Synergistic Combinations between Human-Derived Antimicrobial Peptides and Traditional Antibiotics against *Escherichia coli*

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Abstract

The growing prevalence of antimicrobial resistance to clinical antibiotic treatments has been declared a healthcare crisis by several national and international public health agencies. The emergence of broad-range drug resistance in *Escherichia coli* (*E. coli)*, a bacterium associated with severe infections of the gastrointestinal system, urinary tract, and bloodstream, has been identified as a serious threat by the Centers of Disease Control and Prevention (CDC). A limited pipeline of drug development and discovery demands that novel and creative antibiotic solutions be explored to combat multi-drug resistant bacterial infections.

The human-derived antimicrobial peptide, LL37, and its synthetic short-chain analogues, FK16 and FK13, have been proposed as novel therapeutic treatments in combination with traditional antibiotics. Antimicrobial peptides (AMPs) are being widely explored as a new class of antibiotics with a wide range of efficacy against gram-positive and gram-negative bacteria. Their proposed mechanism of action, permeabilization of bacterial membranes, is particularly well-suited for combinations with antibiotics that are rendered ineffective by the outer membrane barrier of gram-negative bacteria. In addition, drug-combinations have lower risk of generating antimicrobial resistance than monotherapies. The combinatory benefits may be amplified when synergistic combinations are utilized.

A checkerboard assay was used to explore antimicrobial combinations, and synergism was analyzed using the fractional inhibitory combination index (FICI). Synergistic combinations of LL37, FK16, and FK13 have been identified with the traditional antibiotics, vancomycin, polymyxin B, and colistin, against three strains of clinically isolated *E. coli*. These synergistic combinations were further investigated in medium with cationic concentrations resembling human blood. Vancomycin was shown to be an ineffective treatment for *E.* coli due to its inability to diffuse through the outer membrane. However, pre-treatment of *E. coli* with sub-inhibitory concentrations of LL37, FK16, or FK13 was shown to significantly decrease the minimum inhibitory concentration of vancomycin, demonstrating their membrane-permeabilizing capabilities. Synergistic combinations were particularly apparent between the antimicrobial peptides and two other membrane-targeting antibiotics, polymyxin B and colistin.

The interaction of LL37, FK16, and FK13 with the outer membrane of bacteria was investigated by measuring the zeta potential of bacterial suspensions. The cationic peptides were shown to neutralize the membrane potential of bacteria at much lower concentrations than an intracellular-targeting antibiotic (vancomycin) with lower net charge. Neutralization efficiency was directly correlated to the net charge of the AMP, with LL37 having a +6 net charge and both FK16 and FK13 having a +4 net charge. The neutralization of *E. coli* liposaccharide molecules, the endotoxin primarily responsible for septic shock, was also investigated via zeta potential.

This thesis explores and introduces information about antimicrobial resistance mechanisms, *E. coli* anatomy and infections, existing treatments, combinatory antibiotic methods, and antimicrobial peptides that was used to guide the research performed. The outcomes of this research suggest further investigation of LL37, FK16, and FK13 in synergistic combination with traditional antibiotics as a promising therapeutic method. Results from this research are compared to related studies in the field. Current hurdles facing the implementation of antimicrobial peptides in clinical settings are discussed. Finally, intentions for future research are presented.

Acknowledgments

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Contents

List of Figures

List of Tables

List of Abbreviations

Term	Abbreviation
Antimicrobial peptides	AMPs
Centers of Disease Control and Prevention	CDC
Colony forming unit	CFU
Extended spectrum beta-lactamase	ESBL
Extra-intestinal pathogenic E. coli	ExPEC
Food and Drug Administration	FDA
Fractional inhibitory concentration index	FICI
Hemolytic uremic syndrome	HUS
Liposaccharides	LPS
Meningitis-associated E. coli	MNEC
Minimum inhibitory concentration	MIC
Muller-Hinton agar	MHA
Muller-Hinton broth	MHB
N-phenyl-1-napthylamine	NPN
Organisation for Economic Cooperation and Development	OECD
Propidium iodide	PI
Shiga toxin-producing E. coli	STEC
Urinary tract infection	UTI
Uropathogenic E. coli	UPEC
World Health Organization	WHO

Chapter 1: Introduction/Background

1.1 Antimicrobial Resistance

Development of antimicrobial resistance in pathogenic species has been declared a health crisis by several national and international public health agencies such as the Centers of Disease Control and Prevention (CDC) and the World Health Organization (WHO).[1,2] Antimicrobial resistance is the process by which pathogens including bacteria, fungi, and viruses adapt to antimicrobial treatments that were once effective in inhibiting their growth and infection. Several factors influence the spread of antimicrobial resistance including the misuse or overuse of antibiotics in healthcare, agriculture, and industry. In 2013, the CDC reported greater than two million cases of antibiotic-resistant infections, resulting in over 23,000 deaths and $20 billion dollars in healthcare costs.[3] In 2014, 700,000 deaths were attributed to resistant microbe strains globally with estimations predicting an increase to as high as 10 million deaths in 2050.[4]

The main cause for concern is the manifestation of resistance towards antimicrobials which were previously effective and upon which modern medicinal practices rely. This adaptive resistance is observed after exposure to antibiotic drugs via the processes of mutation, selection, and genetic exchange.[5,6] Mutation may lead to resistance towards a certain antibiotic by altering the target protein of the antibiotic, increasing production of enzymes which can deactivate the antibiotic, or decreasing permeability and accumulation of the antibiotic within the cell.[7] Upon exposure to antibiotics, cells which contain beneficial mutations have higher viability and the bacterial population is effectively selected towards resistant strains. Development of resistance within a population can happen quite quickly – even over the course of one antibiotic regimen to treat an infection in a single patient.[5]

However, antimicrobial resistance can also be transferred between bacteria through the process of horizontal gene transfer. Through several mechanisms, genetic material can be passed from one bacteria to another where it can be acquired and incorporated into the genome of a new cell.[8] In this way, bacteria can gain resistance to a certain antimicrobial without ever being exposed to it. In some cases, this genetic transfer can even be observed between different species of bacteria.

The discovery and implementation of antibiotics was one of the hallmarks of the 20th century, enabling revolutions in medicinal science and procedures. As antibiotic resistance grows, infections that were once considered mild are more frequently becoming life threatening as effective treatments become limited. The current pipeline for development and implementation of new drugs with efficacy against resistant strains is unable to keep up with emerging bacterial resistance across the globe. As a result, international experts are calling for global surveillance of drug resistance, reduction of unnecessary antibiotic use, investment towards the development of new treatments, and research into the optimization of existing treatments. [4]

1.2 *Escherichia coli*

1.2.1 *E. coli* anatomy

E. coli is a rod-shaped, gram-negative bacterium and, as such, shares several distinctive structural features with other species in this class. This includes the presence of a membrane envelope formed by three main structures: 1) the outer membrane, 2) the periplasmic space, and 3) the inner membrane.[9] A schematic representation of the membrane envelope is provided in Figure 1. The size of a typical *E. coli* is about 2µm in length and 0.5µm in diameter.[10]

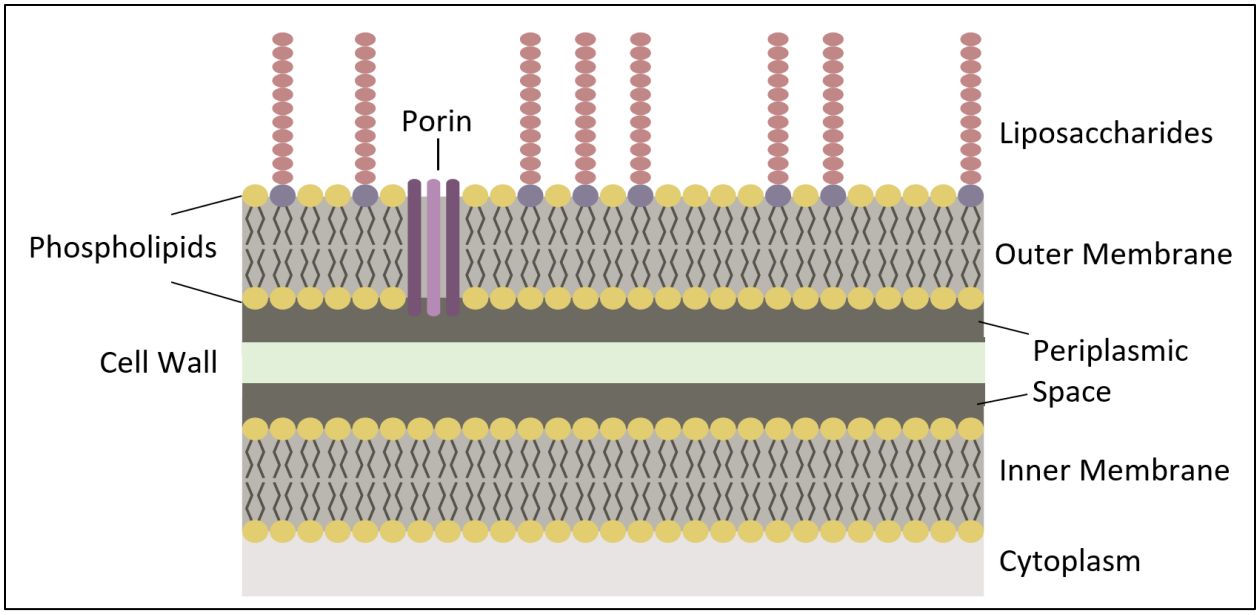

Figure 1. Structure of the cell envelope present in *E. coli* and other gram-negative bacteria. "202209 Gram Negative Bacteria" by DataBase Center for Life Science (DBCLS) licensed under CC BY 4.0/adapted from original.[11]

The outer membrane regulates interaction between the bacteria and its external environment. This membrane serves as a selective barrier, keeping out antibiotics, hydrophobic molecules, and other toxins while allowing for the passage of small molecules critical for cellular processes. It is due to this impermeable membrane that gram-negative bacteria can survive in harsh conditions such as the intestines and are generally more resistant to antibiotics compared to gram-positive bacteria.[12,13] Liposaccharides (LPS), which are present only on the exterior face of the outer membrane, are generally attributed towards many of the properties of the outer membrane. These tightly packed, negatively charged molecules contribute to the barrier properties of the membrane. LPS-deficiency has long been associated with increased susceptibility towards antibiotics.[14–16] Additionally, LPS is an endotoxin which produces a strong immunological response towards human infections. At high enough concentrations during an infection, LPS may result in septic shock. The outer membrane also contains transport structures called porins or β-barrels. These porins facilitate diffusion of small, hydrophilic molecules typically less than 500-600 Da.[17]

Moving inward, the periplasm is comprised of a viscous fluid area located between the outer membrane and inner membrane. The periplasm helps to regulate diffusion of molecules into the cell and houses degradative enzymes. Also located within the periplasmic space is the peptidoglycan cell wall. This rigid protein layer acts as an exoskeleton, giving *E. coli* its signature rod-like shape, and stabilizes the cell by preventing lysis when the bacteria is in dilute medium.[13]

The inner membrane is a phospholipid bilayer within which are located several proteins enabling small-molecule transport and other cellular functions. Functions performed within the inner membrane include those associated with energy production, protein transport, and lipid biosynthesis.[9,13] The cytoplasm comprises most of the inner-most volume of bacteria. The cytoplasm facilitates diffusion of signaling molecules and solutes. It is within the cytoplasm that nucleoid DNA is stored, and ribosomes produce proteins. It is also within the cytoplasm that binary fission initiates the bacterial reproductive process.[18]

1.2.2 *E. coli* infections

Non-pathogenic *E. coli* is naturally found in the gastrointestinal tract of humans. However, several strains are known to be pathogenic and are attributed to infection of the urinary tract (UTI), bloodstream, and central nervous system leading to such conditions as cystitis, diarrhea, hemolytic uremic syndrome (HUS), and sepsis.[19–21] The severity of *E. coli* infections varies according to the patient, the *E. coli* strain, and the strain's resistance to antibiotics. Pathogenic strains of *E. coli* typically possess phenotypical differences from non-pathogenic strains which facilitate adhesion and colonization in infection sites such as the intestines or urinary tract. These structures, called adhesins, are usually small, fibrillar, hair-like forms ranging from 2-10 nm in diameter called fimbriae or fibrillae. Factors dictating the symptoms of infections are determined by the release of a variety of toxins produced by pathogenic *E. coli* strains [20]

Most commonly, *E. coli* is associated with infections of the gastrointestinal tract acquired from contaminated food and water. Shiga toxin-producing *E. coli* (STEC) are among the most virulent strains targeting the intestinal system with symptoms including vomiting, stomach pain, and bloody diarrhea. Persistent infections and the release of large quantities of Shiga-toxin may even progress to the life threating condition of HUS. In general, gastrointestinal infections are common but not particularly fatal. In 2010, WHO estimated that STEC strains caused over 1 million infections resulting in 100 deaths.[22] In the period of 2003 – 2012, the most common and severe transmissions in the United States were foodborne; mostly originating from consumption of beef.[19] Transmission is commonly attributed to unhygienic consumption of contaminated food through the fecal-oral route.[23] In addition to STEC strains, there are six other pathotypes associated with diarrheagenic infection of the intestines or colon.[21]

Extra-intestinal pathogenic *E. coli (ExPEC)* strains target areas outside of the gastrointestinal tract. Some strains of *E.coli,* known as meningitis-associated *E.coli* (MNEC), cause meningitis or sepsis via infection of the central nervous system or the blood.[20] Sepsis is a serious condition categorized by an uncontrolled inflammatory response towards infection of the bloodstream. LPS is the endotoxin primarily responsible for septic responses towards gram-negative bacteria. Significant infection of the blood is not common unless the host is immunocompromised, but may occur when *E. coli* enters the bloodstream from existing infection sites (such as a UTI) or when introduced from an external source (such as contaminated surgical tools). The mortality rate for sepsis is high (>30%) and fast identification and treatment is critical. Mortality rate increases by approximately 6% for every hour without administration of an antimicrobial treatment.[24] Thus, the prevalence of resistant strains severely limits the ability to timely and effectively treat sepsis. *E. coli* is responsible for the majority of sepsis cases in infant and elderly populations.[25]

Neonatal meningitis is associated with inflammation of the membranes of the brain and spinal cord. It is particularly fatal in newborn children. In the US, 20-40% of neonatal meningitis cases are caused by ExPEC strains. Neonatal infections are typically acquired during birth via transmission of bacterial colonies from the mother or during post-natal surgical procedures.[26,27]

Uropathogenic *E. coli* (UPEC) strains target the urinary tract and are responsible for an estimated 80-90% of UTI cases and excess of $1.6 billion in yearly medical expenses in the United States[23,27,28] One study estimates that almost 50% of women and 12% of men will experience at least one UTI, with 25% of infections resulting in a recurrent case.[29] UTIs typically begin with colonization of the urethra, where UPEC colonies can then ascend to the bladder, which is the most common site of infection. UPEC strains adhere to epithelial cells and often form persistent biofilms.[30] Even after successful treatment of an infection, reservoirs of uninhibited UPEC may re-emerge and cause recurrent infections. In fact, recurrent infections

are not uncommon, with approximately 27% of women experiencing a recurrent infection within 6 months and 44% within one year. It is suspected that reservoirs of UPEC strains may persist within the fecal flora and become a source of reinfection after successful elimination from the urinary tract.[31] Particularly serious infections may progress to the kidneys.

1.2.3 *E. Coli* Resistance

As a gram-negative bacterium, *E. coli* is intrinsically resistant towards antibiotics with intracellular targets that cannot permeate the outer membrane. For example, vancomycin and penicillin G are both antibiotics which target proteins essential for peptidoglycan cell wall synthesis. These antibiotics are generally effective for treatment of gram-positive bacteria, but are ineffective at therapeutic levels against *E. coli.* [5,12,23] In the case of vancomycin, its large structure (1450 Da) exceeds the size exclusion limit of porins in the outer membrane (approximately 500-600 Da).[17,32] On the other hand, penicillin G has a smaller structure (334 Da) but it's hydrophobic nature limits permeation through the outer membrane. In general, hydrophobic molecules are repelled by LPS, and thus have limited ability to diffuse through the phospholipid bilayer.[33] Development of penicillin analogues containing amphiphilic substitutions (such as ampicillin) improves outer membrane permeation, but adaptive resistance has begun to limit their effectivity as well.[34]

Antibiotics effective against *E. coli* must be able to either permeate or target the outer membrane. Small scaffold hydrophilic or amphiphilic antibiotics are able to access the interior of the cell through porins. An example of this class includes ampicillin, but, as discussed further below, its efficacy is threatened by the emergence of bacteria strains producing beta-lactamase enzymes.[33] Membrane targeting drugs are generally less common, but act by disrupting the barrier properties of the outer membrane. Cationic peptides such as polymyxin B and colistin (polymyxin E) belong to this family of antibiotics.[35]

In 2019, the CDC categorized extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae, including *E. coli*, as serious infectious threats due to growing antibiotic resistance.[1] Beta-lactamase is associated with resistance towards a broad-range of beta-lactam antibiotics including penicillins, cephalosporins, and monobactams. Up-regulation of beta-lactamase results in enzyme mediated cleavage of beta-lactam based antibiotics. Another pathway towards beta-lactam resistance has been identified as a suppression of the expression of porins, effectively limiting permeability through the outer membrane.[33]

Identification of multi-drug resistant *E. coli* has been on the rise across the globe with presence in clinical environments, animal populations, food products, and municipal waste systems.[23,36,37] Growing resistance in *E. coli* has also been identified for most classes of therapeutic antibiotics including carbapenems, aminoglycosides, fluoroquinolones, and polymyxins.[38] These findings are alarming due to the overwhelming reliance of these antibiotic drugs to treat infections from *E. coli* and other gram-negative bacteria. Thus, identifying strategies to both limit the spread of antibiotic resistance and combat strains with increased viability is paramount.

Antimicrobial resistance level in a particular country has been strongly correlated with the country's participation in the Organisation for Economic Cooperation and Development (OECD). Countries participating in the OECD are often more developed, and access to antibiotics is typically regulated by medical prescription. Countries outside the OECD may permit unrestricted sale of antibiotics over the counter. A pediatric study performed in 2015 revealed that the prevalence of *E. coli* resistance was higher

for every measured antibiotic in non-OPEC countries than in OPEC countries, highlighting the importance of regulation to limit antibiotic resistance.[39]

1.3 Treatments for infections

1.3.1 Current Treatments

The use of antibiotics in clinical treatments varies according to the type and severity of infection as well as regional regulations, availability, and cost. Factors dictating administration include patient age, sex, existing allergies, identification of the microbial isolate, potential interactions with other drugs, and regional antimicrobial resistance. Some common prescriptions for *E. coli* include β-lactams (penicillin and related compounds), trimethoprim, quinolones, aminoglycosides, tetracyclines, and nitrofurantoin.[40,41] Most treatments are administered orally, but some can be introduced via intravenous injection for serious cases or for patients in hospital environments.

UPEC strains are among the most commonly treated with antibiotics, as they are responsible for approximately 80% of UTIs, and 50% of hospital acquired infections.[40] Selection of the proper antibiotics and regimen is very important, as insufficient treatment can leave reservoirs of UPEC in the bladder becoming a source for recurrent infection.[41] Typical prescription regimens of uncomplicated UTIs last about 3-7 days depending on the selection of antibiotic. Antimicrobial prophylaxis has been administered for patients with histories of recurrent infections for periods of months to years.[30,31] Interestingly, consumption of cranberry juice has been shown to impede the function of adhesins in UPEC strains which may decrease the risk of UTI infections.[42,43]

Unfortunately, because antibiotic use is systematic in the global treatment of UTIs, antimicrobial resistance for most first-line treatments is on the rise. Resistance to ampicillin was found in 36 – 54% of cases in the United States. Co-trimoxazole (a combination of trimethoprim and sulfamethoxazole) is among the most commonly prescribed treatment for UTIs worldwide, but resistance was found in 30% – 70% of pediatric infections. [39]

Antibiotic use for gastrointestinal infections, including those caused by STEC, is more controversial. The CDC does not recommend antibiotics for STEC infections, as it may stimulate production or release of shiga-like toxins and increase the risk of life-threating HUS.[44] Despite this, antibiotic treatment is not uncommon, occurring in approximately one-third of HUS cases.[45] Unfortunately, although clinical antibiotic use is less common than with UTI infection, antimicrobial resistance is still on the rise for STEC strains due to overuse of antibiotics in the food industry. Common STEC strains can be found in the gastrointestinal tracts of livestock, thus antibiotic use in animal produce has been found to lead to resistance in human pathogens.[46]

The membrane targeting drugs, polymyxin B and colistin, are considered last resort drugs due to their ability to combat *E. coli* infections with multiple drug resistances. Their use is considered dire due to significant and adverse nephrotoxicity and neurotoxicity effects.[47] Despite the significant risks, use of polymyxin B and colistin is growing in recent years due to the rise of carbapenem-resistant Enterobacteriaceae. Since 2015, the Food and Drug Administration (FDA) has approved four new drugs targeting these resistant strains with increased efficacy and lower toxicity compared to the polymyxins, yet polymyxin B and colistin were still used in 28% of treatments in 2019. The adoption of these new drugs is likely inhibited by cost, with a 1580% - 4800% price increase for a 14-day regimen compared to colistin or polymyxin B.[48] This highlights the societal and economic need for the exploration and development of

new therapeutic strategies to combat antibiotic resistant *E. coli* strains. Alternatives to traditional antibiotics have been proposed in the forms of immunotherapy, bacterial vaccines, biofilm inhibitors, antimicrobial peptides (AMPs), and combination strategies.[40]

1.3.2 Combination Strategies

Combination therapies offer a promising route to combating antibiotic resistant bacteria. One reason is that it offers the potential to use already existing and developed antibiotic treatments in new and effective ways. This is significant, both because drug development and approval are lengthy and costly procedures, but also because the rate of new antibiotic development is slowing.[49] Secondly, successful combination strategies, particularly synergistic combinations, show several advantages over traditional therapies: 1) synergistic combinations lead to higher antimicrobial efficacy at lower dosages; 2) it is much more challenging for bacteria to acquire resistance therapies with multiple cellular targets; 3) lower concentrations of toxic compounds lead to lower cytotoxic effects; 4) in cases where the infecting species has not been identified, combination therapies provide a wider range of efficacy.[50] Thus, synergistic combinations have been a growing research focus for antibiotic resistant therapies in recent years.

Antibiotic combination is not a new concept. Synergistic combinations still in use for *E. coli* infections today, such as the combination of trimethoprim and sulfonamide (co-trimoxazole), were developed in the 1950s and 60s, and show increased efficacy compared to either drug alone.[39,50,51] However, advances in the understanding of antibiotic mechanisms have led to proposals for smarter design in combination development. For example, it has been found that treatments are generally less susceptible to antibiotic resistance when they target multiple genes or the cellular products of multiple genes, rather than a single gene or protein.[52] Antimicrobial combinations approved by the FDA in recent years for the treatment of infections caused by gram-negative bacteria include Zerbaxa and Avycaz. Zerbaxa is a combination of ceftolozane (a beta-lactam antimicrobial) and tazobactam (a beta-lactamase inhibitor) approved for use in 2014.[53] Avycaz is a similar antimicrobial combination between ceftazidime (a beta-lactam antimicrobial) and avibactam (a beta-lactamase inhibitor) that was approved in 2015.[54]

Several reviews have adopted nomenclature to describe the interactions between drug combinations as congruous, syncretic, coalistic, additive, or antagonistic.[50,55,56] Congruous, syncretic, and coalistic combinations are all examples of synergistic combinations via differing mechanisms. Congruous combinations are those between drugs which target separate essential genes or cellular components. An example of this is co-trimoxazole, which targets two different genes associated with folate synthesis.[52] Congruent combinations are by far the most commonly used and historically successful antibiotic combinations. Coalistic combinations are those between a compound that targets an essential cellular component, and one that targets a non-essential cellular component. In other words, a synergistic combination between one compound with intrinsic antibiotic efficacy and one compound without intrinsic antibiotic efficacy. These combinations are recently receiving attention in addressing β-lactamase producing *E. coli* strains showing multi-drug resistance. In this case, molecules inhibiting β-lactamase production or function are able to re-sensitize *E. coli* to β-lactam drugs (such as the cases of Zerbaxa and Avycaz).[50] Finally, coalistic combinations are antibiotic combinations between two compounds which generally do not produce antibiotic effects alone. These combinations are very difficult to identify, and therefore are relatively uncommon. In terms of non-synergistic combinations, additive combinations are generally less desirable as they provide essentially no interaction or advantage. Antagonistic combinations should be actively avoided, as they can partially or fully suppress the antibiotic properties of one or both compounds.

Combinations between two previously separate, but well understood and clinically approved antibiotic drugs have dominated the practice of drug combination historically. Now, in efforts to expand and improve antibiotic approaches, new combinations are being investigated between traditional antibiotics and new classes of treatments including antimicrobial peptides,[57] bacteriophages,[58] and inorganic nanoparticles.[59] However, antibiotic combinations still need to be heavily monitored to prevent unwanted negative effects. In regard to patient safety, unexpected toxicological consequences of antibiotic combinations must be thoroughly scrutinized. In addition, regulatory approval must be strongly administered, as ineffective antimicrobial combinations may lead to increased rates of sub-inhibitory antibiotic exposure and accelerated antibiotic resistance in bacteria.[50]

1.3.3 Immunotherapy

Many of the current concerns surrounding the prevalence of antimicrobial resistance can be attributed to the reliance of therapeutic treatment on antibiotics and the lack of effective treatment alternatives. Therefore, there is an interest in developing therapeutic approaches which have significantly diversified mechanisms of action. Immunotherapy is an approach whereby the intervention mechanism does not directly kill bacteria, but boosts or restores the natural immune response to infection.[60] This can be achieved through various strategies. For example, some bacteria can take advantage of immunosuppressive signaling pathways (e.g. immune checkpoints) to reduce and subvert the immune response during infection. An immunotherapy strategy called an "immune checkpoint blockade" works to reduce this effect and maintain a proper immune response during the infection period by introducing blocking antibodies or other inhibitor molecules to repress this inhibition.[61]

Immunotherapy may also take the form of cytokine modulation. Cytokines are messenger molecules which play an important role in immunomodulation. Cytokine therapies are recently receiving attention in the treatment of sepsis, where ensuring proper function of white blood cells while simultaneously preventing hyper-inflammation is critical.[60,62] A third and relatively recent immunotherapy strategy may be termed as cellular therapy. In this approach host immune cells (including T cells and macrophages) are genetically modified in order to impart attributes that promote bacterial recognition and interaction, or aid against the immunosuppression pathways of bacteria. These modified cells are then introduced into the infected host to boost the immune response. Already, modified T cells and macrophages have shown increased ability to combat infections of *S. Typhimurium*, *K. pneumoniae*, MRSA, and *P. aeruginosa.*[60]

Immunotherapy represents a novel treatment pathway towards bacterial infections. It is attractive, in part, due to its fundamental difference from antibiotic treatment and subsequent possibility to subvert antimicrobial resistance. However, there are still several hurdles that impede its adoption. Clinical studies using immunotherapy for the treatment of cancer are currently very active, however, very few trials are underway in the treatment of sepsis or bacterial infection.[63] In addition, more research is needed to better understand the use of immunotherapy for bacterial infections, including potential interference or interaction with traditional antibiotics treatments and the potential to exacerbate the risk of sepsis through increased inflammatory responses.[60]

1.4 Antimicrobial Peptides

AMPs are innate components of the immune systems shared by a multitude of organisms including humans. They are short, amphipathic, cationic molecules that are present in a variety of structures

depending on their environment and function. Primarily, AMPs function as antimicrobial agents or serve a role in the modulation of inflammation in areas of infection.[64,65] Interest in AMPs mostly stems from their interactions with gram-negative bacteria. The antimicrobial mechanism of most AMPs is largely attributed to their binding and subsequent alteration of the permeability and stability of the outer membrane.[17,57,66,67]

Positively charged AMPs are electrostatically attracted to negatively charged LPS and other lipids on the outer membrane of bacteria, allowing for efficient binding to the membrane surface. Here, owing to the amphipathic nature of AMPs, they are able to interact, penetrate into, and disrupt the phospholipid bilayer. This suppresses the barrier function of the outer membrane, rapidly leading to increased permeability and cell lysis.[68] Due to performance against a wide range of microbes including gram-positive and gram-negative bacteria, fungi, and even viruses, many AMPs are now being subjected to clinical trials as therapeutic agents.[65,69]

The interaction with the cell membrane is what makes AMPs promising as a class of antibiotics. Unlike traditional antibiotics, which often target specific metabolic processes within the cell, AMPs perform a nonspecific interaction with the membrane. It is for this reason that many researchers propose bacteria have less threat of developing antibiotic resistance to AMPs.[57] In combination with traditional antibiotics, the mechanism whereby AMPs disrupt the barrier performance of the outer membrane and promote greater uptake of traditional antibiotics has led to identification of several synergistic pathways.[65] A general mechanism for this is displayed in Figure 2. This mechanism will be explored in greater detail in section

1.4.2 Membrane-Acting Mechanisms.

AMPs do have some disadvantages which must be understood and addressed before widespread adoption. AMP selectivity and toxicity against bacteria cells versus host organism cells (eukaryotic cells) is not well understood and potentially threatening. AMPs can be unstable in physiological conditions, especially in the presence of proteases. In addition, the manufacture and cold storage requirements of AMPs are expensive.[57,65,69,70] Due to cytotoxicity concerns and degradation in vivo, many clinical studies have been limited to topical applications.[71] While many AMPs are being studied in clinical trials, very few have reached the market due to these significant limitations. Steps are being taken to address these issues including chemical modification of AMPs to increase stability and reduce toxicity. Synthetic manufacture of shorter AMP derivatives over full length AMP compounds is one strategy to assist in scalability and affordability.[65]

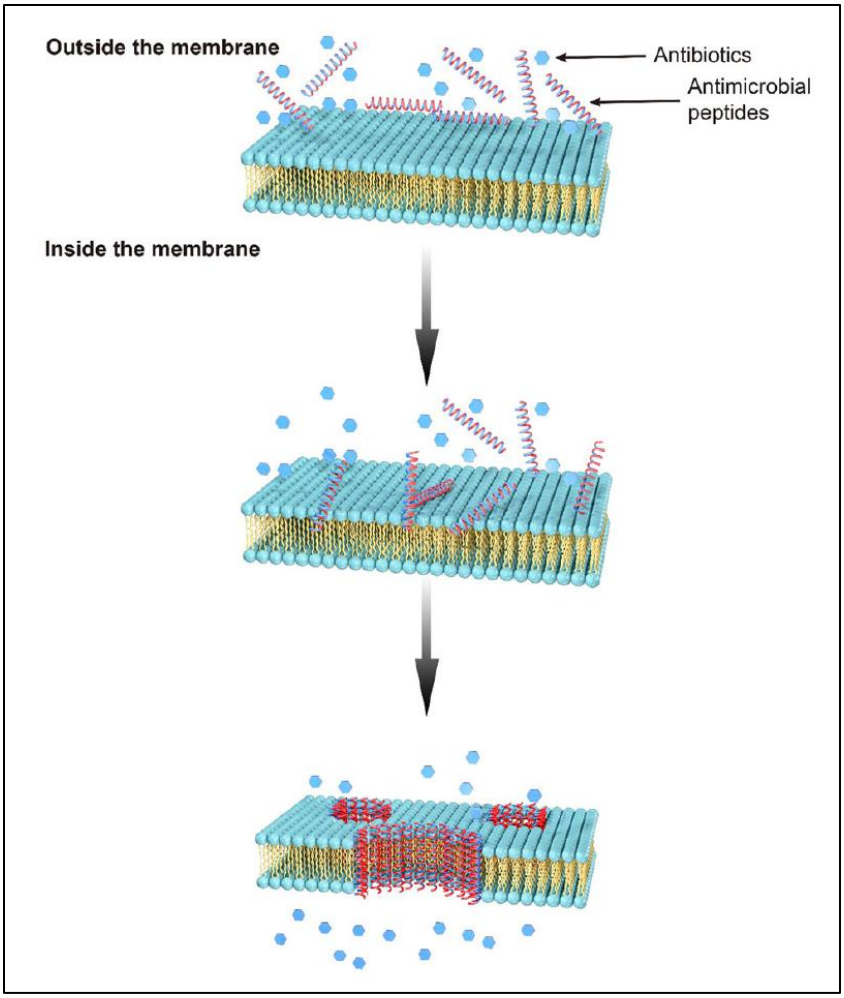

Figure 2. Depiction of a proposed mechanism of interaction between AMPs and the cell membrane resulting in increased diffusivity of antibiotic compounds into the cell. Reproduced with permission of John Wiley and Sons.[65]

1.4.1 LL37

LL37 is an α-helical structured AMP named for its 37 amino acid sequence with two leading leucine residues. It is the only human AMP derived from the cathelicidin family of peptides. At physiological pH, LL37 has a net charge of +6 and 70-80% α-helical structure with a disordered region on the N-terminus of the peptide. α-helical structure is generally attributed towards the mechanism of interaction with the membrane and antimicrobial efficacy.[72,73]

LL37 has garnered attention from researchers due to its natural participation in human immunity and dual role as both an antimicrobial agent and inflammation modulator. The precursor gene of LL37 is found in high concentrations following skin infections and LL37 has been proven to display broad spectrum antimicrobial activity, high LPS binding affinity, and synergistic activity with traditional antibiotics.[73,74] In addition to destabilizing the outer membrane of gram-negative bacteria, recent research indicates that LL37 also targets intracellular compounds, inhibiting gene expression and protein transport.[75,76] This behavior may contribute to antimicrobial efficacy, but is overshadowed by the membrane permeabilizing

and lysis mechanisms. In order to study this phenomenon, induction of LL37 into the cell must be accomplished via shuttle vectors in order to avoid membrane destruction.[75]

Limitations of LL37 include its large size (leading to high costs of synthesis) and low cell selectivity (leading to host cytotoxicity). These drawbacks have limited the clinical development and testing of LL37. Only two clinical trials in the United States have attempted to use LL37 as an interventional therapeutic for various conditions. A Phase 1-2 study (NCT02225366) attempted intratumoral injections of LL37 to patients with melanoma to stimulate an immune response to the disease.[77] The trial only consisted of three patients, with 2/3 treatments showing efficacy and 2/3 patients showing non-serious adverse effects. A different Phase 2 study (NCT04098562) is evaluating the efficacy of topical LL37 creams on controlling infection and inflammation of diabetic foot ulcers.[78] No results have been posted for this study.

In order to overcome the drawbacks of LL37 for therapeutic applications, fragments and chemically modified analogues of LL37 have been developed.[74] Short chain AMP fragments are advantageous because they can preserve the functionality of the full-length peptide, but the synthesis pathway is less costly to manufacture. Studies on LL37 fragments have revealed that antimicrobial efficacy in LL37 is primarily attributed to amino acid residues 17-32.[73] FK16 and FK13 are two LL37 fragments derived from residues within this region. FK16 corresponds to amino acid residues 17-32 while FK13 corresponds to amino acid resides 17-29. Both structures adopt a nearly perfect α-helical structure at physiological pH.[73] The amino acid sequence of LL37 and two derived fragments, FK16 and FK13, studied in this work are given in Table 1.

Indeed, FK16 and FK13 have been shown to display antimicrobial efficacy towards gram-negative bacterial and, additionally, display higher cell selectivity towards bacteria and lower cytotoxicity towards mammalian cells compared to LL37.[74,79] LL37 has a hemolytic concentration causing 50% lysis of human blood cells (HC_{50}) of 176 μg/mL, while FK13 does not display this behavior at concentrations >250 μg/mL.[74] Furthermore, LL37 was shown to have toxicity towards human corneal epithelial cells with an EC_{50} value of 43 μg/mL while the EC_{50} values for FK16 and FK13 were both >200 μg/mL.[79] Further chemical modification of LL37 fragments has been successful in improving cell selectivity and LPS binding while preserving membrane permeabilization performance of the full-length peptide.[74] FK16 and FK13 have shown synergistic effects against gram-negative *P. aeruginosa* when combined with vancomycin, an antibiotic which is generally not effective against gram-negative bacteria due to low permeability through the outer membrane.[79] All of these properties make LL37 fragments particularly promising as therapeutic agents against bacterial infections.

Table 1. LL37 and LL37-derived fragment amino acid sequences. Red text indicates a positively charged residue and blue text indicates a negatively charged residue.[72,73]

Peptide	LL37 Fragment	Sequence	Net Charge
LL37	LL37 (1-37)	**LLGDFFRKSK-EKIGKEFKRI-VQRIKDFLRN-LVPRTES**	+6
FK16	LL37 (17-32)	**FKRIVQRIKDFLRNLV**	+4
FK13	LL37 (17-29)	**FKRIVQRIKDFLR**	+4

1.4.2 Membrane-Acting Mechanisms

α-helical AMPs interact with bacterial membranes in several different ways. In the following sections, some of the more well-defined mechanisms will be explored. Starting from low concentrations, AMPs bind to a lipid bilayer in a parallel direction, with the hydrophobic portion of the amphiphilic structure orienting towards the lipid head.[80] As the concentration increases, the AMPs interact with each other and with the lipid bilayer according to several defined mechanisms.

Barrel-Stave

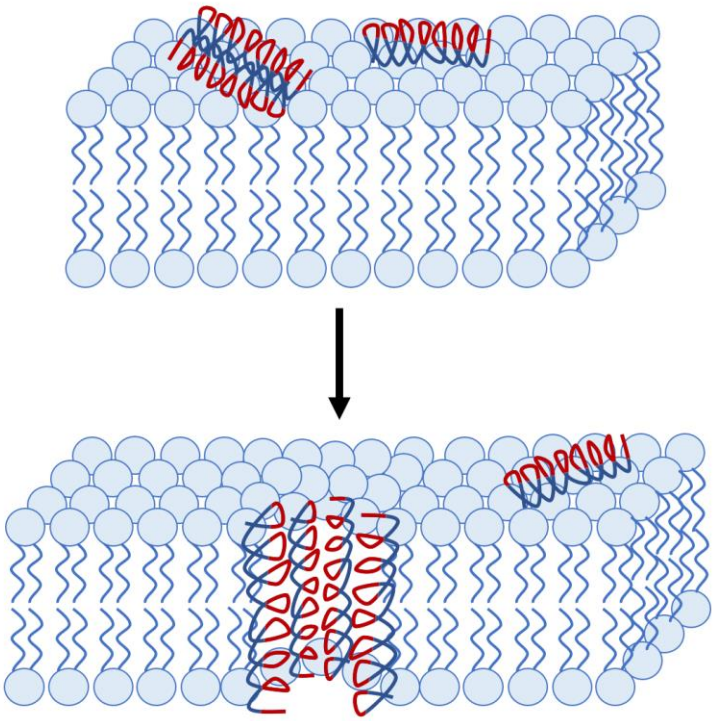

Figure 3. Barrel-stave model. Bundles of AMPs insert into the membrane and create a perpendicularly oriented transmembrane pore. Blue and red colors represent hydrophobic and hydrophilic portions of the AMP, respectively. Figure adapted from reference [66].

The barrel-stave mechanism is defined by the formation of "stave-like" transmembrane pores with the antimicrobial peptide aligned perpendicularly to the lipid bilayer. In this case, the hydrophobic portion of the AMP is aligned towards the lipid bilayer, creating a hydrophilic channel which increases the permeability of the membrane.

Carpet Model

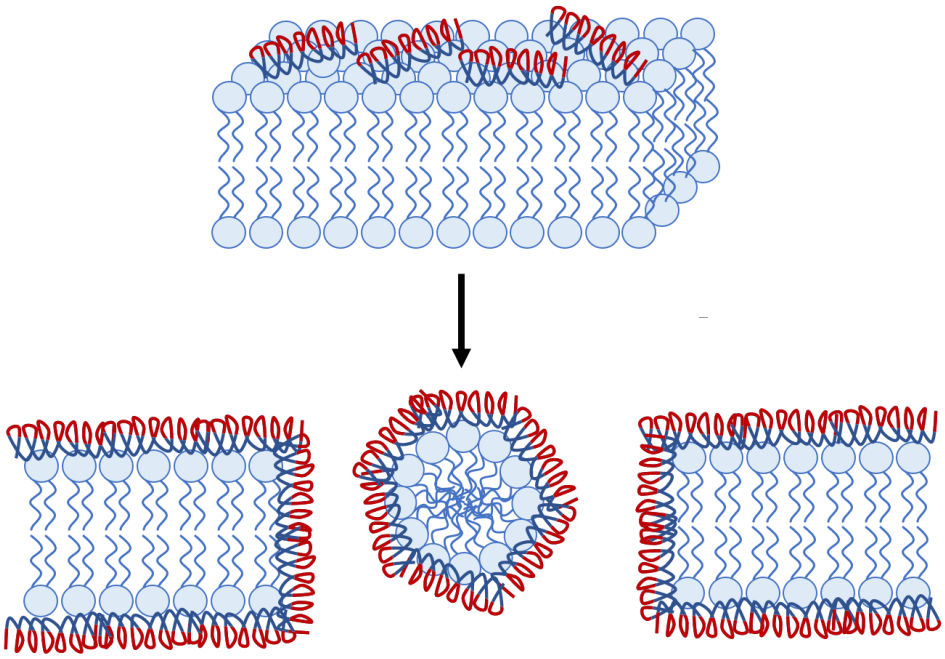

Figure 4. Carpet model. High concentrations of AMPs blanket the surface of the membrane. Disruption of the membrane surface leads to the formation of micelles. Blue and red colors represent hydrophobic and hydrophilic portions of the AMP, respectively. Figure adapted from reference [66].

In the carpet model, AMPs are described as having a "detergent-like" effect.[81] Peptides are prone to align parallel to the membrane surface. At high enough concentration, AMPs begin to reorient, disrupting the underlying membrane and allowing for further penetration. At critical concentrations, micelles form and begin to break away from the larger bilayer structure, forming defects and holes. This mechanism can be identified by the presence of phospholipid fragments in bacterial solution.

Toroidal Model

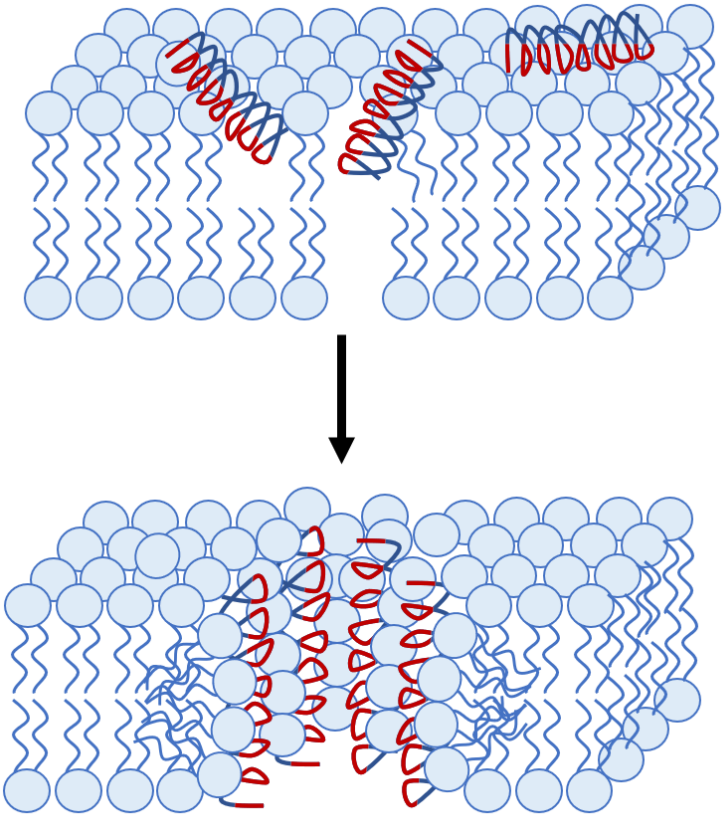

Figure 5. Toroidal pore model. AMPs penetrate and distort the lipid bilayer, creating toroidal channels lined by both hydrophobic lipid heads and hydrophilic portions of the AMP. Blue and red colors represent hydrophobic and hydrophilic portions of the AMP, respectively. Figure adapted from reference [66]

The toroidal pore model is similar to the barrel-stave model, except that the hydrophobic portion of the AMP interacts exclusively with the lipid head of the membrane. Insertion of the AMP into the lipid bilayer results in bending of the monolayer in order to form a curved pore.[65] The toroidal pore is lined by the hydrophobic lipid head and the hydrophilic region of the AMP. It is via this method that the human-derived cathelicidin AMP, LL37, is proposed to interact with bacterial membranes.[82]

Chapter 2: Materials/Methods

2.1 Materials

Three *E. coli* strains were obtained from clinical isolates for study. *E. coli* B37, *E. coli* B73, and *E. coli* B78 were obtained from UTI patients at VA Medical Center (Minneapolis, MN) and stored at -80^{o}C. *E. coli* B37 and *E. coli* B78 are known to have intermediate resistance to treatments of ampicillin / sulfamethoxazole. *E. coli* B73 had shown resistance to treatments of trimethoprim / sulfamethoxazole.[43]

E. coli strains were cultured in Muller-Hinton broth (MHB) purchased from Sigma-Aldrich (St. Louis, MO) and on Mueller-Hinton agar (MHA) purchased from Hardy Diagnostics (Santa Maria, CA). LL37, FK16, and FK13 were purchased from Anaspec, Inc. (Fremont, CA). Vancomycin, polymyxin B, and colistin were purchased from Sigma-Aldrich (St. Louis, MO).

0.2 µm sterilization filters (Whatman™ Puradisc™ 30/0.2 CA S, Cytiva, Marlborough, MA) and 1 mL syringes (Luer-Lock™, BD, Franklin Lakes, NJ) were used in preparation of sterile stock solutions. 0.5 McFarland Standard (Hardy Diagnostics, Santa Maria, CA) was used for the standardization of bacterial suspensions. Assays were performed in 96 well polypropylene cell culture plates obtained from Corning Incorporated (Somerville, MA). LPS from *E. coli* O55:B5 was purchases from Sigma-Aldrich (St. Louis, MO).

2.2 Methods

2.2.1 Preparation of media

MHA plates were prepared according to manufacturer specifications. 38 g/L of dehydrated MHA media was completely dissolved in deionized water. The suspension was autoclaved at 121 ^{o}C for 15-30 minutes. Upon cooling, the media was poured into petri dishes to a thickness of approximately 4mm. Prepared agar plates were stored at 4 ^{o}C.

E. coli strains were removed from storage at -80 ^{o}C for inoculation. Bacteria was spread evenly across the agar surface. The inoculated agar plate was incubated at 37 ^{o}C for 18-24 hours. Inoculated plates were stored at 4 ^{o}C until use.

For suspensions, MHB media was prepared according to manufacturer specifications. 21 g/L of dehydrated MHB media was dissolved in deionized water. Cation-adjusted MHB medium was also prepared to better reflect cation concentrations in human blood. For cation-adjusted MHB medium, $CaCl_2$ and $MgCl_2$ were added to reach final concentration of 20 mg/L Ca^{2+} and 10 mg/L Mg^{2+}. The suspensions were autoclaved at 121 ^{o}C for 15 minutes. MHB media was stored at 4 ^{o}C until use.

2.2.3 Broth microdilution assay

Minimum inhibitory concentration (MIC) of antibiotics and AMPs was determined according to published methods.[83] MHB media was inoculated via colonies from MHA plates. Inoculated MHB was incubated overnight at 37 ^{o}C overnight to achieve log phase growth. Incubation was facilitated in a Tissue Culture Roller Drum TC-7 (New Brunswick Scientific Co., Inc) at 40 rpm.

AMPs and antibiotics were dissolved in autoclaved deionized water and filtered with a 0.2 µm filter to create sterile stock solutions. The solutions were stored at the appropriate temperatures according to manufacturer guidelines. Antimicrobial stock solutions were used to prepare two-fold serial dilutions in 96-well polypropylene microtiter plates. 10 µl of antimicrobial solutions were placed in each well and the serial dilution was performed. The serial dilution was accomplished by pipette mixing the stock solution

in a 1:1 ratio with water, using the diluted solution to pipette mix the next well in a 1:1 ratio, and progressing as such so that each successive well contains an antibiotic concentration equal to ½ the concentration of the previous well.

A spectrophotometer (Genesys 20, Thermo Scientific™) was used to measure the optical density of the bacterial suspensions at 600 nm (OD_{600}). Using McFarland Standard No. 0.5, bacterial suspensions were diluted in MHB to an initial concentration of approximately 1.5 x 10^8 CFU/mL followed by subsequent dilution to the desired concentration. 90 µl of diluted bacterial suspension was added to each 10 µL antimicrobial solution within the 96-well plate and mixed thoroughly via pipette mixing. The final concentration of bacteria in each well was approximately 5 x 10^5 CFU/mL.

The inoculated plates were incubated at 37 ^oC for 20 hours. The plates were visually inspected, and the MIC was determined as the lowest antimicrobial concentration at which no visible *E. coli* growth could be identified. A representation of this method is provided in Figure 6 below. Each experiment was repeated in triplicate. If the MIC results differed between experiments, or if contamination of wells within the plate was suspected, additional repetitions were added for analysis.

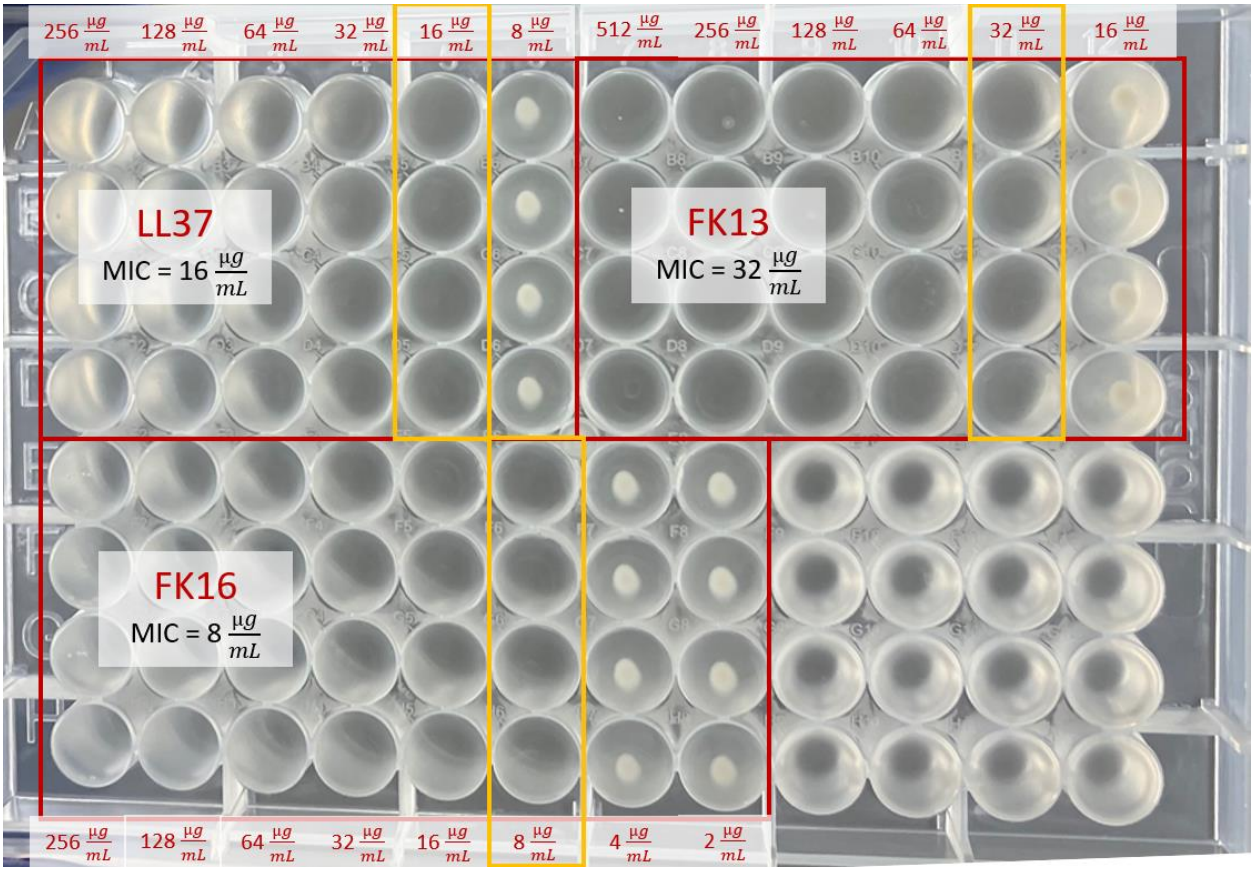

Figure 6. Example of a broth microdilution assay used to determine MICs of LL37, FK16, and FK13 against *E. coli* B78 in standard MHB medium. Two-fold serial dilutions were performed from stock solutions. The MIC was determined as the lowest concentration for which no visible growth of *E. coli* could be observed (highlighted in orange boxes).

2.2.4 Checkerboard assay

A checkerboard assay was used to investigate the effects of AMP-antibiotic combinations. The fractional inhibitory concentration index (FICI) was used to determine whether combinations displayed synergistic effects.[50,84] A two-fold serial dilution of antibiotic solutions was performed to prepare 10 µL of antibiotic solutions in decreasing concentrations to a 96-well polypropylene plate. The two-fold serial dilution was performed according to the method described in **Section 2.2.3**. To each well containing antibiotic solution, 10 µL of AMP solution at a concentration of ¼ MIC was added. *E. coli* suspensions were prepared as described in **Section 2.2.3**. 80 µL of *E. coli* suspensions were added to the AMP-antibiotic combination wells for a final bacteria concentration of 5 x 10^5 CFU/mL. The inoculated plate was then incubated at 37 ^{o}C overnight and the new MIC was determined as the lowest antimicrobial concentration at which no visible *E. coli* growth could be identified. Experiments were performed in triplicate.

Synergism of AMP-antibiotic combinations were determined via visual assessment. Synergy was determined using the FICI, the equation for which is shown below: [85]

$$FICI = \frac{MIC\ of\ A\ in\ combinaton\ with\ B}{MIC\ of\ A} + \frac{MIC\ of\ B\ in\ combination\ with\ A}{MIC\ of\ B}$$

FICI of ≤ 0.5 indicates a synergistic combination. FICI between 0.5 – 4.0 indicates no interaction. FICI > 4.0 indicates an antagonistic combination. [84] An example of a checkerboard assay experiment is given in Figure 7.

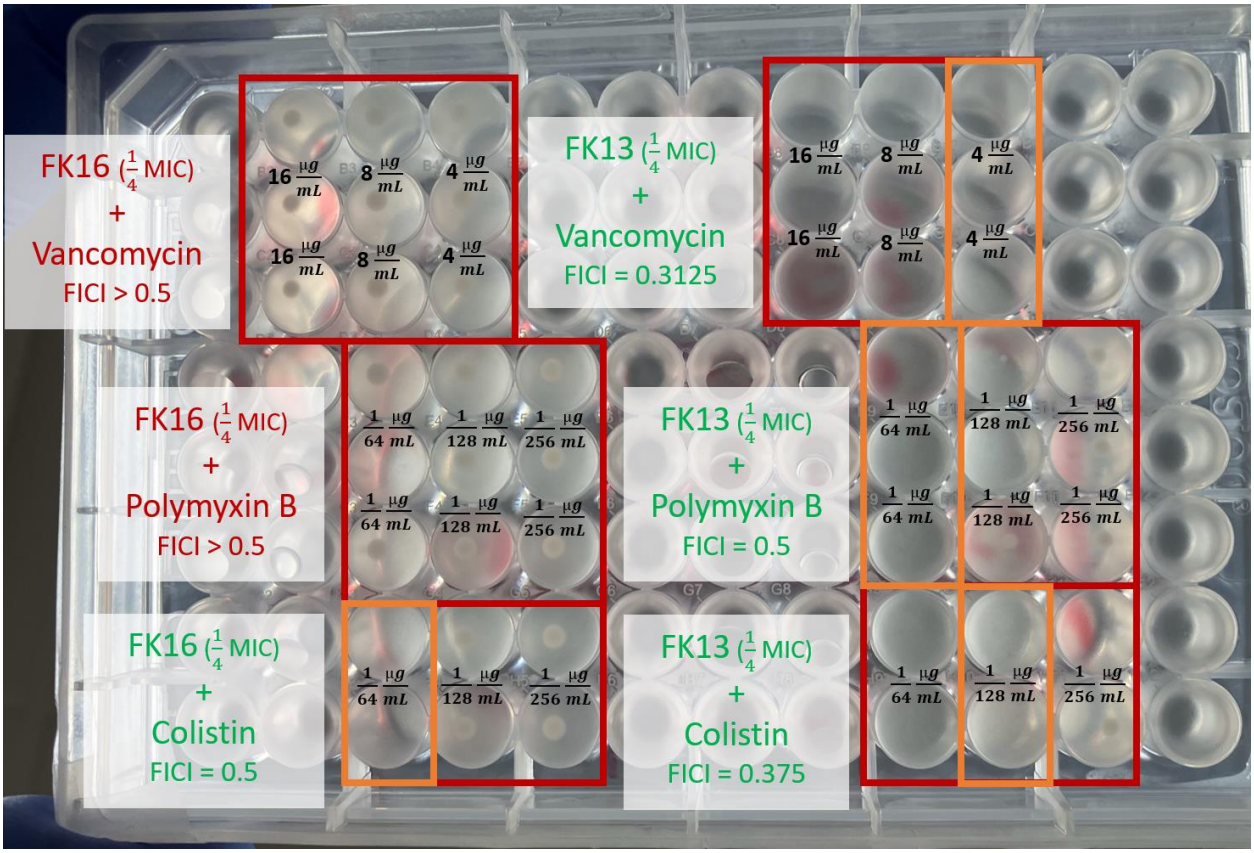

Figure 7. Example of a checkerboard assay used to determine FICI of AMP-antibiotic combinations. FK16 and FK13 combinations shown for *E. coli* B73. MICs are shown in orange boxes.

2.2.5 Vancomycin sensitization

Sensitization of *E. coli* strains to vancomycin after treatment with AMPs was investigated to support synergistic findings. *E. coli* suspensions were prepared in MHB medium according to the procedure described in **Section 2.2.3**. *E. coli* suspensions were diluted to a concentration of 1 x 10^6 CFU/mL. AMPs were introduced into this suspension at a concentration equal to ½ MIC. The AMP-treated *E. coli* suspensions were incubated at 37 ^{o}C for one hour alongside untreated controls. A two-fold dilution of vancomycin was performed to add 10 µL solutions of decreasing concentrations to a 96-well plate. 90µL of AMP-treated and untreated controls were added to the vancomycin wells for a final bacteria concentration of 5 x 10^5 CFU/mL. The new MIC of vancomycin after treatment with AMPs was determined as previously described. The experiment was performed in triplicate.

In order to investigate the impact of the three AMP treatments on the sensitization of vancomycin, an ordinary one-way ANOVA was performed on the resulting MIC values. This type of analysis is useful in investigating the effects of one factor (AMP treatment) on one dependent variable (MIC). The null hypothesis of this test is that the mean MIC value of all AMP treatments and the control are identical. However, this analysis has a limitation in that it cannot distinguish which treatment, if any, is different from the others. Therefore, Dunnett's multiple comparison was used as a post hoc test to compare the mean MIC value of each AMP treatment against the mean MIC of the control. The result of this analysis

reports a P value, with a low P value indicating a high probability of statistical difference between the treated group and the control group.

2.2.6 LPS addition

The role of LPS on cell viability was investigated by adding LPS extract to *E. coli* suspensions and determining the effect on MIC. LPS was dissolved directly into deionized water. The procedure for determination of MIC was repeated, however, LPS was added to each well in the 96-well plate at a concentration of 100 μg/mL. All other steps remained the same. The MICs of AMPs were determined after the addition of LPS via visual assessment. Experiments were performed in triplicate.

2.2.7 Zeta potential

The zeta potential of bacterial solutions and LPS solutions before and after additions of antibiotics were measured to investigate LPS binding and neutralization potentials. *E. coli* strains were incubated in MHB or cation-adjusted MHB medium at 37 ^{o}C overnight to reach log phase growth. The suspensions were washed before zeta potential measurements. The suspensions were centrifuged at 4000 rpm for 5-minutes before the supernatant was removed and the bacteria was re-suspended in deionized water or cation-adjusted deionized water. This washing procedure was repeated two times. Following washing, the *E. coli* suspensions (now dispersed in water) were diluted to 1 x 10^6 CFU/mL. Zeta potential was determined by adding *E. coli* suspensions to a folded capillary cell (DTS1070, Malvern Instruments, Inc.) and measured using a Zetasizer Nano-ZS ZEN 3600 (Malvern Instruments, Inc.). The effect of AMPs and antibiotics on the zeta potential of *E. coli* strains was investigated by adding various concentrations to the bacterial suspensions. Similarly, *E. coli* LPS extract was dissolved in deionized water or cation-adjusted water to a concentration of 100 μg/mL and zeta potential was measured according to the same procedures. Experiments were performed in triplicate.

Chapter 3: Results

3.1 MIC

3.1.1 Standard MHB Medium

Table 2. MICs of antimicrobials against *E. coli* strains in MHB medium.

Anti-microbials	MIC (μg/mL)		
	E.coli B37	*E.coli* B73	*E.coli* B78
LL37	32	16	16
FK16	4	2	8
FK13	16	16	32
Vancomycin	64	64	64
Polymyxin B	0.0625	0.0625	0.125
Colistin	0.0625	0.0625	0.0625

The MICs of each antibiotic as determined via the broth microdilution assay in standard MHB media are given in Table 2. Vancomycin is the only antibiotic tested that does not target the outer membrane and it is generally ineffective against gram-negative bacteria.[17] The antimicrobial efficacy of vancomycin relies upon binding to Lipid II, a component essential in the synthesis of the bacterial cell wall.[12,86] However, vancomycin cannot effectively permeate the outer membrane, rendering it ineffectual. Therefore, it is unsurprising that vancomycin displays the highest MIC against all *E. coli* strains. Polymyxin B and colistin are structurally similar, differing only by one amino acid substitution.[35] They are both effective in binding to LPS and disrupting membrane function. This is facilitated in part by the net positive charge (+5) for each molecule enabling interaction with negatively charged LPS.[87,88] Polymyxin and colistin have a higher affinity for LPS than the divalent cations, Ca^{2+} and Mg^{2+}, which form stabilizing bridges between LPS molecules. Thus, these drugs displace the cationic bridges, destabilize the outer membrane, increase permeability, and ultimately lead to cell death.[35] The effectivity of this mechanism is highlighted by the low MIC values for polymyxin and colistin in the broth microdilution assay.

As cationic peptides, the initial mechanism of action for LL37, FK16, and FK13 is similar to that of polymyxin B and colistin. FK16 displays the lowest MIC among the three AMPs, with the efficacy of LL37 and FK13 being roughly equivalent. The differences in efficacy between LL37 and its fragments may have stem from differences in charge and structure. LL37 is the more positively charged than FK16 and FK13 (+6 versus +4), which may promote stronger affinity and interaction with LPS. Structural differences can also define the interaction of an AMP with the outer membrane. The ability of an AMP to form an amphiphilic α-helical structure has been strongly correlated with its antimicrobial behavior.[72,89–91] LL37 forms approximately 70-80% α-helical structure, with a disordered region on the N-terminus while FK16 and FK13 fragments truncate this terminus to adopt near-perfect α-helical structures.[73,74] An NMR study on

LL37 and its fragments investigated the structure of peptides when bound to a membrane-mimicking micelle. It was found that the last three residues of FK16 were not essential for membrane binding, leading to the development of FK13. However, deletion of these residues increased the MIC of FK13 towards *E. coli*, revealing that this hydrophobic terminal group is in some way responsible for antimicrobial efficacy. The MIC results against *E. coli* in that study are in good alignment with the results presented here.[73]

3.1.2 Cation-Adjusted MHB Medium

As discussed, cations such as Ca^{2+} and Mg^{2+} play a significant role in the stabilization of the outer membrane by providing electrostatic salt bridges between negatively charged LPS molecules.[13,33,92] The effectivity of cationic antibiotics relies, in part, on their ability to bind to LPS and displace these divalent cations. Increasing the local concentration of Ca^{2+} and Mg^{2+} creates a dual effect by increasing the competition with antibiotics for negatively charged binding sites and stabilizing the outer membrane. Thus, the broth microdilution assay was also used to determine the MICs of antibiotics in adjusted MHB medium having cationic concentrations mimicking those of human blood. Table 3 displays the MIC of antibiotics in cation-adjusted medium.

Table 3. MICs of antimicrobials against *E. coli* strains in Mg^{2+} and Ca^{2+} cation adjusted MHB medium.

Anti-microbials	**MIC (µg/mL)**		
	E.coli B37	*E.coli* B73	*E.coli* B78
LL37	> 256	64	64
FK16	64	16	32
FK13	64	64	128
Vancomycin	128	128	128
Polymyxin B	0.125	0.125	0.125
Colistin	0.125	0.0625	0.125

The MIC of almost every antibiotic increased upon addition of divalent cations (the lone exception being colistin versus *E. coli* B73). The MIC of polymyxin B and colistin increased approximately 2-fold, but still remained relatively low, indicating that the *E. coli* outer membrane was stabilized, but the drugs were still able to competitively displace Mg^{2+} and Ca^{2+}. The MIC of vancomycin is also increased 2-fold, highlighting continued inability to reach Lipid II targets.

The effect of increased cation concentration is most drastic on the AMPs. MICs of AMPs increased a minimum of 4-fold, but also as high as 16-times the original value. The salt-sensitivity of LL37 and its analogues towards antimicrobial efficacy is a drawback that is also witnessed in literature.[72,74,91] Among the AMPs, FK16 generally remained the most effective with the lowest MICs across the three *E. coli* strains. However, the two-fold serial dilution method used to prepare antibiotic solutions is a noted limitation in the accuracy of these types of comparisons.

3.1.3 LPS Enriched MHB Medium

In order to investigate the interaction between AMPs and their proposed target molecules, MIC was also determined for AMPs in MHB medium with an exogenous LPS concentration of 100 μg/mL. In much the same way that additional covalent cations increase the MICs of AMPs via membrane stabilization, higher concentrations of LPS may increase MIC by decreasing the availability of AMPs targeting active bacterial cells. In other words, interaction with exogenous LPS molecules would decrease the availability for AMPs to interact with LPS molecules at membrane surfaces. In fact, an increase in MIC is seen for LL37 and both analogues after introduction of exogenous LPS as shown in Table 4. This shows that AMPs do in fact interact with LPS, and LPS is likely a target molecule for AMP antibiotic activity. A similar effect has been shown for a different outer membrane permeabilizing peptide upon exposure to exogenous LPS in literature.[12]

Table 4. MICs of peptides against *E. coli* strains with concentration of 100 μg/mL LPS.

Anti-microbials	**MIC (μg/mL)**		
	E.coli B37	*E.coli* B73	*E.coli* B78
LL37	> 256	> 256	> 256
FK16	16	16	64
FK13	128	128	128

FK16 remains the most effective AMP across all three broth microdilution assays followed by FK13. These results may highlight the relative importance of an α-helical structure (FK16 and FK13 are more structured) for *E. coli* antibacterial activity over charge effects and binding potential (for which LL37 has the higher charge difference).

3.2 AMP Synergism

3.2.1 Standard MHB Medium

LL37, FK16, and FK13 were combined with the antibiotics vancomycin, polymyxin B, and colistin. Vancomycin is used clinically to treat infections of gram-positive bacteria while polymyxin B and colistin are considered last-resort drugs for gram-negative infections. The results of the checkerboard assay in standard MHB medium are shown in Table 5. Synergism was determined by using the equation to calculate FICI for each combination. LL37 showed the most synergistic effects among all AMPs with FICI ≤ 0.5 for 7/9 combinations. LL37 displayed synergism with polymyxin B and colistin against all three *E. coli* strains, however, only displayed synergism with vancomycin against *E. coli* B37. FK13 had the next most synergistic combinations with antibiotics (5/9) followed by FK16 (2/9).

There were more synergistic combinations identified for the membrane targeting antibiotics, polymyxin B (6/9) and colistin (5/9), than there were for the lipid II targeting drug, vancomycin (3/9). This result is interesting, as a literature survey conducted in 2017 indicated that membrane targeting antibiotics are most often studied in combination with antibiotics that target intracellular compounds.[93] This line of reasoning has guided the research of many successful combinations between membrane-targeting AMPs

and intracellular targeting antibiotics.[57,65] However, the results here indicate that combinations between two or more classes of membrane-targeting antimicrobials may be worth deeper investigation. A recent article also found many synergistic combinations between short cationic peptides and polymyxin B against gram-negative *P. aeruginosa* strains. Synergistic combinations were more frequent between AMPs and polymyxin B than any other intracellular-targeting antibiotic in the trial. However, in this study, a synergistic effect was not identified for LL37.[94]

Table 5. Peptide-antibiotic combinations against *E. coli* strains in MHB medium. Bolded numbers indicate synergistic combination.

Strain	∑ FICI			
	Antimicrobial	Vancomycin	Polymyxin B	Colistin
E.coli B37	LL37	**0.5**	**0.5**	**0.5**
	FK16	> 0.5	> 0.5	> 0.5
	FK13	> 0.5	> 0.5	> 0.5
E.coli B73	LL3-7	> 0.5	**0.5**	**0.5**
	FK16	> 0.5	> 0.5	**0.5**
	FK13	**0.3125**	**0.5**	**0.5**
E.coli B78	LL37	> 0.5	**0.5**	**0.5**
	FK16	> 0.5	**0.5**	> 0.5
	FK13	**0.5**	**0.5**	> 0.5

3.2.2 Cation-Adjusted MHB Medium

Synergistic combinations were more frequently identified in cation-adjusted MHB medium than standard MHB medium (17/27 versus 14/27). The results for these tests are shown in Table 6. Every AMP tested displayed a synergistic combination with polymyxin B against all *E. coli* strains. Each AMP also had synergistic combinations with colistin against *E. coli* B37 and *E. coli* B78, but no AMPs showed synergism with colistin against *E. coli* B73. Once again, synergistic combinations with vancomycin were least common (2/9) and only against one strain, *E. coli* B37.

The results here suggest that in environments that stabilize the outer membrane, such as cation-adjusted medium or human blood, combinations of two membrane-targeting antimicrobials may offer a promising pathway for treatment. In these cases, the presence of a higher concentration of cationic antimicrobials leads to higher likelihood for displacement of divalent cations and destabilization of the cell membrane.

Table 6. Peptide-antibiotic combinations against *E. coli* strains in Mg^{2+} and Ca^{2+} cation adjusted MHB medium. Bolded numbers indicate synergistic combinations.

Strain	∑ FICI			
	Antimicrobial	Vancomycin	Polymyxin B	Colistin
E.coli B37	LL37	**< 0.375**	**< 0.5**	**< 0.5**
	FK16	**0.5**	**0.375**	**0.5**
	FK13	> 0.5	**0.5**	**0.5**
E.coli B73	LL37	> 0.5	**0.5**	> 0.5
	FK16	> 0.5	**0.375**	> 0.5
	FK13	> 0.5	**0.5**	> 0.5
E.coli B78	LL37	> 0.5	**0.375**	**0.375**
	FK16	> 0.5	**0.5**	**0.5**
	FK13	> 0.5	**0.375**	**0.375**

3.2 Vancomycin Sensitization

In order to further investigate the interaction of LL37 and derived fragments and to test the theory that AMP treatment results in membrane permeation, bacterial uptake of vancomycin after AMP treatment was studied. As a large scaffold antibiotic (molecular weight of 1450 Da), vancomycin is well over the 600 Da exclusion limit of the *E. coli* outer membrane.[12,17] As such, it is ineffectual in penetrating the membrane and reaching its Lipid II target, as shown by the high MIC values from the broth microdilution assays. By treating the membrane with sub-inhibitory concentrations of AMPs, membrane permeability may be correlated to increased uptake and efficacy of vancomycin.

E. coli suspensions were treated with LL37, FK16, and FK13 at concentrations of ½ MIC for one hour. The AMP treated suspensions were subjected to a broth microdilution assay using two-fold serial dilutions of

vancomycin. The vancomycin MIC was determined after AMP treatment and compared to an untreated control. The results are depicted in Figure 8. Reduction in vancomycin MICs are compared in Table 7.

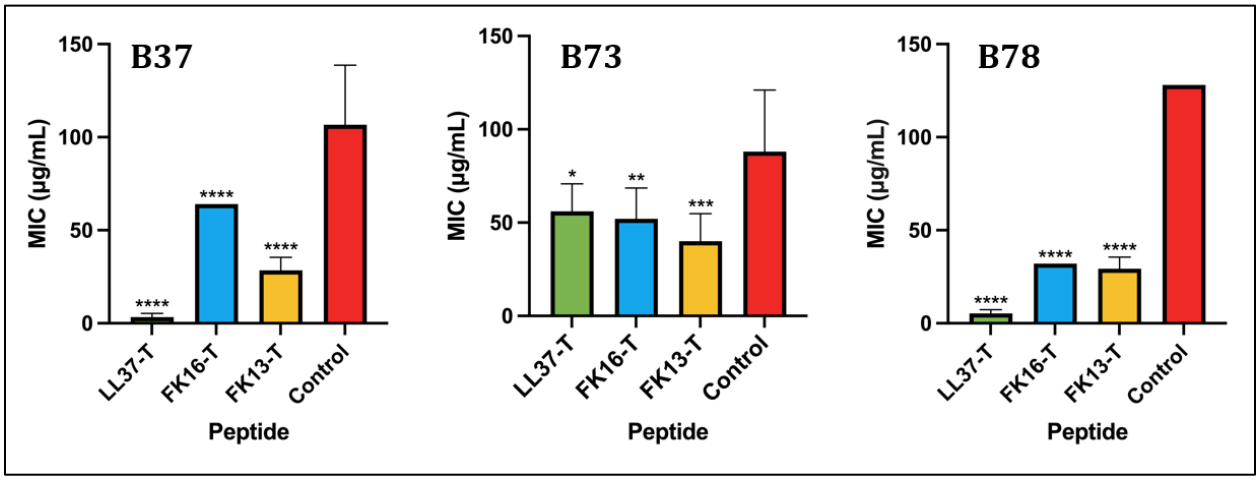

Figure 8. Vancomycin MIC after treatments with concentrations of ½ MIC of LL37, FK16, or FK13 at 37°C for one hour. Control groups were not treated with AMPs. (Left) *E. coli B37*, (Middle) *E. coli B73*, (Right), *E. coli B78.* **** P < 0.0001, *** P < 0.001, ** P< 0.01, * P < 0.05.

Table 7. Reduction in vancomycin MIC after 1-hour treatment with various peptides at ½ MIC. Synergistic combinations identified via FICI are shown in bold.

Strain	Vancomycin MIC Reduction		
	LL37	FK16	FK13
E. coli B37	**96.9%**	40.0%	73.3%
E. coli B73	36.4%	40.9%	**54.5%**
E. coli B78	95.8%	75.0%	**77.1%**

All AMP treatments reduced the vancomycin MIC for each *E. coli* strain. This is an indication that AMPs do indeed increase the permeabilization of *E. coli* outer membranes. Vancomycin had a synergistic combination with LL37 against *E. coli* B37 according to the FICI values in Table 5. Indeed, the sensitization results reveal that LL37 treatment resulted in the greatest uptake in vancomycin with an MIC reduction over 35-fold. Although the FICI calculation did not reveal a synergistic combination against *E. coli* B37 with FK16 or FK13, these peptides were still able to reduce the MIC significantly (Figure 8, Left).

This trend continued for the treatment of *E. coli* B73 (Figure 8, Middle). FK13 was found to be synergistic with vancomycin and results in the largest MIC decrease. Overall, treatments against *E. coli* B73 were not as effective as against *E. coli* B37, with MIC reductions between 36-55%.

All AMPs were quite effective in reducing the vancomycin MIC of *E. coli* B78 (Figure 8, Right). However, this strain did not show a noticeable MIC decrease for the identified synergistic combination (FK13) as compared to the non-synergistic combinations.

The results are in line with other published studied investigating combinations of membrane sensitizing agents with intracellular targeting antibiotics. MAC13243, an antimicrobial agent that inhibits lipoprotein transfer to the outer membrane, was able to sensitize *E. coli* towards several intracellular antibiotics.[17] In a study investigating the effects of eight natural and synthetic AMPs with vancomycin, six were found to be synergistic against *E. coli.*[12] In similar studies, treatment with LL37, FK16, and FK13 was shown to decrease the MIC of vancomycin towards gram-negative *P. aeruginosa.*[67,79]

3.3 Zeta Potential

Zeta potential is a measurement that can be used to indirectly characterize the surface potential of bacteria. The zeta potential, or electrokinetic potential, represents the net charge of molecules exposed on the surface of a material. The zeta potential of a bacteria cell influences adhesion towards host surfaces, biofilm formation, and proper maintenance of cellular function.[95] In recent years, several studies have used zeta-potential to characterize the interactions of membrane targeting antibiotics and peptides with the outer membrane of gram-negative bacteria, including *E. coli*.[96–98] A schematic displaying a zeta potential measurement is provided in Figure 9. In this section, the zeta potential of *E. coli* strains and LPS are measured, as well as the effect of AMP addition on zeta potential.

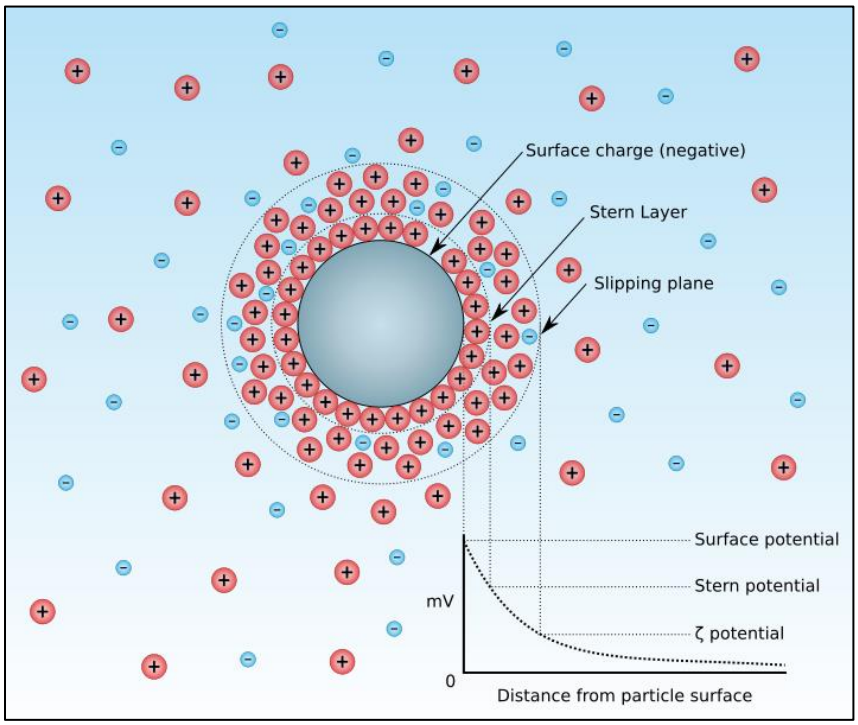

Figure 9. Schematic representation of the zeta potential of a particle surface. Source: [99]

3.3.1 Bacteria Strains and LPS

The zeta potentials of *E. coli* B37, *E. coli* B73, *E. coli* B78, and LPS extracted from *E. coli* are shown in Figure 10. The concentration of bacteria suspensions is 1 x 10^6 CFU/mL while the concentration of LPS is 100 µg/mL. As can be seen, the zeta potential measurements confirm that the net charge of each suspension is quite negative. In cation-adjusted water, the zeta potential of each suspension increases as divalent cations create salt bridges between LPS molecules.

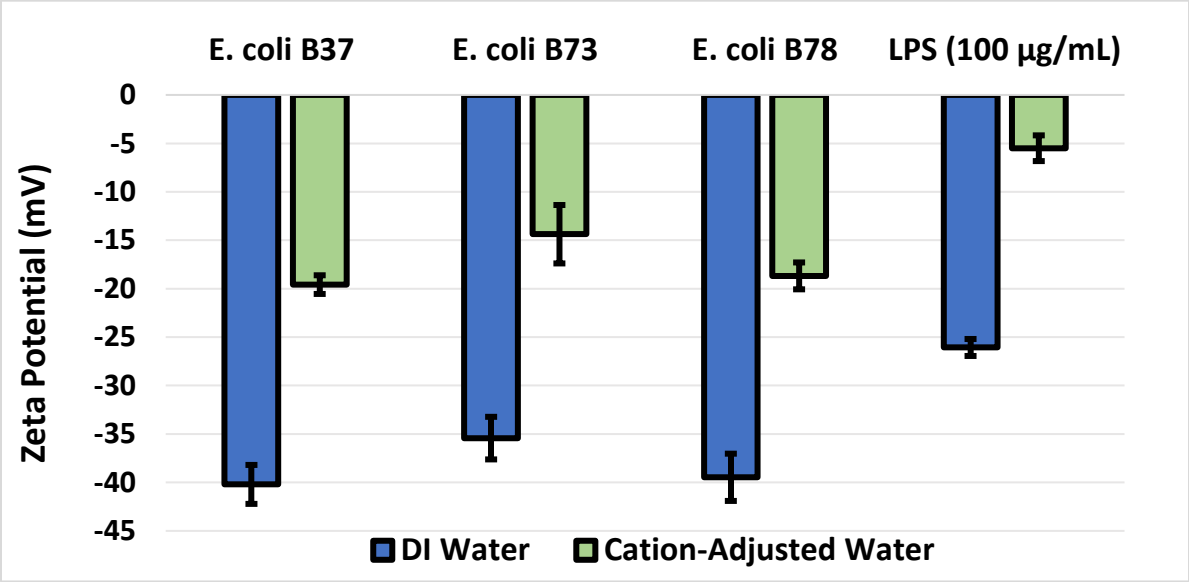

Figure 10. Zeta potential of various strains of *E. coli* and LPS suspensions in Mg^{2+} and Ca^{2+} cation adjusted water.

3.3.2 *E. coli* neutralization

The affinity of a certain antibiotic towards bacteria cells has often been correlated with a change in zeta potential in the presence of the antibiotic. Here, the effects of LL37, FK16, FK13, polymyxin B, and vancomycin on the zeta potential of bacterial solutions was investigated. The surface charge of the antimicrobial will have a significant effect on the interaction with a given bacteria and the resulting membrane permeabilization potential. LL37 has net charge of +6 while FK16 and FK13 have net charges of +4. As a membrane acting agent, polymyxin B serves as a positive control in this study with a surface charge of +5 at physiological conditions.[100] As an intracellular targeting antibiotic, vancomycin serves as negative control with a surface charge of +0.65 at physiological conditions.[101]

The influence of increasing concentration of LL37, FK16, FK13, and polymyxin B is shown in Figure 11. As can be seen, the concentration of antimicrobial needed to neutralize the bacteria suspension is directly correlated to the surface charge of the antimicrobial. For all three strains, LL37 (+6) neutralized the bacteria at the lowest concentration, followed by polymyxin B (+5), and lastly by FK16 and FK13 (+4) which displayed roughly equivalent neutralization properties.

In addition, the initial charge of the *E. coli* suspension dictated the amount of antimicrobial needed to neutralize the suspension. *E. coli* B37 and *E. coli* B78 suspensions had roughly equivalent zeta potentials

(-40.2 mV and -39.5 mV respectively) and the neutralization concentration of each antimicrobial was also roughly equivalent. However, *E. coli* B73 has a higher zeta potential (-35.4 mV), thus the amount of each antimicrobial needed to neutralize the suspensions were dramatically reduced.

The zeta potential of *E. coli* strains upon introduction of vancomycin is shown in Figure 12. As might be expected by the low surface charge (+0.65), the neutralization concentrations of vancomycin are 1-2 orders of magnitude higher than the membrane targeting antibiotics. Still, the neutralization concentration of *E. coli* B73 is less than half of the concentration required to neutralize *E. coli* B37 or *E. coli* B78.

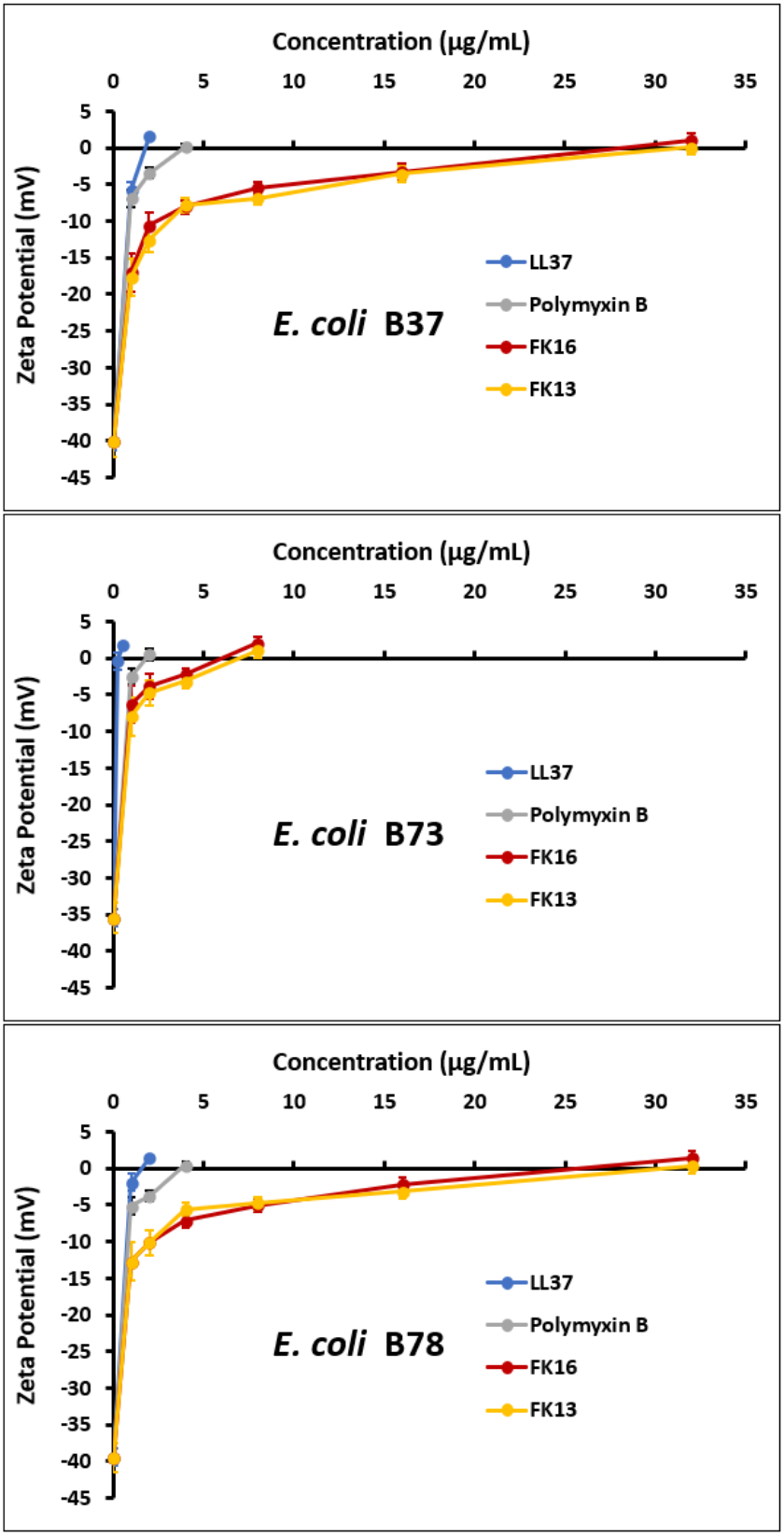

Figure 11. Zeta potential of various strains of E. coli upon introduction of increasing concentrations of AMPs and polymyxin B. *E. coli* B37 (top), *E. coli* B73 (middle), *E. coli* B78 (bottom).

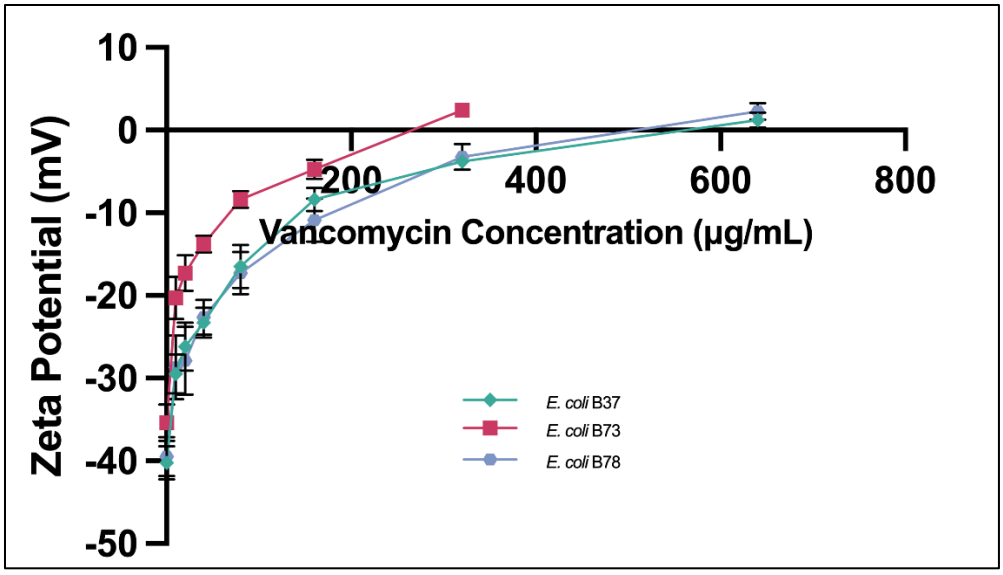

Figure 12. Zeta potential of various strains of E. coli upon introduction of increasing concentration of vancomycin.

3.3.3 LPS Neutralization

Zeta potential of LPS solutions upon addition of LL37, FK16, and FK13 was similarly studied. The results are shown in Figure 13. Interestingly, although the initial zeta potential of the LPS solutions was less negative than the bacteria suspensions, the concentrations of AMPs needed to neutralize the solution were significantly higher. In addition, the differences in zeta potential between equivalent concentrations of LL37 and the less charged fragments, FK16 and FK13, were much lower than observed in the bacteria suspensions. These two observations indicate that there is a fundamental difference in the way that AMPs interact with free LPS molecules as compared to those bound to the outer membrane. It has been shown that introduction of AMPs to LPS solutions causes LPS molecules to aggregate, effectively blocking AMPs from accessing all the negatively charges sites on the LPS molecules.[102,103] The ability of AMPs to dissociate LPS aggregates and neutralize the endotoxin is seen as an important property for modulating immune responses and inhibiting septic shock. In successful cases, the surface adsorption of AMPs onto LPS molecules blocks the ability of LPS to bind to host cells. This is one reason why LL37 is said to have dual antimicrobial and immunomodulatory roles in nature, and is being investigated as an anti-sepsis therapeutic agent.[72,103] A similar mechanism of LPS aggregation and dissociation may be occurring upon introduction of AMPs in this study.

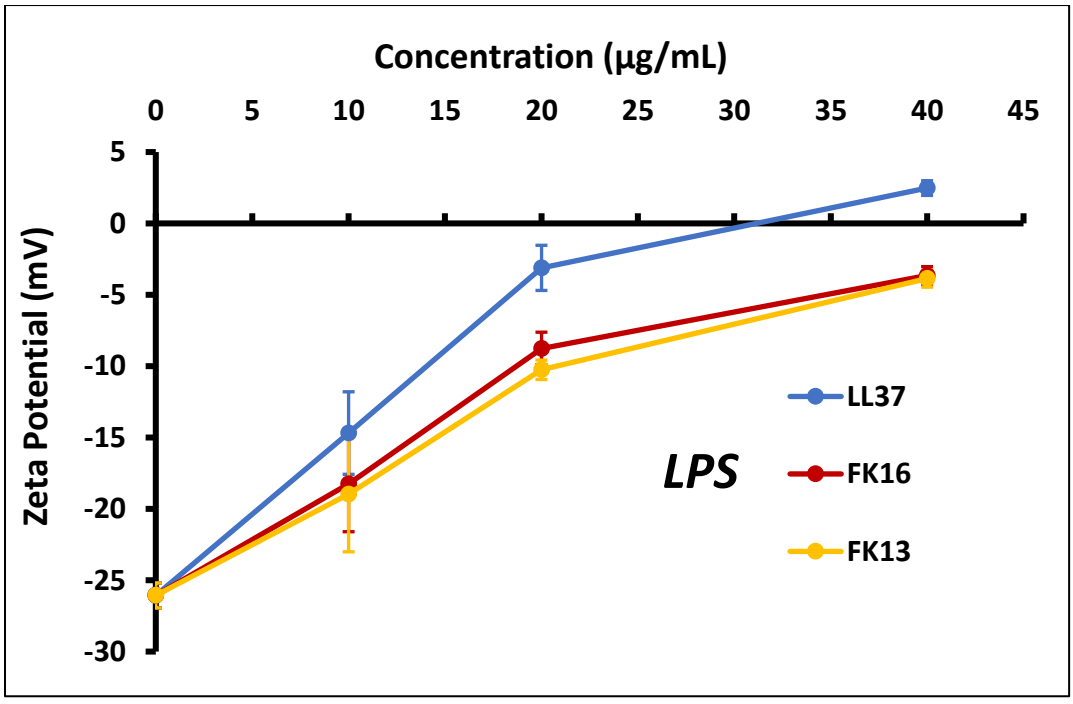

Figure 13. Zeta potential of LPS solution (100 µg/mL) with addition of increasing concentration of AMPs.

Chapter 4: Discussion

The rise of antibiotic resistance in *E. coli* raises concern for the long-term effectiveness of treatment for conditions such as UTIs, gastrointestinal tract infections, and sepsis.[23,30,39,41,46] This comes in front of a backdrop of growing antimicrobial resistance for many bacterial species across the globe.[1–4] Thus, it is imperative to identify new solutions to combat bacterial infections before the current supply of antibiotic treatments becomes obsolete.

LL37 and its shorter peptide derivatives, FK16 and FK13, were chosen for this study based on previous findings from our own laboratory, but also in published literature.[67,79,89] LL37 is a human derived, α-helical AMP from the cathelicidin family of peptides. It has natural roles in the human immune system as a broad range antimicrobial and immunomodulatory agent.[72,89] FK16 and FK13 are synthetic AMPs derived from LL37 which isolate the α-helical structure and preserve the antimicrobial functionality of LL37 towards gram-negative bacteria. These synthetic peptides have additional advantages of lower cost of synthesis and lower cytotoxicity towards human cells.[73,74,79] LL37 and its derivates have been studied as standalone antimicrobial agents, but recently have been highlighted in combination with traditional antibiotics. The membrane permeabilizing properties of these antimicrobial peptides has been of particular interest for introducing efficacy of intracellular targeting antibiotics towards gram-negative bacteria.[67,79] The identification and use of synergistic combinations has many advantages including improved antimicrobial efficacy, broader spectrum of treatment, lower dosages and toxicities, and less chance of developing resistance.[50] Thus, identification and evaluation of synergistic combinations with LL37 and its shorter synthetic peptide derivatives is the primary focus of this research.

In this work, the MIC of FK16 against the three *E. coli* strains was generally lower than those of LL37 or FK13 in both standard medium and cation-adjusted medium as presented in Table 2 and Table 3. This result generally agrees with a previous study which evaluated the MICs of LL37, FK16 and FK13 against *E. coli*.[73] Interestingly, this was not the case when LL37, FK16, and FK13 were studied against gram-negative *P. aeruginosa*. Against *P. aeruginosa,* LL37 showed higher antibacterial efficacy compared to either FK16 or FK13 showing that the specific antimicrobial behavior may be dependent on bacteria strain.[79]

Several synergistic combinations were identified between the AMPs and traditional antibiotics against three strains of pathogenic *E. coli*. Traditional antibiotics with synergistic combinations included vancomycin, polymyxin B, and colistin. The sensitization of *E. coli* to intracellular targeting antibiotics, including vancomycin, upon exposure to AMPs is a promising method that can expand the use-case for antibiotics that are traditionally only effective for gram-positive bacteria. The sensitization of gram-negative bacteria to vancomycin after treatment with LL37 and its derivatives has been shown in gram-negative bacteria in previous literature (particularly against *P. aeruginosa)*, but this is the first time it has been shown for *E. coli*.[67,79] Treatment with sub-inhibitory concentrations (½ MIC) of all AMPs was shown to reduce the MIC of vancomycin regardless of whether a synergistic combination was identified via the FICI method. In some cases, the MIC of vancomycin was reduced below current recommended therapeutic concentrations in human serum (15-20 μg/mL).[104] These results indicate that LL37 and derived AMPs in combination with vancomycin may have promise as a novel therapeutic treatment for *E. coli* infections.

Synergistic combinations between AMPs and either polymyxin B or colistin were identified more frequently than for vancomycin, especially when the bacteria strains were stabilized in cation-adjusted medium. In fact, every AMP in this study displayed a synergistic combination with polymyxin B (FICI ≤ 0.5) against all three strains of *E. coli*. This synergism is interesting as AMPs, polymyxin B, and colistin are all

known to target the outer membrane of *E. coli*. Additionally, AMPs, polymyxin B, and colistin are all limited in clinical practice due to hemolytic and cytotoxicity concerns.[35,65,66,72] The use of AMPs and other antibiotics in synergistic combination has been shown to dramatically decrease toxicity compared to monotherapies, lending credence to this approach.[105] The reduction in concentration needed for antimicrobial efficacy of these synergistic antibiotics may help overcome cytotoxicity concerns from their respective monotherapies.

AMPs may display synergism with the polymyxins if they are able to target different areas of the outer membrane. The exact antimicrobial mechanism for polymyxins is not well defined, but it is proposed that polymyxins initially bond to the lipid A portion of liposaccharide, displace divalent cations between LPS molecules, penetrate the membrane through a self-promoted uptake mechanism, and continue into the cell to disrupt the inner membrane.[35,88,106,107] The proposed pathway for α-helical AMPs is similar, but an interaction with the phospholipid bilayer is proposed to occur in one of three ways as discussed in **Section**

1.4.2 Membrane-Acting Mechanisms**.** In addition, LL37 has been found to have secondary antimicrobial mechanisms whereby intracellular targets responsible for metabolism and protein transport are inhibited.[75,76] The synergistic combination of LL37 with polymyxin or colistin against *E.* coli was also observed in two recent publications.[108,109] The combination of LL37 and polymyxin B was even effective against *E. coli* biofilms.[108] Our results agree with these findings and also extend the potential for synergistic combinations to the LL37 fragments, FK16 and FK13, for the first time.

Investigation of the zeta-potential of *E. coli* and LPS suspensions may reveal details about the mechanisms of interaction between AMPs and bacteria. For example, LL37 contains a higher net charge (+6) compared to FK16 or FK13 (+4). Via zeta potential, it can be seen that LL37 neutralizes the bacterial suspensions at much lower concentrations than FK16 and FK13. This phenomenon is quite indicative of LPS binding, and likely related to membrane permeabilization ability (to be investigated) but does not directly translate to antimicrobial efficacy according to our MIC results. A study on the LPS binding affinity via chromogenic LAL assays determined that FK13 lost nearly all its LPS binding efficacy compared to LL37.[74] However, our results clearly show that the antimicrobial efficacy of FK16 and FK13 are preserved. Therefore, the factors dictating antimicrobial efficacy of LL37 and its derivatives could be separated into two main steps: 1) LPS attraction/binding, and 2) interaction with the phospholipid bilayer. LL37 has the superior binding and LPS neutralizing capability, which is quite important in endotoxin neutralization and inflammatory modulation.[103] However, FK16 and FK13 can overcome decreased LPS affinities to display low MIC values, likely due to their nearly perfect α-helical structure which is associated with antimicrobial activity.[72,89–91] LL37 only has 70-80% α-helical structure, which may limit its ability to interact with and penetrate the phospholipid bilayer.[73]

The results of this study highlight the potential of LL37 and its derivatives for use in synergistic combination with traditional antibiotics against drug-resistant, gram-negative bacteria including *E. coli*. LL37, FK16, FK13 have been shown to synergize with both intracellular targeting and membrane targeting antimicrobials. The use of FK16 or FK13 over the full-length peptide offer the advantage of lower cytotoxicity,[79] improved cell selectivity,[74] and more cost-efficient synthesis pathways[65,70] while maintaining or improving antimicrobial efficacy against *E. coli*. LL37 may pose a higher threat in terms of cytotoxicity, but has the benefit of strong LPS binding and neutralizing potential which can aid in suppression of the endotoxin and lower risk of septic shock.[103]

That is not to say there aren't significant hurdles that must be overcome in order for LL37 and other AMPs to be effective in clinical environments. Manufacturing costs for traditional aminoglycoside antibiotics can be as low as $0.8/gram while AMPs range between $50-400/gram.[70] The issues of toxicity, decreased antimicrobial efficacy, and instability in vivo have limited the scope of clinical trials for treatment of infections to topical applications.[65] Synergistic combinations offer to lower therapeutic AMP dosages, but evaluating the toxicology of drugs in combination needs to be carefully screened for unexpected and adverse host effects.

Furthermore, bacterial mechanisms for building resistance to LL37 and other AMPs need to be studied in more detail to ensure the longevity and efficacy of peptide treatments. Many studies claim AMPs have low risk of inducing drug resistance because of their nonspecific interactions, multiple targets, and low rate of mutation of their primary target location (the cell membrane).[65,70,89] Indeed, 30 exposures of *P. aeruginosa* to sub-inhibitory concentrations of an aminoglycoside antibiotic increased resistance 190-fold, but the same exposure to an AMP only resulted in a 2-4 fold resistance increase.[70] However, instances of AMP resistance, including towards LL37, have been identified and may occur through several mechanisms. For example, modifications that increase the net charge of LPS result in lower LL37 affinity and inhibit antibacterial efficacy.[110,111] Similar mechanisms of action shared between LL37, colistin, and polymyxin B have led to concerns for the development of cross-resistance between the three antibiotics, and this has been occasionally identified, though with conflicting results.[110] A particularly dire outlook in the development of bacterial resistance towards LL37 considers LL37's role in the innate human immune system, and the possibility of compromising human's natural pathogenic defenses.[112,113] These possibilities further increase the attractiveness of using AMPs in synergistic combinations, rather than monotherapies, where development of antimicrobial resistance is less likely.

Chapter 5: Conclusions

LL37 and its derivates, FK16 and FK13, represent a promising new class of antimicrobial peptides for the combat of drug resistant bacterial infections. Synergism of AMPs with traditional antibiotics offers a promising therapeutic path with advantages of enhanced efficacy, reduced dosages, lower toxicity, and reduced possibility of acquired antimicrobial resistance. Synergistic combinations of these peptides with vancomycin, colistin, and polymyxin B have been shown to be effective against three strains of *E. coli*, even in the presence of cation-enriched medium which stabilized the outer membrane target. Sub-inhibitory concentrations of each peptide, regardless of the identification of synergism, was able to significantly reduce the MIC of vancomycin against all *E. coli* strains. The ability for antibiotics to neutralize suspensions of *E. coli* colonies and LPS was directly correlated to the net charge of the antimicrobial. Hurdles of manufacturability, toxicity, and in vivo stability must be overcome in order to introduce LL37 and its derivates as therapeutic agents. A wider study into the potential of acquired resistance towards these peptides is imperative for its long-term success as an antimicrobial agent.

Chapter 6: Future Work

Future work on this topic is intended to study the interactions of LL37, FK16, and FK13 with *E. coli* and human cells in more detail. Zeta potential measurements have demonstrated the ability for the cationic peptides to neutralize *E. coli* and LPS suspensions. In addition, pre-treatment of *E. coli* with each AMP resulted in a reduction in the MIC of intracellular-targeting vancomycin. Both of these results indicate disruption or permeation of the outer membrane barrier, however, there are methods to investigate this more discreetly. Outer and inner membrane permeability assays are planned according to published methods.[114] These methods investigate membrane permeability via bacterial uptake of fluorescent probes measured by a fluorescence spectrophotometer.

Outer membrane permeabilization can be measured using the fluorescent probe N-phenyl-1-napthylamine (NPN). NPN is a hydrophobic molecule which has low fluorescent emission when dissolved in water. However, when NPN binds with nonpolar molecules (such as phospholipids) the intensity of the fluorescent emission increases significantly. Binding with non-polar intracellular targets is usually blocked by the outer membrane, but in the case of membrane permeabilization the degree of NPN permeation can be correlated to an increase in the fluorescent emission. This method will be used to study the membrane permeabilization ability of each AMP and antibiotic used in this study. Further, effects of cation-adjustment medium on membrane stability can be studied in more detail using this method. Preliminary results in our laboratory indicate a strong connection between the net charge of the antibiotic and the magnitude of NPN uptake. Antibiotics with a high net positive charge, such as LL37, lead to higher uptake and fluorescent emission of NPN.

The effect of AMPs on the inner membrane of bacteria is less commonly studied, but recent literature has indicated that intracellular targets of LL37 may contribute to its antimicrobial efficacy.[75,76] Inner membrane permeability can be studied using very similar methodology with propidium iodide (PI) as the fluorescent probe. PI is another fluorescent dye, and the fluorescent emission of PI sharply increases upon binding to nucleic acids found in RNA and DNA. Since RNA and DNA are only found within the inner membrane of bacteria, increases in fluorescent emission may be indicative of inner membrane disruption. This study could further illuminate the role of AMPs after permeating the outer membrane.

Finally, in order to address one of the main challenges preventing AMPs from therapeutic applications, experiments are planned to examine the cytotoxicity of LL37, FK16, and FK13 and their combinations towards human cells. Several studies claim decreased cytotoxicity and increased therapeutic potential of FK16 and FK13 compared to LL37.[74,79] Still more claim that synergistic combinations may lead to lower cytotoxicity compared to monotherapies.[50,65,105,108] However, more research is needed to study AMP-eukaryotic cell interactions as well as the cytotoxic effects of specific AMP-antibiotic combinations in order to recommend the treatments for viable clinical study. We hope to provide further insight on this topic for the synergistic combinations identified in this study.

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