colicin E7 by the host microorganism in order to determine the optimal variables required to produce an optimized yield of colicin E7. Conditions evaluated include the method of induction used to enhance colicin production, the amount of time the host microorganism was incubated before induction, the volume of induction agent used, and the growth media used to grow the host microorganism.

The first condition evaluated was the growth media used for the host microorganism that produces colicins. Components of the growth media, such as ion concentration, pH, source of nitrogen, and source of carbon, have all been demonstrated to substantially influence the production of colicins (Cascales *et al.*). In this evaluation, four different growth media were investigated: Luria Broth, Nutrient Broth, Brain Heart Infusion Broth, and Tryptic Soy Broth. The components of each growth media are presented in Table 5. As seen in Table 5, the sources and the quantities of nitrogen and carbon varied among the growth media evaluated. Luria Broth was the simplest media tested with only three components while Brain Heart Infusion was the most complex, consisting of six components. The results of the activity drop tests (results not shown) demonstrated that cellular lysates of microorganisms that produce colA, col10, and colN exhibited the greatest activity when the microorganisms were prepared using Luria Broth and

Nutrient Broth. This result suggests that both of these growth media promote more active or higher production of the antimicrobial peptides, relating to greater antimicrobial activity observed in a given volume. Luria Broth was chosen for future studies due to its simple composition and cheaper cost.

Table 5. Components of Growth Media Evaluated for Effects on Colicin Production.

Luria Broth	Nutrient Broth	Tryptic Soy Broth	Brain Heart Infusion Broth
10.0 g Casein Enzymic Hydrolysate	5.0 g Peptic Digest of Animal Tissue	17.0 g Pancreatic Digest of Casein	6.0 g Brain Heart, Infusion from (solids)
5.0 g Sodium Chloride	5.0 g Sodium Chloride	5.0 g Sodium Chloride	5.0 g Sodium Chloride
5.0 g Yeast Extract	1.5 g Yeast Extract	3.0 g Papaic Digest of Soybean Meal	6.0 g Peptic Digest of Animal Tissues
pH 7.0	1.5 g Beef Extract	2.5 g Dextrose	3.0 g Dextrose
	pH 7.4	2.5 g Dipotassium Phosphate	14.5 g Pancreatic Digest of Gelatin
		pH 7.3	2.5 g Disodium Phosphate

The induction agent used to enhance the colicin synthesis was another condition evaluated to optimize colicin production in a safe, cost-effective manner. Mitomycin C is used extensively throughout literature to induce augmented colicin production (Hardy et. al.,Iijima *et al*.). Mitomycin C functions by cross-linking DNA, thereby causing damage to the genetic molecule (Tomasz *et al.*). In response to environmental stress, such as the induced DNA damage, a coordinated cellular process is initiated to regulate the expression of genetic information through the activation of the cellular SOS system. A transcriptional repressor associated with the SOS response has been found to regulate the expression of colicins. Thus, the mitomycin C-induced DNA damage promotes the production of colicins (Butala *et al.*, Gillor *et al.*). Although mitomycin C is traditionally applied to enhance production of colicins, this induction agent is cost prohibitive and hazardous for use in large-scale or industrial production of colicins. Because of these reasons, an alternative method for induction of colicin production was investigated.

In communities of bacteria, interaction among competing species is regulated by the production of bacteriocins, a family of narrow-spectrum antimicrobial peptides that include colicins. Investigation of the relationship between bacterial strains that produce the lethal antimicrobial proteins led to the discovery that not only is bacteriocin production by one microorganism fatal to opposing bacteria in the community but, additionally, bacteriocin production by one microorganism induces expression of the opposing bacteria's bacteriocin (Majeed, 2011). This finding could provide a different method to enrich the production of colicin without the use of a hazardous or costly induction agent.

To investigate this alternative approach, several of the ATCC uropathogenic strains were evaluated for their ability to induce increased production of colE7. The strains selected for evaluation were ATCC 25922, ATCC 700414, and ATCC 700417. These strains were chosen

because results of previous experiments demonstrated that no activity was observed for these three strains against the uropathogenic target strain, ATCC 53498, used in the activity drop test assay (results not shown). Because of this, any activity observed from the combination of the cell lysate of the colE7 host microorganism and the cell lysate of ATCC 25922, ATCC 700414, or ATCC 700417 may be inferred to be due to the production of colE7. To investigate the effects of the chosen ATCC strains on the production of colE7, varying amounts of ATCC strain cellular lysates were incubated with a culture of colE7-producing microorganism. The arbitrary activity units were determined to assess the resulting production of the antimicrobial peptide.

Figure 6 shows a summary of results for the evaluation of enriched colE7 production in the presence of ATCC 25922, ATCC 700414, and ATCC 700417. The x-axis represents the unique induction agents assessed while the y-axis displays the arbitrary activity units (AU/mL). The production of colE7 can be inferred from the magnitude of AU/mL observed, where the greater value of AU/mL signifies a greater quantity of antimicrobial activity which implies a greater yield of antimicrobial peptide. With no addition of induction agent (negative control), a baseline level of colE7 production by the host microorganism was observed. The addition of mitomycin C yielded the greatest activity, suggesting a large increase in the production of colE7. The application of the ATCC strains as an induction agent provided modest enhancement in activity, suggesting that the production of colE7 was only minimally effected. The results of this study indicate that the addition of mitomycin C yielded the greatest enhancement in production of colE7 while the ATCC strains evaluated provided minimal enrichment of colE7 production.

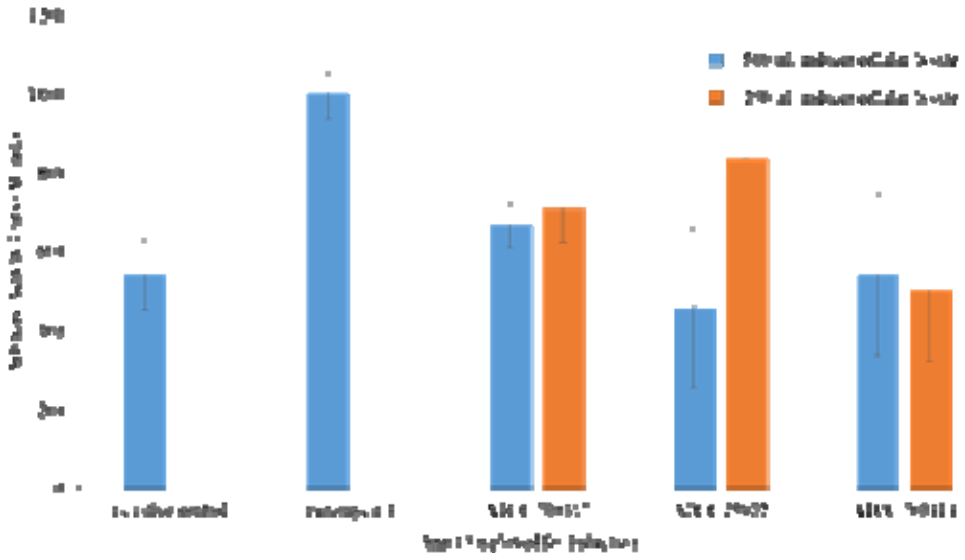

Figure 6. Evaluation of Induction Agents on the Production of Colicin E7.

Additional studies are required to further assess the potential of employing alternative methods to induce enhanced expression of colicin E7 in the future application of the antimicrobial peptide. For example, production of colE7 in the presence of competing whole cells could be assessed instead of just the competing cellular lysate. Also, other strains of bacteria or other colicins could be investigated for their effects on the production of colE7. Furthermore, an engineered bacterial system could be designed and constructed using recombinant DNA

technologies to yield a high expression of colE7 without the need of additional induction agents to enhance production of the antimicrobial peptide.

Growth conditions of the host microorganism of colE7 were investigated to yield optimized production of colE7, which could enable future characterization and application of the antimicrobial peptide. The incubation time before addition of the induction agent to enrich colE7 production was assessed to determine conditions that yield maximum production of the antimicrobial peptide. Increasing the amount of time the culture is incubated before addition of mitomycin C will allow the population of cells to increase, potentially yielding more sources of colE7 production. To assess the effects of pre-induction incubation time on colE7-induced antimicrobial activity, which could relate to the quantity of colE7 produced, a time course of pre-induction incubation times was evaluated. The chosen time points of incubation were as follows: 30 min, 60 min, 70 min, 90 min, 120 min, and 150 min. The enhanced production of colE7 was then induced by the addition of mitomycin C. The cellular lysate of the colE7 host microorganism was assessed for activity using an activity drop test assay. In addition, the total protein concentration was also determined for the cellular lysate of each pre-induction incubation time point using the BCA protein assay.

Figure 7 shows a summary of the results of the arbitrary activity units for colE7 and the total concentration of protein as determined by BCA for each pre-induction incubation time point. The x-axis represents the time points evaluated. The y-axis on the left represents the arbitrary activity units (AU/mL), while the y-axis on the right represents the total protein concentration (mg/mL). As expected, the total protein concentration increased over time (orange line, Figure 7). The longer the colE7 host microorganism was permitted to grow before induction, the greater the population of cells and the greater the quantity of total cellular protein produced. This finding is also supported by an observed increase in OD for the cell culture over time. However, the antimicrobial activity of colE7 over the incubation time course evaluated before induction with mitomycin C remained constant (blue line, Figure 7). This indicates that increasing the incubation time before the addition of a colE7 production enhancing agent has minimal effect on the resulting antimicrobial activity of colE7, suggesting that the production of colE7 remains constant.

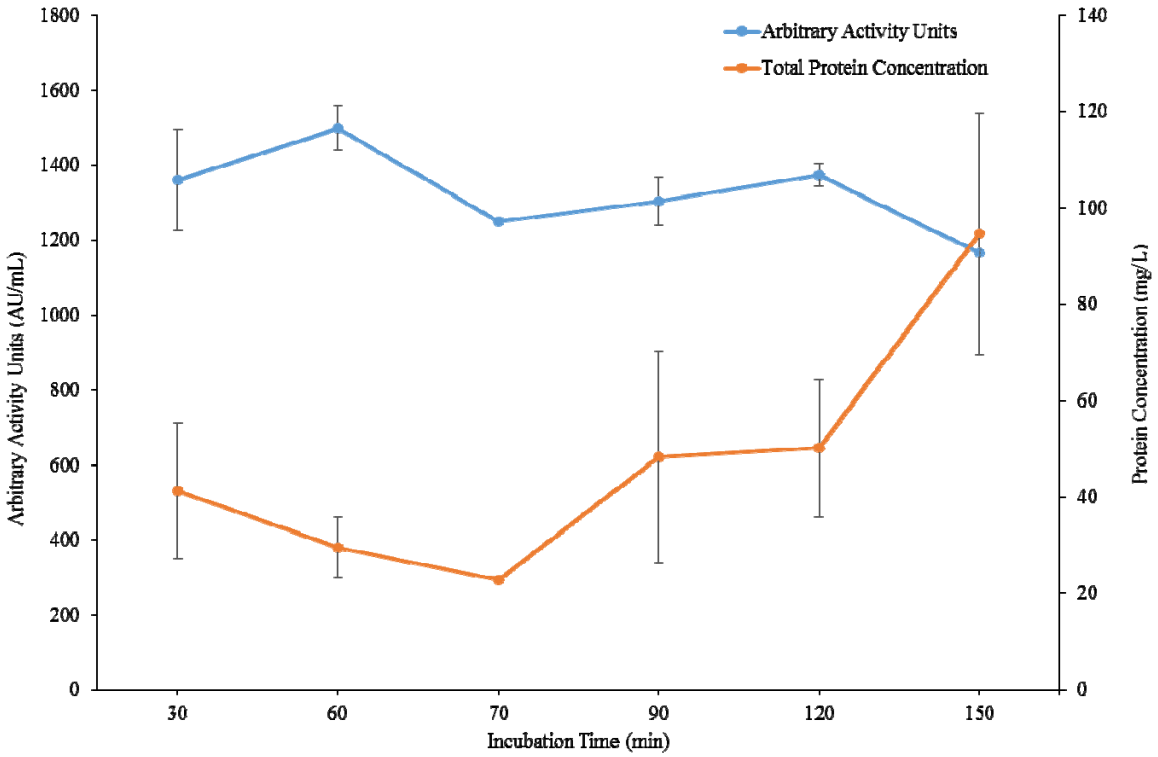

Figure 7. Effect of Pre-Induction Incubation Time on Total Protein Concentration and Activity of Colicin E7.

In conjunction with the observed increase of total protein concentration, the constant activity or production of colE7 over time is unexpected. This may indicate that although the total production of protein increases, the production of colE7 does not increase or that production of colE7 does increase but the resulting antimicrobial peptide is not active. Another possibility may be that the maximum yield of colE7 has been achieved. Further work to elucidate these findings is necessary. For example, in addition to monitoring the OD as the incubation time increases, the number of cells in the culture could be counted to ascertain the extent by which the cellular population increases. The quantity of colE7 produced could be isolated and characterized to determine the concentration of colE7 in comparison to the total protein concentration in the cellular lysate. The information garnered from these additional studies would yield greater insight into the effects of incubation time of the colE7 host microorganism culture before addition of mitomycin C to promote production of the antimicrobial protein.

The concentration of mitomycin C used to enhance the production of colE7 was investigated to determine if an optimal concentration of the induction agent could be identified to yield the greatest quantity of colE7 possible. In previous assays, 3 µL of 0.5 mg/mL (0.15 µg/mL) mitomycin C was added to a 10 mL culture of colE7-producing microorganism to yield a final concentration of 0.15 µg/mL of mitomycin C. To determine if the addition of more mitomycin C would positively affect the enhanced production of colE7, increased concentrations of 0.25 µg/mL and 0.5 µg/mL of mitomycin C were assessed. The incubation time before addition of mitomycin C was also evaluated at 1 h and 2 h for each concentration of mitomycin C tested.

The resulting activity of colE7 was determined by activity drop test assay by which the concentration of colE7 produced could be inferred.

Figure 8 shows a summary of the results of antimicrobial activity of the lysate of the host microorganism of colE7 when varying the concentration of mitomycin C after pre-induction incubation times of 1 h (blue bar) and 2 h (orange bar). The x-axis represents the final concentration of mitomycin C applied to induce enhanced expression of colE7, and the y-axis represents the arbitrary activity units (AU/mL) as determined by activity drop test assay. As seen in Figure 8, the concentrations of mitomycin C evaluated do not affect the AU/mL of the colE7-containing lysate for both pre-induction incubation times tested. As the increased concentration of mitomycin C did not correlate to increased activity of colE7, it is possible to infer that increasing the amount of induction agent does not lead to greatly enhanced production of colE7 in comparison to the baseline. It is possible that an even higher concentration of mitomycin C may significantly promote the production of colE7. However, due to the hazards associated with mitomycin C, investigations with greater amounts of mitomycin C are not feasible.

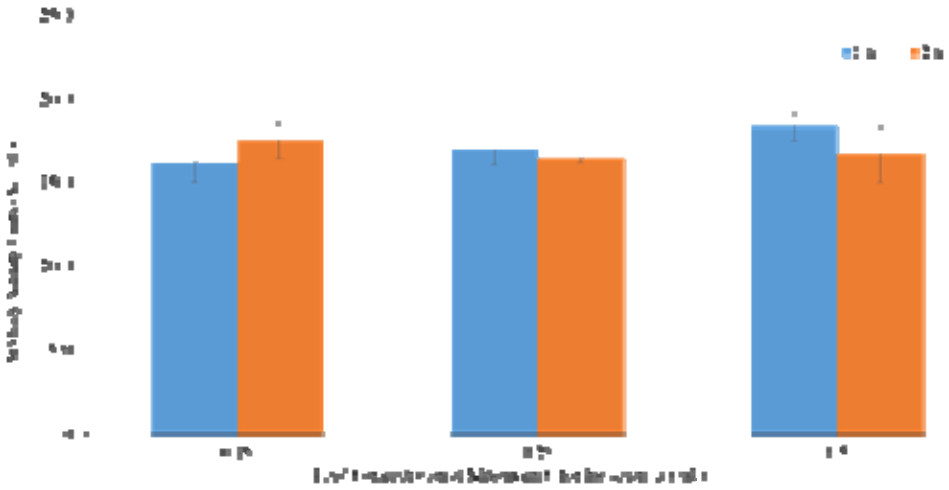

Figure 8. Effect of Mitomycin C Concentration for Induction of Enhanced Production of Colicin E7 on Activity.

3.3 Purification of Colicin E7

Colicin E7 was purified by FPLC. Figure 9A shows a representative FPLC chromatograph with increasing column volume along the x-axis and absorbance values at 280 nm along the y-axis. The observation of peaks in the chromatograph represent the presence of protein being eluted from the column. The numerous peaks observed confirm that after TFF additional purification steps, such as separation by FPLC, is necessary to isolate colE7. To identify where colE7 eluted from the column, elution fractions were collected and evaluated by antimicrobial activity using the activity drop test assay against *E. coli* DH5α. *E. coli* DH5α was selected as an alternative target lawn for screening because the stock of *E. coli* ATCC 53498 was compromised. *E. coli* DH5α has been successfully applied previously as a target lawn for evaluating antimicrobial activity (Birkemo *et al.* 2009). Figure 9B shows representative results of the activity drop test assay for fractions 15–33. The prominent zones of inhibition for fractions 15–26 (highlighted in the red box in Figure 9A) indicate that colE7 is present in these fractions due to the observation

of antimicrobial activity. Additional elution fractions before and after the range presented in Figure 9B were also tested for antimicrobial activity. No activity was observed outside of elution fractions 15–26 which suggests that colE7 consistently elutes in these fractions. The overlapping, multiple peaks that represent elution fractions 15–26 suggest that colE7 is not the only protein present in these elution fractions. Thus, in order to isolate colE7 from other contaminating proteins of the cell lysate, further methods of purification should be employed.

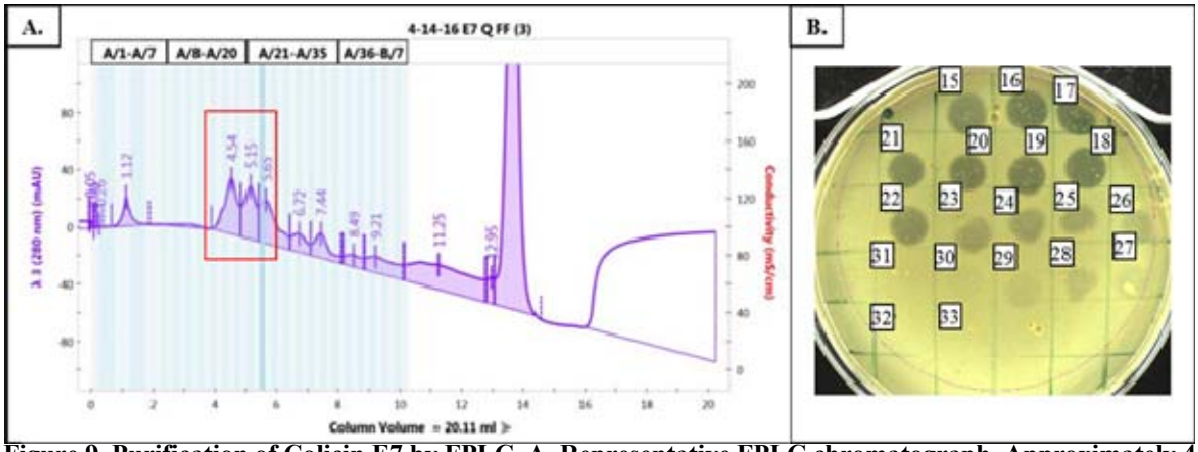

Figure 9. Purification of Colicin E7 by FPLC. A. Representative FPLC chromatograph. Approximately 4 mL elution fractions were collected and assessed for antimicrobial activity using the activity drop test assay. B. Results of activity drop test assay against DH5α for fractions 15 – 33. Prominent zones of inhibition observed for fractions 15 – 26 (highlighted by the red box in A.) suggest that colE7 is present in these eluted fractions.

To evaluate the purity of the FPLC purified colE7, samples of colE7 before and after purification by FPLC were characterized by gel electrophoresis. Figure 10 presents a representative gel. Lane 1 shows purified colE7 from pooled elution fractions of FPLC that were lyophilized to increase the concentration of the protein (240 µg total protein loaded). Lane 2 shows 20 µg of BSA. Lane 3 shows FPLC purified colE7 in stabilizing solution (160 µg total protein loaded). The smearing of the protein bands in Lane 3 may be due to the components of the stabilizing solution, such as Tween. Lane 4 shows purified colE7 from pooled elution fractions of FPLC (24 µg of total protein loaded). Lane 5 shows colE7 before purification by FPLC as the retentate from TFF (240 µg total protein loaded). Lane 6 shows the protein standard with the corresponding molecular weights listed on the right in kDa. Total protein concentration for each sample was determined by BCA. The arrow placed in the left of the figure near Lane 1 highlights the protein band speculated to be colE7. This speculation is based on the size of the antimicrobial peptide, 61 kDa. Further substantiating evidence to determine if the speculated protein band is colE7 is necessary.

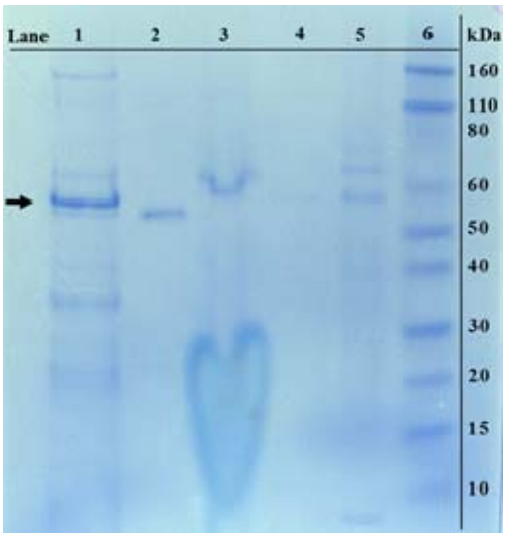

Figure 10. Characterization of Purified Colicin E7 by Gel Electrophoresis.

For instance, the protein in the band could be evaluated for antimicrobial activity. Additionally, the protein in the band could be eluted from the gel and sequenced to ascertain the identity of the protein. As expected, numerous protein bands are present in Lane 1 for the purified colE7 from the pooled FPLC elution fractions, signifying that further purification is necessary to isolate the antimicrobial peptide. Silver stain or fluorescently tagged antibodies specific to colE7 could provide a more sensitive technique to better visualize the protein bands that are present in each sample and provide more information about the composition of each sample. As this gel is not a denaturing gel, it is possible that some of the protein bands observed are aggregates of colE7. Interestingly, although the same amount of total protein was loaded in Lane 1 (concentration FPLC purified colE7) and Lane 5 (FPLC *un*purified colE7 from TFF retentate), more protein bands are visualized in Lane 1 than in Lane 5. This effect may be due to the TFF only providing a cursory, preliminary purification of the cellular lysate to eliminate some of the contaminating proteins. The pooled fractions of protein eluted by FPLC further removed contaminating proteins, yielding a few number of proteins present at a greater concentration in comparison to the TFF sample with a greater number of proteins present, each at a more dilute concentration.

To isolate colE7 from the remaining contaminants of the cell lysate, the colE7 present in the pooled elution fractions by FPLC was further purified by HPLC. Using HPLC as an additional purification measure enabled separation of colE7 from other proteins. HPLC purified colE7 was eluted as one protein peak (results not shown). Analysis of the HPLC chromatograph suggests that the average yield of colE7 is 12.2 mg/L (±0.35) with an average purity of 70% (±5.5). Reported yields of purified colicins and bacteriocins in the literature range from ~30 mg/L for colA (Gokce *et al.*) to 100 mg/L for nisin (Parente *et al*. 1999). While the yield and purity of colE7 obtained in this study is substantial for the preliminary work conducted, increased yield of the antimicrobial peptide would be necessary in order for colE7 to be developed in future applications. Increasing the yield of colE7 may be achieved by continuing the work initiated in this study to optimize the yield of the antimicrobial peptide by evaluating effects of growth conditions. Additionally, the process for scaling up colE7 production by using larger volumes of colE7-producing microorganism cultures should be assessed and optimized to ensure that the greatest yield of peptide is achieved. Further, any contaminating proteins that remain with colE7

should be characterized and assessed for resulting effects on the future development of colE7-based technology.

3.4 Investigation of Colicin E7 Stability

In order for a colicin-derived technology to be used for future applications, the stability of the colicin must be considered. As colicins are biological molecules, they are susceptible to degradation and diminished function due to changes in environmental conditions (e.g., temperature, pH, etc.). As the intended application of the developed colicin is a hygiene wipe to prevent the formation of UTIs, the wipe solution that incorporates the colicin must be carefully considered to ensure that the stability and function of the colicin is retained. With this motivation, the stability of colicin E7 was evaluated in the presence and absence of a stabilizing solution over a time course. The composition of the stabilizing solution was based on a patent related to the company Immucell (Portland, ME) that presents data to support increased stability of the bacteriocin nisin in a stabilizing solution (Blackburn *et al.*). A stabilizing solution with similar composition was investigated for the effects on the stability of colE7 as determined by evaluation of antimicrobial activity by the activity drop test assay. In order to determine the individual effects of each unique component of the stabilizing solution, several variations of the stabilizing solution were tested, where an individual component was omitted to evaluate the effects of that unique component.

Initially, to gain a baseline understanding of the stability of colE7 over time, the antimicrobial activity of colE7 against uropathogenic *E. coli* strains ATCC 53498 and DH5α was monitored before and after incubation at 4 °C for up to 5 weeks. Unpurified colE7 in the crude lysate of the microorganism that produces colE7 and the retentate of the cellular lysate containing colE7 after purification by TFF were tested. Resulting antimicrobial activity was assessed by determining arbitrary activity units (AU/mL) by activity drop test assay against a lawn of *E. coli* strain ATCC 53498 and *E. coli* strain DH5α. Figure 11 presents the arbitrary activity units for colE7 in the crude lysate and colE7 in tangential flow purified retentate over time. Arbitrary activity units are displayed on the y-axis and incubation time in days is displayed on the x-axis. The activity of unpurified colE7 in the cellular lysate retained its activity against the target ATCC 53498 up to the maximum incubation time tested of 30 days (blue data points) and *E. coli* DH5α up to the maximum incubation time tested of 44 days (grey data points). Conversely, colE7 in the retentate collected from purifying cellular lysate by TFF lost activity over time when evaluated against *E. coli* ATCC 53498 (orange data points) and *E. coli* DH5α (yellow data points). When evaluated against *E. coli* ATCC 53498, the AU/mL of colE7 from the TFF retentate decreased by 33% in comparison to the AU of colE7 in the crude cell lysate. The AU/mL of colE7 from the TFF retentate also decreased (by 53%) in comparison to colE7 in the crude cell lysate when tested against *E. coli* DH5α. The difference in AU/mL observed may be due to differences in purity of the antimicrobial peptide. Tangential flow filtration removes impurities from the cellular lysate which may function as stabilizing agents for colE7. The elimination of such stabilizing agents may reduce the stability of colE7, which correlates to reduced activity of the antimicrobial peptide. In general, the higher values of AU/mL observed against *E. coli* DH5α in comparison to *E. coli* ATCC 53498 is expected as *E. coli* DH5α is a bacterial strain that is more susceptible to antimicrobial activity.

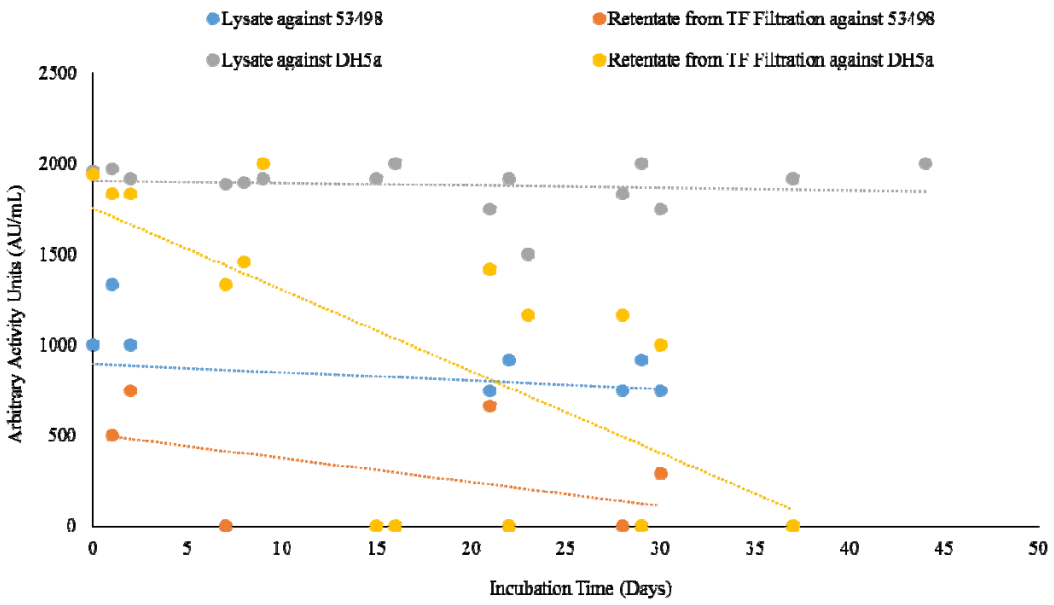

Figure 11. Time Course of Colicin E7 Activity.

Next, the stability of colE7 in a stabilizing solution was investigated over time. ColE7 purified by FPLC was added to a stabilizing solution containing the components listed in Table 2 and incubated at 4 °C. The resulting stability of colE7 was assessed by determining AU/mL using the antimicrobial activity drop test assay against *E. coli* DH5α. In addition to the complete stabilizing solution, colE7 stability was also investigated in several variations of the stabilizing solution where an individual component was omitted in order to evaluate the effects of the unique components of the solution on colE7 stability.

Figure 12 displays the activity against *E. coli* DH5α of FPLC purified colE7 in stabilizing solution over time. The x-axis presents incubation time in days while the y-axis presents AU/mL. Each different colored data bar represents colE7 in a variation of the stabilizing solution. For example, the blue bar represents data for colE7 in the complete stabilizing solution, while the yellow bar represents data for colE7 in the stabilizing solution with glycerol absent. AU/mL at 0 days was determined immediately after combining colE7 with the desired variation of the stabilizing solution. The AU/mL of colE7 in the complete stabilizing solution did not diminish significantly over the timeline of this study. This result suggests that all the components that comprise the stabilizing solution do not exhibit deleterious effects on the antimicrobial peptide. Variants of the stabilizing solution with omitted components were investigated for up to 8 days. During this time, the AU/mL of colE7 did not significantly change for any of the compositions tested.

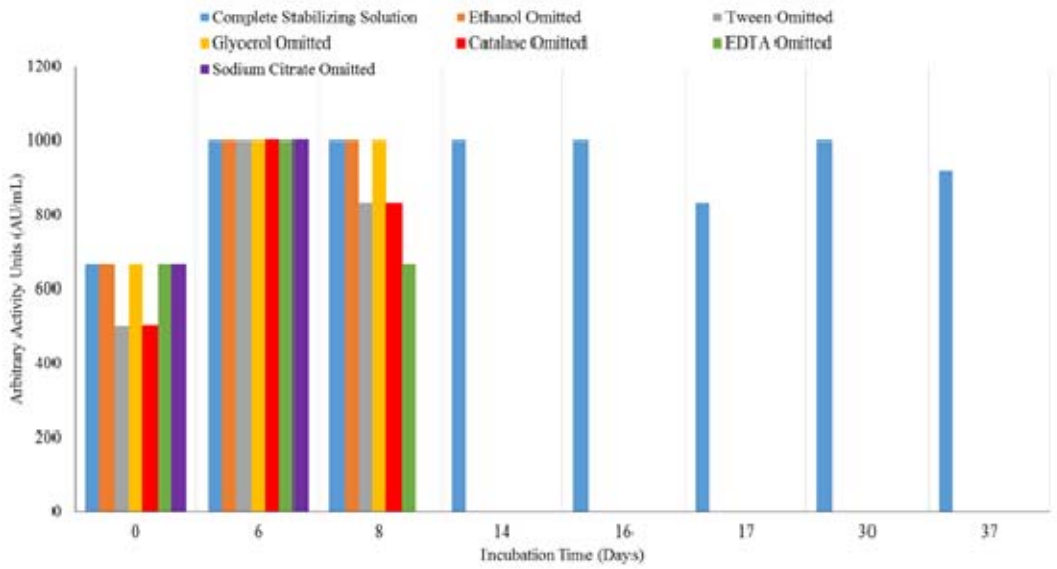

Figure 12. Time Course of Colicin E7 Activity in Stabilizing Solution Against DH5α.

While these preliminary data suggest that not one unique component of the stabilizing solution contributes to the stability of colE7, this study should be repeated in triplicate and executed over a longer time course to generate more data and greater understanding. In order to more accurately assess the effects of the stabilizing solution on the stability of colE7, these data should be directly compared to colE7 purified by FPLC without addition of the stabilizing solution and incubated under the same conditions of this study. Further, additional characterization of purified colE7 over the time course of this study by FPLC or gel electrophoresis may provide insight into possible degradation of the antimicrobial peptide over time, which would contribute to its stability and affect observed activity.

4 Conclusions

The objectives of the work, detailed in this technical report, were to: (1) identify a narrow-spectrum antimicrobial, a colicin, that demonstrates selective activity against uropathogenic bacteria; and (2) investigate the production and purification of the selected antimicrobial peptide. This work was motivated by two factors. The first was the ongoing investigation of narrow-spectrum antimicrobial technologies as an alternative to broad-spectrum antimicrobials, which are speculated to contribute to the rising incidence of drug-resistant bacteria. Additionally, the emergence of females on the battlefield has precipitated the need to address female-related events that could deleteriously effect mission performance, such as the inception of UTIs.

As described in this report, a collection of colicins was screened for activity against a panel of representative uropathogenic bacteria and representative skin and vaginal commensal microorganisms. Analysis of the results led to the selection of colicin E7 for further analysis. Growth conditions of the host microorganisms, such as incubation time, were explored. Conditions used to promote the enhanced production of colE7 were also investigated, including the induction agent and the concentration used to induce enhanced production of the antimicrobial peptide. The resulting yield of colE7 was purified by FPLC and characterized by gel electrophoresis. FPLC purified colE7 was further purified by HPLC. Analysis of the HPLC data determined the yield of colE7 to be 12.2 mg/L (±0.35) with a purity of 70% (±5.5). Further, the stability of colE7 was evaluated in a stabilizing solution to prevent the loss of activity of the antimicrobial peptide over time.

This work was executed with the vision that a colicin could be developed to fabricate a hygiene wipe to help prevent the development of UTIs for female Soldiers in the field, leading to less time lost due to UTIs. The application of a narrow-spectrum antimicrobial to a hygiene wipe was the long-term focus as previous work by BSTT determined that encapsulation of an antimicrobial for textile treatment is not trivial; it would require more studies to ascertain the necessary methods to apply an antimicrobial to a textile, which was outside the scope of this study. The data and analysis collected on colE7 may provide the foundational studies necessary to develop a colicin-based, narrow-spectrum antimicrobial technology. Though due to the limited number of colicins evaluated and the narrow collection of bacteria screened against, further studies should be conducted to assess a more comprehensive range of colicins or combinations of colicins as well as commensal and uropathogenic strains of bacteria.

Studies have shown that colicins tested for antimicrobial activity in combination exhibited greater activity than when tested individually (Riley *et al*. 2012). Future efforts should seek to elucidate the antimicrobial activity of colE7 and other colicins against a broader array of uropathogenic and commensal microorganisms. Testing against a greater number of bacterial strains would provide more data to generate a comprehensive understanding of the extent of activity of a chosen colicin. Production of colE7 at a higher scale should be explored to gain knowledge of limitations of the quantity of colE7 that can be acquired, which may be applicable to the development of an alternative colicin(s). Development of a colicin-based technology will require high yields of the antimicrobial peptide.

Additionally, the impurities present in the colE7 sample should be identified and characterized to ensure that they do not elicit safety concerns for human application. Moreover, the stability of

the chosen colicin must be investigated further. The storage conditions (e.g., temperature, humidity, storage time, etc.) of the colicin in the intended application should be considered while conducting stability studies. A longer, more comprehensive stability study would be critical toward evaluating the feasibility of developing a narrow-spectrum antimicrobial technology based on colicin E7 or an alternative colicin.

This document reports research undertaken at the U.S. Army Natick Soldier Research, Development and Engineering Center, Natick, MA, and has been assigned No. NATICK/TR- 17/024 in a series of reports approved for publication.

5 References

Birkemo, G. A., O'Sullivan, O., Ross, R. P., Hill, C.; "Antimicrobial Activity of Two Peptides Casecidin 15 and 17, found Naturally in Bovine Colostrum" *J. Appl. Microbiol.* **2009**, *106*, 233-240.

Blackburn, P., de la Harpe, J.; "Stabilized Lanthionine Bacteriocin Compositions" *U. S. Patent No. 5,763,395* Washington, DC: U.S. Patent and Trademark Office (1998).

Branck,T.A., C.M Cowell, J.M. Rego and R.E. Stote "Bactericidal Coatings on Textiles for Remediation of Intermicrobe Activity (BaCTeRIA) Summary Report"No NATICK/TR17/017, ARMY NATICK SOLDIER RESEARCH DEVELOPMENT AND ENGINEERING CENTER MA NATICK United States, July 2017.

Butala, M., Zgur-Bertok, D., Busby, S. J. W.; "The Bacterial LexA Transcriptional Repressor" *Cell. Mol. Life Sci.* **2009**, *66*, 82-93.

Cascales, E., Buchanan, S. K., Duche, D., Kleanthous, C., Lloubes, R., Postle, K., Riley, M., Slatin, S., Cavard, D.; "Colicin Biology" *Microbiol. Mol. Biol. Rev.* **2007**, *71*, 158-229.

Cotter, P. A., Hin, C., Ross, R. P. "Bacteriocins: Developing Innate Immunity for Food". *Nat. Rev. Microbiol*. **2005**, *3*, 777-778.

Cox, C. P., Coburn P. S., Gilman, M. S.; "Enterococcal Cytolysis: a Novel Two Component Peptide System that Serves as a Bacterial Defence Against Eukaryote and Prokaryote Cells". *Curr. Protein Pept. Sc*. **2005**, *6*, 77-84.

Cursino, L., Chartone-Souza, E., Nascimento, A.; "Recent Updated Aspects of Colicins of Enterobacteriaceae" *Braz. J. Microbiol.* **2002**, *33.3*, 185-195.

Doherty, M. E., Scannell-Desch, E.; "Women's Health and Hygiene Experiences During Deployment to the Iraq and Afghanistan Wars, 2003 through 2010" *J. Midwifery Women's Health*. **2012**, *57,* 172-177.

Dorit, R., Riley, M. A.; "Engineered Bacteriocins and Bacteriocin Combinations and Methods for Treating Bacterial Based Infections" *U. S. Patent Application Publication 20060229244A1* **2006** Washington, DC: U.S. Patent and Trademark Office.

Field, D., Gaudin, N., Lyons, F., O'Connor, P. M., Cotter, P. D., Hill, C., Ross, R. P.; "A Bioengineered Nisin Derivative to Control Biofilms of Staphylococcus pseudintermedius" *PloS O*ne, **2015**, *10*, e0119684.

Flores-Mireles, A. L., Walker, J. N., Caparon, M., Hultgren, S. J.; "Urinary Tract Infections: Epidemiology, Mechanisms of Infection and Treatment Options" *Nat. Rev. Microbiol.* **2015**, *13*, 269-284.

Galvz, A., Ahriovel, H., Lopez, R. L., Omar, N. B.; "Bacteriocin-based Strategies for Food Biopreservation" *Int. J. of Food Microbiol*. **2007**, *120*, 51-70.

Gillor, O., Vriezen, J. A. C., Riley, M. A.; "The Role of SOS Boxes in Enteric Bacteriocin Regulation" *Microbiol.* **2008**, *154*, 1783-1792.

Gokce, I., Lakey, J. H.; "Production of an *E. coli* Toxin Protein; Colicin A in *E. coli* Using an Inducible System" *Turk J. Chem.* **2003**, *27*, 323-331.

Gulberg, E., Cao, S., Berg, O.G., IIback, C., Sandegren, L., Hughes, D., Andersson, D.L.; "Selection of Resistant Bacteria have Very Low Antibiotic Concentrations". *PLOS pathogens* **2011**, *7*, 1-9.

Hancock, V., Nielsen, E.M., Krag, L., Engberg, J., Klemm, P.; "Comparative Analysis of Antibiotic Resistance and Phylogenetic Group Patterns in Human and Porcine Urinary Tract Infectious Escherichia coli" *APMIS* **2009**, *117*, 786-790.

Hardy, K. G. and Meynell, G. G.; " 'Induction' of Colicin Factor E2-P9 by Mitomycin C" *J. Bacteriol.* **1972**, *112*, 1007-1009.

Herschman, H. R. and Helinski, D. R.; "Comparative Study of the Events Associated with Colicin Induction" *J. Bacteriol*. **1967**, *94*, 691-699.

Iijima, T.; "Studies on the Colicinogenic Factor in *Escherichia coli* K12. Induction of Colicin Production by Mitomycin C. *Biken's J.* **1962**, *5*, 1-8.

Immucell Corporation. [online]. Available from: http//www.immucell.com/pf/whats_new.php. 11 December 2008.

Johnson, J.R.; "Virulence Factors in Escherichia coli Urinary Tract Infections" *Clin. Microbiol. Rev.*" **1991**, *4*, 80-128.

Johnson, J.R., Kuskowski, M.A., Gajewski, A., Sahm, D.F., Karlowsky, J.A.; "Virulence Characteristics and Phylogenetic Background of Multi-Drug Resistant and Antimicrobial-Susceptible Clinical Isolates of Escherichia coli from across the United States 2000-2001" *J. Infect. Dis*. **2004**, *190*, 1739-1744.

Levy, S. B., Marshall, B.; "Antibacterial Resistance Worldwide: Causes, Challenges and Response" *Nat. Med.* **2004**, *10*, S122-S129.

Majeed, H., Gillor, O., Kerr, B., Riley, M. A.; "Competitive Interactions in *Escherichia coli* Populations: The Role of Bacteriocins" *ISME J.* **2011**, *5*, 71-81.

Mueller, Ralf. "Topical Antimicrobial Therapy." *Sheila MF Torres*, **2012,** *323*.

Nickerson, Stephen C. "Choosing the Best Teat Dip for Mastitis Control and Milk Quality." *NMC-Milk Quality Conf. Proceed.*, April **2001**, *43*.

Parente, E., Brienza, C., Moles, M., Ricciardi, A.; "A Comparison of Methods for the Measurement of Bacteriocin Activity" *J. Micobiol. Meth.* **1995**, *22*, 95-108.

Parente. E., Ricciardi, A.; "Production, Recovery and Purification of Bacteriocins from Lactic Acid Bacteria" *Appl. Microbiol. Biotechnol.* **1999**, *52*, 628-638.

Pugsley, A. P. "*Escherichia coli* K12 Strains for Use in the Identification and Characterization of Colicins" *J. Gen. Microbiol.* **1985**, *131*, 369-376.

Riley, M. A., Cadavid, L., Collett, M. S., Neely, M. N., Adams, M. D., Phillips C. M, Neel, J. V., Friedman, D.; "The Newly Characterized Colicin Y Provides Evidence of Positive Selection in Pore-former Colicin Diversification" *Microbiology* **2000**, *146*, 1671-1677.

Riley, M. A., Robinson, S. M., Roy, C. M., Dennis, M., Liu, V., Dorit, R. L.; "Resistance is Fultile: The Bacteriocin Model for Addressing the Antibiotic Resistance Challenge" *Biochem. Soc. Trans.* **2012**, *40*, 1438-1442.

Rijavec, M., Erjavec, M.S., Avgustin, J.A., Reissbrodt, R., Fruth, A., Krizan-Hergouth, V., Zgur-Bertok, D.; "High Prevalence of Multidrug Resistance and Random Distribution of Mobile Genetic Elements among Uropathogenic Escherichia coli (UPEC) of the Four Major Phylogenetic Groups" *Curr. Microbiol.* **2006**, *53*, 158-162.

Salvatore, S., Salvatore, S., Cattoni, E., Siesto, G., Serati, M., Sorice, P., Torella, M.; "Urinary Tract Infections in Women" *Eur. J. Obstet. Gynecol. Reprod. Biol.* **2011**, *156*, 131-136.

Smajs, D., Micenkova, L., Smarda, J., Vrba, M., Sevcikova, A., Valisova, Z., Woznicova, V.; "Bacteriocin Synthesis in Uropathogenic and Commensal Escherichia coli: Colicin E1 is a Potential Virulence Factor" *BMC Microbiol*. **2010**, *10*, 288.

Tomasz, M., Lipman, R., Chowdary, D., Pawlak, J., Verdine, G. L., Nakanishi, K.; "Isolation and Structure of a Covalent Cross-Link Adduct Between Mitomycin C and DNA" *Science* **1987**, *235*, 1204-1208.

Trautner, B. W., Hull, R. A., Darouiche, R. O.; "Colicins Prevent Colonization of Urinary Catheters" *J. Antimicrob. Chemother.* **2005**, *56*, 413-415.

Vejborg, R.M., Hancock, V., Schembri, M.A., Klemm P.; "Comparative Genomics of Escherichia coli Strains Causing Urinary Tract Infection" *App. Environ. Microbiol*. **2011**, *77*, 3268-3278.