ABSTRACT

Design and Delivery of Novel Antimicrobial Peptides (Amps) Targeted Towards Specific Microbial Pathogens

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Mentor: Christopher Michel Kearney, Ph.D.

Targeted therapies selectively eliminate a pathogen without disrupting the native microbiota. Traditional antibiotics, in contrast, destroy the native microbiota along with pathogens, causing adverse health outcomes for the patient. In my research, antimicrobial peptides (AMPs) were synthesized by fusing a guide-peptide that makes them selective towards a target pathogen. *Staphylococcus aureus* was chosen as a preliminary pathogen and a previously published guiding peptide (A12C) was selected. A12C was fused to AMPs, eurocin and plectasin, and the guided-AMPs (gAMPs) were expressed in *E. coli*. The gAMPs showed strong selective inhibition of *S. aureus* in vitro but were significantly less toxic towards several off-target bacteria. This selective bactericidal effect was observed in both planktonic culture and bacterial film formations.

To optimize the in vivo delivery of gAMPs through oral route, I used engineered *Lactococcus lactis*, a probiotic bacterium and native resident of the human stomach flora. *Helicobacter pylori*, a main causal factor for peptic ulcers and gastric cancer, was the target pathogen. I targeted the VacA protein, an important virulence factor of *H. pylori*, with a guide peptide from a portion of Multimerin-1 (MM1), a human receptor for VacA.

Three different AMPs, each fused to the MM1 guide, were tested. In vitro, co-culture of the engineered probiotic expressing gAMPs strongly inhibited *H. pylori* while being significantly less toxic to off-target bacteria. In vivo tests in mice were completed by introducing the *H. pylori* and engineered probiotic by oral gavage. Probiotics delivering gAMPs as a therapy reduced the *H. pylori* stomach titer by 1860-fold compared to untreated infected mice. As a prophylactic, gAMP probiotics effectively inhibited *H. pylori* colonization of the stomach. Microbiome analysis showed that the recovery or preservation of taxonomic diversity of the stomach microbiota was much greater with the use of gAMP probiotics than with AMP probiotics or antibiotics.

My research shows that guided AMPs can be a novel and useful approach for combating pathogens without endangering the natural microbial flora and that bioengineered probiotics can be used to secrete gAMPs at the site of infection. Given the wealth of AMPs and potential guide peptides, both natural and synthetic, this approach can be adapted to develop a diverse array of chimeric guided AMPs and can be cloned into probiotics to create a safe and effective alternative to conventional chemical antibiotics.

Design and Delivery of Novel Antimicrobial Peptides (AMPs) Targeted towards Specific Microbial Pathogens

by

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A Dissertation

Approved by the Department of Biology

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Submitted to the Graduate Faculty of
Baylor University in Partial Fulfillment of the
Requirements for the Degree
of

Doctor of Philosophy

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ACKNOWLEDGMENTS

First, I would like to acknowledge my mentor Dr. Christopher Kearney and my lab members Patrick Ortiz, Grace Pruett, Mishu Islam, Meron Ghidey and Andrew Cox who has taught me and helped me through the laboratory skills and scientific expertise that I earned over the years and which helped me in the completion of all the research that built this dissertation. Their constant support, advice and most importantly the freedom I had to curate my research around my scientific interest and passion towards neglected diseases.

I would also love to acknowledge the members of my Ph.D. committee, Dr. Joseph Taube, Dr. Bessie Kebaara, Dr. Leigh Greathouse and Dr. Jason Pitts for all their invaluable suggestions, advice and scientific perspectives that has shaped my dissertation to its final form.

The Baylor Biology Department and the Baylor Graduate School deserves a special mention for all their help in making the journey in Graduate School a fruitful one and their constant advocacy for making the of all Graduate students as smooth as possible and making conducting research an enjoyable experience. Them along with the other Graduate students in my cohort has been the constant source of support one needs for having a functional working and social life as a Graduate student.

My gratitude also goes towards the entire scientific community and the healthcare professionals who have been tirelessly working towards ending the ongoing pandemic across the globe and sharing their knowledge and research freely with the rest of the world

so that aspiring scientists can build upon their achievements and progress the tapestry of the science.

Lastly, it would be remiss of me to not thank my family, my parents Animesh Choudhury and Chaitali Choudhury, my brother Ayan Choudhury, my uncle Dr. Somesh Choudhury, who inspired me to become a scientist like him, my aunt Satabdi Choudhury and my cousins Spreeha and Sompurna Choudhury who all have made my life possible in a country across the globe from my own and have incessantly urged me to better myself both as a person and as a researcher.

ATTRIBUTIONS

Ankan Choudhury is the primary author of these manuscripts and compiled the reference material and data, wrote the body of the manuscripts, and created the figures and tables. For Chapter Two, he developed the project and statistical analyses along with Dr. Christopher Michel Kearney, S.M. Ashiqul Islam and Meron Ghidey and he also developed the methodologies with the help of S.M. Ashiqul Islam, Meron Ghidey and Patrick Ortiz . For Chapters Three and Four, Ankan Choudhury developed the project with Dr. Christopher Kearney and with the help of Patrick Ortiz. Ankan Choudhury performed the *in vitro* and *in vivo* experiments, collected, and analyzed the data with constant help of Patrick Ortiz and applied for a patent based on the techniques with Dr. Kearney and Patrick Ortiz. For Chapter Four, Ankan Choudhury, with help from Patrick Ortiz, performed all the animal experimentations, as per addressed by the IACUC approved protocols, including collection of samples, extraction of DNA, Next-Gen sequencing, and subsequent statistical analyses of the microbiome sequencing data.

DEDICATION

To my family and members of my laboratory and to the late, great Pete Seegers who made me believe that “We shall always overcome”

CHAPTER ONE

Introduction

Indiscriminate killing of commensal microflora by conventional antibiotics allows opportunistic pathogens to invade commensal niche spaces and multiply (Institute of Medicine (US) Forum on Microbial Threats, 2010, Ferreyra et al., 2014). Furthermore, the ensuing imbalance in the microflora population in the host has many other health side effects including lowered immunity and improper metabolism (Ferreyra et al., 2014; Kau et al., 2011; Nicholson et al., 2012). This drives the need for producing antimicrobial agents that target only specific pathogens and spare other commensal bacteria, the need which I addressed in this dissertation.

Antimicrobial peptides (AMPs), are an alternative to antibiotics with high potential for rapid commercial product development, in contrast to the slow pace of commercial development of traditional antibiotics (Hancock and Sahl, 2006; Mygind et al., 2005). There are thousands of AMPs already fully characterized in the literature, and, being peptides, can be easily modified genetically, such as adding peptide tags that confer specificity. AMPs are found across bacterial, animal and plant taxa, and these inhibit or destroy bacteria, viruses and/or fungi. (Ganz, 2003; Hancock and Diamond, 2000; Hancock and Sahl, 2006, Lei et al., 2019). Recombinant AMPs can be heterologously expressed in bacterial (Li, 2011; Zorko and Jerala, 2010), fungal (Cao et al., 2018; Mygind et al., 2005; Oeemig et al., 2012) or plant (Ghidey et al., 2020; Company et al., 2014; Holaskova et al., 2015; Morassutti et al., 2002) bio-factories for mass screening and production. This is in

contrast to optimizing the synthesis of each single chemical antibiotic, which, at present, slows down the process of antibiotics development. AMPs exhibit a broad mechanism of action that forms pores in bacterial membranes, and thus it has been suggested that it may be more difficult for bacteria to evolve resistance mechanisms to these peptides than traditional antibiotic drugs, though resistance can still occur (Assoni et al., 2020; Di et al., 2020; El Shazely et al., 2020)**.**

Due to the ease of making recombinant modifications, AMPs can address the central flaw of gut microbiome destruction caused by antibiotics due to broad spectrum action. The addition of a guide peptide to the AMP has been shown to make normally broad-spectrum AMPs highly specific, targeting only single pathogen species or genera (Devocelle, 2012; Eckert et al., 2006; Malanovic and Lohner, 2016). These targeting strategies are already in use in cancer therapy and gene therapy. In cancer therapy applications, a guide peptide is made from the external viral peptides which bind to specific cellular receptors found on the target cell. Other targeting strategies include RNAi, CRISPR Cas9, and antibody fragments such as scFvs (Chen et al., 2010; Marty and Schwendener, 2005; Zha et al., 2016). Moving outside of cancer and gene therapy, only a handful of targeting strategies have been used to target AMPs to specifically destroy pathogens. scFv targeting domains fused to AMPs have been successfully produced in transgenic plants and found to be active against fungal plant pathogens (Peschen et al., 2004). Another approach to targeting AMPs involves the "pheromone" peptides used by bacteria for quorum-sensing, which signal other bacteria of the same species to form biofilms (Li et al., 2010). Eckert et al. have, in this manner, chemically synthesized short AMPs sequences preceded by pheromone sequences specific for *Streptococcus mutans*

(Kaplan et al., 2011). This technology has advanced to Phase II clinical trials. Quorum-sensing peptide conjugates have also been used to target the larger AMP, plectasin, produced in an *E. coli* expression system, against methicillin resistant *Staphylococcus aureus* (MRSA) (Xiong et al., 2011). The receptor binding peptides of viruses that have been used in cancer (Cripe et al., 2009) and gene therapy (Buchholz et al., 2015; Norian et al., 2011) have not yet been used with AMPs. However, this strategy has been used successfully in guiding an insecticidal toxin to the targeted insect cell. A peptide derived from the coat protein of the aphid-vectored Pea enation mosaic virus strain has been fused to a broadly insecticidal spider toxin to create an aphid-specific toxin (Bonning et al., 2014). Thus, there are several successful technologies which point to the feasibility of targeted AMPs being produced at commercial scales in *E. coli*, but the full demonstration of this approach remains unproven.

Helicobacter pylori was one of the model bacterial pathogens I used to test the guided AMP concept, in part due to its ease of use but also because of its medical importance on a global basis. *H. pylori* is the source of one of the most prevalent infections in the world, with over 50% prevalence in many countries but often over 90% in Africa and East Asia (Salih, 2009). Over 60% of the cases of gastric cancer can be attributed to *H. pylori* infection (Correa and Piazuelo, 2011), making it one of the most widespread cancers caused by an infectious agent (Wroblewski et al., 2010). Multidrug resistant strains of *H. pylori* constitute an increasing portion of *H. pylori* infections, from >10% in European countries to >40% of infections in Peru (Boyanova et al., 2019). The most recent recommended treatment regimens for *H. pylori* infection include triple and quadruple antibiotic therapies to match the growing challenge of antibiotic resistance. Such

therapeutic regimens include combinations of amoxicillin, tetracycline, bismuth, metronidazole, clarithromycin, and other antibiotics and adjuvants. As a consequence, quadruple, quintuple, and sextuple antibiotic-resistant strains have been detected (Boyanova et al., 2019). This escalation of antibiotic resistance in *H. pylori* has heightened the need for new therapeutic strategies to combat infection. The modes of action of these antibiotics, such as rRNA inhibition, β-lactams, and nucleic acid inhibitors, typically are broadly effective across many bacterial taxa. This causes dysbiosis of the native human microbiota by killing off-target bacteria (Becattini et al., 2016; Langdon et al., 2016; Zarrinpar et al., 2018). Antibiotic-associated dysbiosis can culminate in intestinal inflammatory diseases like colitis (Strati et al., 2021), or worsen neuro-immune mechanisms and viscerosensory functionalities (Aguilera et al., 2015) and often makes way for bloom of pathogens (Vangay et al., 2015) creating other possibly more serious infectious diseases. This presents a dilemma, as stronger small molecule antibiotics are required to kill bacteria with ever-evolving antibiotic resistance mechanisms, but stronger antibiotics kill a wider variety of commensal bacteria (Becattini et al., 2016; Langdon et al., 2016; Zarrinpar et al., 2018).

Several specific types of AMPs have been demonstrated to effectively kill *H. pylori*. Cathelicidins such as LL-37 and its murine homolog Cathelin-related Antimicrobial Peptide (CRAMP) have been demonstrated to effectively kill *Helicobacter pylori* in both in vitro and in vivo experiments (Hase et al., 2003; Zhang et al., 2016, 2013). Bacteriocins are small, stable AMPs released by other bacteria, that have broad bactericidal ability against a variety of gram-positive and gram-negative bacteria including *H. pylori* (Neshani et al., 2019). Among them, Type IId bacteriocins including laterosporulin has been well

documented for their bactericidal activity with well-established mechanisms (Baindara et al., 2016; Singh et al., 2015). As more novel AMPs are discovered, a catalog of AMPs with activity against *H. pylori* has grown, showing promise as potential therapeutics.

Modifying AMPs to make chimeric peptides using a short glycine linker and a guide peptide is the method I used to "target" a specific microbial taxon. Such guided antimicrobial peptides (gAMPs) have been shown to be effective in several settings against a variety of bacteria (Choudhury et al., 2020; Eckert et al., 2012, 2006; Kim et al., 2020). In some cases, such constructs can be made to increase the toxicity of a relatively weak AMP towards a targeted bacterium (Eckert et al., 2006), whereas in others it has been demonstrated to decrease toxicity of a potent AMP towards off-target bacteria (Choudhury et al., 2020)**.** Furthermore, while studies have shown the bactericidal effects of such gAMPs in an in vitro setting, the selectivity of these constructs has not been demonstrated in vivo to ascertain if the native microbiota are relatively undisturbed; nor has a gAMP been utilized against *H. pylori***.**

One of the reasons for the lack of in vivo testing of gAMPs is that delivery of engineered peptides has its difficulties. Antimicrobial peptides, being proteinaceous, are at a greater risk of enzymatic degradation through oral routes (Moncla et al., 2011; Svenson et al., 2008) and the high gastric acidity and peptidolytic enzymes cause breakdown of proteins and peptides when ingested orally. To avoid this gastric degradation, drugs are often delivered through systemic injection. For peptides, this is problematic as the size and high molecular weight of proteinaceous drug make it an easier target for opsonization and neutralization by the blood complement system (Vaucher et al., 2011). Thus, for having the desired therapeutic effect, the peptide drug will have to survive the degradation in gut

and reach the site of action. Encasing the antimicrobial peptide in a delivery system that masks it to survive the journey in the oral delivery and release it once the site is reached would be of great help and would help in microbial infections along the gut for which oral delivery of drugs is necessary.

My solution is the use of lactic acid bacteria as delivery vehicles for AMPs. Lactic acid bacteria like *Lactobacillus* and *Lactococcus* have been a part of the human diet for millennia and are hence considered safe for consumption (Axelsson and Ahrné, 2000). Cloning antimicrobial peptides in such bacteria could be a mode of expressing these peptides in situ. Previous studies have demonstrated the use of lactic acid bacteria for the expression and delivery of vaccines (Mannam et al., 2004; Robinson et al., 1997), interleukins (Bermúdez-Humarán et al., 2003; Steidler et al., 2003, 2000, 1998), and nanobodies (Vandenbroucke et al., 2010) through lactic acid bacteria for treating Crohn's disease (Braat et al., 2006; Steidler et al., 2003), ulcerative colitis (Bermúdez-Humarán et al., 2003; Foligne et al., 2007; Vandenbroucke et al., 2010), respiratory infection 46 among a few others. In contrast, lactic acid bacteria have seldom been used to deliver AMPs in vivo for treatment of gut infections. Thus, there is unexplored space open for the development of a lactic acid bacterial based expression and delivery of targeted antimicrobial peptides for combating gastrointestinal pathogens like *Helicobacter pylori* without harming the rich and beneficial physiological microflora of the gut region.

Employing food grade bacterial systems like the lactic acid bacteria can solve the problem of the peptide's survival through degradative environments such as the gastrointestinal tract (Steidler et al., 2003). These bacteria are adapted to survive, propagate, and produce and secrete their indigenous proteins in low pH conditions of the

stomach. Encoding the chimeric antimicrobial peptide into a secretion vector inside such lactic acid bacteria will ensure that the protein will survive the journey into the gastrointestinal tract and be released from the cell into the site of infection (Jeong et al., 2006; Li et al., 2011). The cells will act as a sustained release platform as the expression of the protein will happen over a time. The cells will also replicate and maintain a colony of drug-releasing bacteria for an extended period (Drouault et al., 1999), unlike conventional drug delivery system. This reduces the number of dosages required to maintain the effective drug level for treatment of the infection. The vector can also be modified to contain an inducible promoter that is pH dependent (de Vos, 1999; Madsen et al., 1999), like the inducible heat shock and nitrogen dependent promoters. A promoter that is induced by low pH, like P1, P2 and P170 (de Vos, 1999; Madsen et al., 2005, 1999), will enable the lactic acid bacteria to express and secrete the encoded peptide only when it is exposed to such conditions at the target location in the stomach. Thus, a lactic acid bacterium containing a secretion vector with a pH inducible promoter driving AMP expression constitutes an excellent sustained release drug delivery system that will protect the peptide drug from the enzymatic degradation in the gastrointestinal tract and deliver it to the proper target site. Lactic acid bacteria in the genera *Lactobacillus* and *Lactococcus* have been a part of the human diet for millennia. There have been numerous strains of lactic acid bacteria that are considered safe to consume and graded by the FDA as such (Nutrition, 2020). Since early 2000s, *Lactococcus lactis* has proven to be an excellent method for delivering engineered peptides *in-situ* from what has since burgeoned to include a variety of engineered probiotic bacteria. These engineered probiotics have been used to deliver signaling peptides, dyes, interleukins, and even unmodified AMPs (Foligne et al.,

2007; Steidler et al., 2003, 1998). *Lactococcus lactis* also holds the distinction of being the first genetically engineered organism to be approved by FDA as a therapeutic application in humans (Braat et al., 2006).

In the following chapters, we will explore the approaches of synthesizing chimeric gAMPs in *E. coli* and *L. lactis* that will have specific targeted antimicrobial effect on the pathogens of our choice. As a proof of principle, I purified AMPs and their gAMP analogues from *E. coli* with a targeting moiety specific for *Staphylococcus aureus* and tested in vitro their efficacy against the pathogen versus off-target bacteria. Then I engineered the probiotic *Lactococcus lactis* to secrete gAMPs specifically active and *Helicobacter pylori*. The efficacy of these AMP-releasing bioengineered probiotics was tested against the targeted pathogen *H. pylori* and other non-target bacteria in vitro. Finally, these gAMPs were tested in a mouse model of *H. pylori* by providing cultures of the bioengineered probiotic bacteria with both AMP and gAMP to C57BL/6J mice by oral gavage. To test the treatment as a therapeutic, the mice were first infected with H. pylori by oral gavage and then treated with the engineered probiotic. For prophylactic tests, the mice were first provided with the engineered probiotic and then challenged with *H. pylori*. Stomach fluid samples were collected by a novel reverse oral gavage method, with qPCR being used to estimate *H. pylori* titer. These samples were sequenced using an Illumina Miseq sequencer with primers specific for the 16S rRNA variable region 4 (Caporaso et al., 2012, 2010). The sequencing data was analyzed within the QIIME2 environment (Bolyen et al., 2019) to determine the microbiota composition. CCREPE (Schwager et al., n.d.) was used for the correlation network analysis on differential taxa abundance and a

dysbiosis index was specially developed to measure the destructive effects of *H. pylori* on the microbiota and the ability of gAMPs to correct this.

CHAPTER TWO

Repurposing A Drug Targeting Peptide For Targeting Antimicrobial Peptides Against *Staphylococcus*

This chapter was published as: Choudhury, A., Islam, S.M.A., Ghidey, M.R., Kearney, C.M., 2020. Repurposing a drug targeting peptide for targeting antimicrobial peptides against *Staphylococcus*. Biotechnol Lett 42, 287–294.

Abstract

Objectives. Targeted therapies seek to selectively eliminate a pathogen without disrupting the resident microbial community. However, with selectivity comes the potential for developing bacterial resistance. Thus, a diverse range of targeting peptides must be made available.

Results. Two commonly used antimicrobial peptides (AMPs), plectasin and eurocin, were genetically fused to the targeting peptide A12C, which selectively binds to *Staphylococcus* species. The targeting peptide did not decrease activity against the targeted *Staphylococcus aureus* and *Staphylococcus epidermidis*, but drastically decreased activity against the nontargeted species, *Enterococcus faecalis*, *Bacillus subtilis*, *Lactococcus lactis* and *Lactobacillus rhamnosus*. This effect was equally evident across two different AMPs, two different species of *Staphylococcus*, four different negative control bacteria, and against both biofilm and planktonic forms of the bacteria.

Conclusion. A12C, originally designed for targeted drug delivery, was repurposed to target antimicrobial peptides. This illustrates the wealth of ligands, both natural and

synthetic, which can be adapted to develop a diverse array of targeting antimicrobial peptides.

Introduction

Two major drawbacks to traditional antibiotics are seemingly contradictory. On one hand, traditional antibiotics cause a broad disruption to the natural human gut microbiota, increasingly recognized as a detriment to health in many ways. On the other hand, traditional antibiotics tend to have highly specific molecular targets which are susceptible to the development of bacterial resistance. One possible solution to this problem would be to use the combination drug approach used to reduce the development of resistance in antibiotic treatment (Worthington and Melander 2013). As an alternative technology, antimicrobial peptides (AMPs) have broad spectrum activity hinged on basic cellular properties, such as membrane charge, or to a multiplicity of potential targets (Nguyen et al. 2011). Thus, AMPs also have a deleterious effect on the general microbiota but are less susceptible to the development of bacterial resistance (Peschel and Sahl 2006).

The antimicrobial activity of AMPs can be narrowed by including a targeting peptide sequence in the AMP which recognizes a specific target on the targeted pathogenic bacterium. However, only a few reports of targeted antimicrobial peptides are found in the literature and these all use derivatives of bacterial pheromones for the targeting peptide (Eckert et al. 2006, Eckert et al. 2012, Mao et al. 2013). There are a diverse array of ligands, both natural and synthetic, that have been extensively characterized and found to specifically bind bacterial pathogens. These can be repurposed as targeting sequences and

constitute a resource for the development antimicrobial peptides with a wide array of targeting mechanisms.

As a simple demonstration of this approach, we have repurposed the A12C peptide, originally designed for drug delivery using a bacteriophage as the delivery vehicle (Yacoby et al. 2006). A12C was developed by selection from random peptide sequences using biopanning (Yacoby et al. 2006). We fused A12C with two commonly used antimicrobial peptides, plectasin (Mygind et al. 2005) and eurocin (Oeemig et al. 2012), which are broadly effective against gram positive bacteria.

Materials and Methods

Reagents

The pE-SUMOstar vector (LifeSensors) was grown in *E. coli* 10-β (DH10B) and *E. coli* BL21-DE3 (New England Biolabs) and AMP was released from expressed fusion/AMP using Ulp1 protease produced in house. The AMPs plectasin (GFGCNGPWDEDDMQCHNHCKSIKGYKGGYCAKGGFVCKCY; MW 4408) and eurocin (GFGCPGDAYQCSEHCRALGGGRTGGYCAGPWYLGHPTCTCSF; MW 4345) were expressed from pE-SUMOstar as were A12C-plectasin (MW 6137) and A12C-eurocin (MW 6074), both of which had the A12C targeting peptide (underlined) plus a short linker (G<u>VHMVAGPGREPT</u>GGGHM) fused to the N-terminus of the respective AMP sequences. As a control, plectasin and eurocin were also conjugated with the AgrD1 bacterial pheromone sequence (YSTCYFIM) (Mao et al. 2013) at the N- terminus. Synthetic A12C peptide (Biosynthesis) was used as a "target peptide only" control.

Expression, Purification and Analysis of Fusion Proteins

The DNA sequences for the AMPs were synthesized (Integrated DNA Technologies) and ligated into the pE-SUMOstar vector (Figure 2.1) and cloned into *E. coli* 10-beta cells. Plasmid from these were used to transform *E. coli* BL21 cells for protein expression. Transformed cultures were grown out and induced with IPTG according to standard procedures. The resulting bacterial pellets were resuspended in PBS/25 mM imidazole/0.1 mg lysozyme /ml and frozen overnight. The cells were then thawed, sonicated, and ultracentrifuged at 80,000 x g for 1 h at 4˚C and the 6his/SUMO/AMP fusion protein in the supernatant was purified by nickel column chromatography. The AMP was separated from SUMO by proteolysis using Ulp1 (1U per 100 µg fusion protein) at 4˚C overnight and the cleavage was evaluated by SDS-PAGE. Yields were calculated from the SDS-PAGE data, using NIH ImageJ to measure band density and the marker lane bands for mass reference.

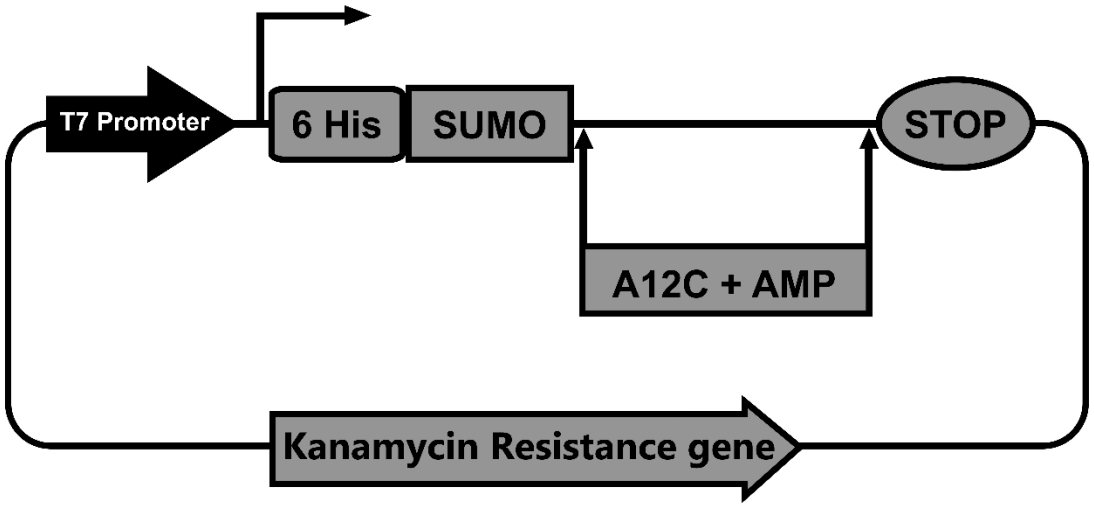

FIGURE 2.1 pE-SUMOstar carrying AMP for expression in *E. coli* BL21-DE3 cells. SUMO protease site is between SUMO and A12C-AMP

Mass spectrometry was used to ensure the proper cleavage of the AMP from the SUMO carrier protein. In-gel tryptic digest (Thermo Fisher) was performed on the AMP excised from the SDS-PAGE gel. The digest was examined by LC-ESI-MS (Synapt G2-S, Waters) at the Baylor University Mass Spectrometry Center. The analysis of the MS data was done by MassLynx (v4.1) The spectra of each protein, both non-targeted and targeted, were peak centered and MaxEnt3 processed and then matched against hypothetical peaks from peptides generated by simulated trypsin digestion of the respective proteins (Supplemental Figures A1-16).

Hemolytic Activity Assay

Targeted AMPs, non-targeted AMPs and synthetic A12C peptide were assessed for human hemolytic activity via exposure to washed human erythrocytes. Whole blood cells were collected a healthy volunteer using standard procedures (Evans et al. 2013) and cells were diluted in phosphate buffered saline to $5x10^8$ cells/ml. To initiate hemolysis, 190 µl of the cells was added to 20 µl of a 2-fold serially diluted peptide/ test reagent in phosphate buffered saline. Wells without peptide were used as negative controls, while wells containing 1% Triton X-100 were used as positive controls.

In Vitro Bactericidal Activity Assay

The Ulp-1 protease-cleaved proteins were tested for antimicrobial assays against four strains of bacteria: *Staphylococcus aureus* (SA113/ATCC® 35556™), *Staphylococcus epidermidis* (ATCC® 14990™), *Enterococcus faecalis* (OG1RF/ ATCC® 47077™), *Bacillus subtilis* (ATCC® 6051™), *Lactococcus lactis* (MG1363/ LMBP 3019) and *Lactobacillus rhamnosus* (ATCC® 7469™) . These 6 species were selected because they are gram positive and the AMPs plectasin and eurocin are specifically active against gram

positive bacteria (Mygind et al. 2005, Oeemig et al. 2012). The component controls were free SUMO protein and synthetically produced A12C peptide. Vancomycin was used as the positive control. The standard protocol for a microtiter plate assay with serial dilution was used in which serial 2-fold dilutions of test peptide were made across a 96-well plate containing uniform bacterial inoculum across the peptide dilutions. After bacterial growth in the presence of peptide, cell viability was assayed with resazurin. Experiments with all peptides against all bacterial species were performed with ≥6 replicates each.

In Vitro Cell Kinetics Study

Ulp-1 protease-cleaved peptides were assayed to determine their dynamic action against the bacteria in a growing culture. The bacteria were grown at 37°C with shaking and diluted to ~$1x10^6$ - $1x10^8$ CFU/ml. To these cultures were added plectasin or eurocin, at 3x the respective minimum inhibitory concentrations, or the A12C-targeted versions at these same respective concentrations. The postive control (Erythromycin for *L. rhamnosus*, Vancomycin for the rest) concentration was also 3x the MIC for each species for the respective antibiotic. Growth was then monitored from 2-8 h after addition of the peptides, diluting 10 µl of culture in medium and plating onto Mueller-Hinton agar plates. The number of colonies was recorded the next day.

In Vitro Biofilm Inhibition Assay

In addition to planktonic cultures, biofilm cultures were used to assay inhibition by the peptides, using standard procedures (O'Toole 2011). Briefly, overnight cultures were diluted 1:100 and added to serially diluted peptides. Biofilms were allowed to grow for 24-36 h of unshaken culture. The liquid was removed and the biofilms were washed, dried and

fixed with methanol and then stained with Crystal Violet, which was later dissolved with 30% acetic acid and the resulting solution measured for absorbance at 540 nm to quantify the amount of biofilm formed. All assays were run in triplicate or greater.

Results

Protein Expression and Purification

AMP/SUMO fusion proteins, with or without the A12C targeting domain, were highly expressed in E. coli BL21 cells. These were successfully cleaved with SUMO protease (Ulp-1) into their component AMP and SUMO carrier protein and were clearly visualized with SDS-PAGE as 4-6 kDa free AMP and ~17 kDa SUMO/AMP fusion proteins (Figure 2.2). The average yields (n>=3) of the proteins plectasin, A12C-plectasin, eurocin and A12C-eurocin were 15-26 mg (3-4 µmoles) per L of culture. For peptide confirmation, peptides were extracted from the SDS-PAGE gel bands, digested by trypsin and analyzed by mass spectrometry. Peptide identities were confirmed using the MassLynx (v4.1) application (Waters). Supplemental Figures A1, A3, A5, A7, A9, A11, A13 and A15 provide the simulated trypsin digestion with the matched peaks appearing in black; A2, A6, A10 and A14 provide the deconvoluted mass spectra; and A4, A8, A12 and A16 provide the mass corrected (green) and peak centered (red) mass spectra.

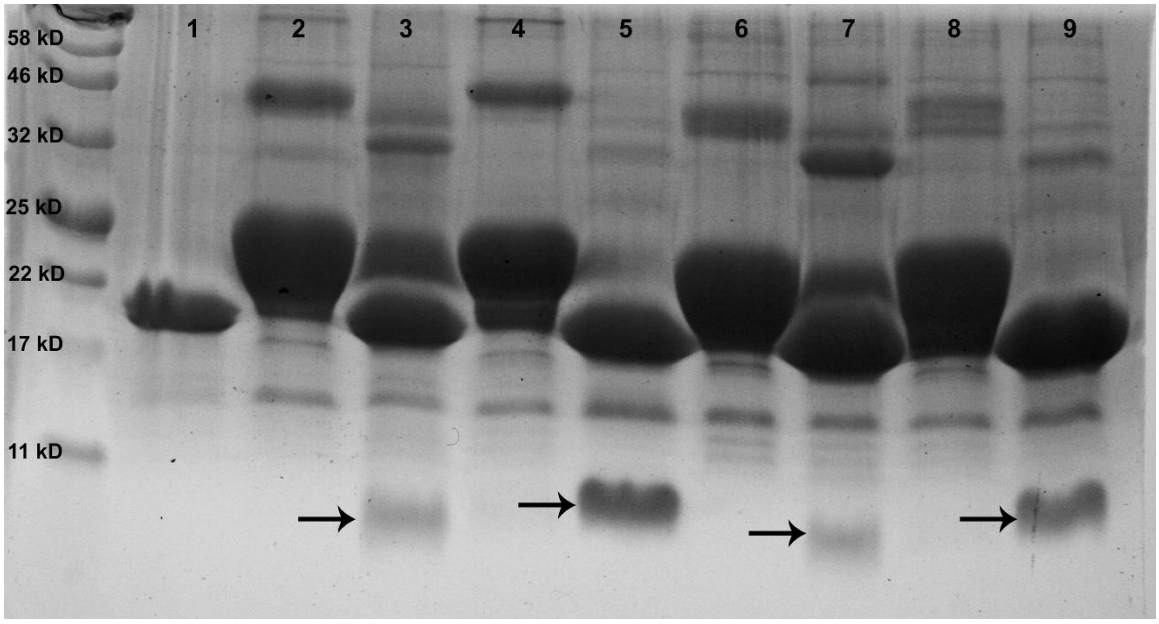

FIGURE 2.2. Expression of SUMO/AMP in *E. coli* and cleavage of AMP free of SUMO fusion partner. Lane 1, free SUMO control. Lanes 2-9: Intact fusion proteins (even lanes) and cleaved products (odd lanes) in the following order: SUMO/plectasin, SUMO/A12C-plectasin, SUMO/eurocin, SUMO/A12C-eurocin. Arrows: free AMP

Hemolytic Activity Assay

In concordance with previously published individual studies on plectasin and eurocin (Mygind et al. 2005, Oeemig et al. 2012, Yacoby et al. 2006), both targeted and nontargeted antimicrobial peptides, along with the free A12C peptide control, displayed no hemolytic effect on human erythrocytes in comparison to a 1% Triton-X positive control (data not shown).

In Vitro Bactericidal Activity Assay

Differential toxicity against off target bacteria was observed with the A12C targeting peptide added to the AMPs. A12C-AMPs retained their toxicity against both the targeted staphylococci bacterial species but showed a dramatic decrease in toxicity against the off target bacterial species relative to unmodified AMPs (presented

logarithmically in Figure 2.3). This data is presented in tabular format in Supplemental Table A1.

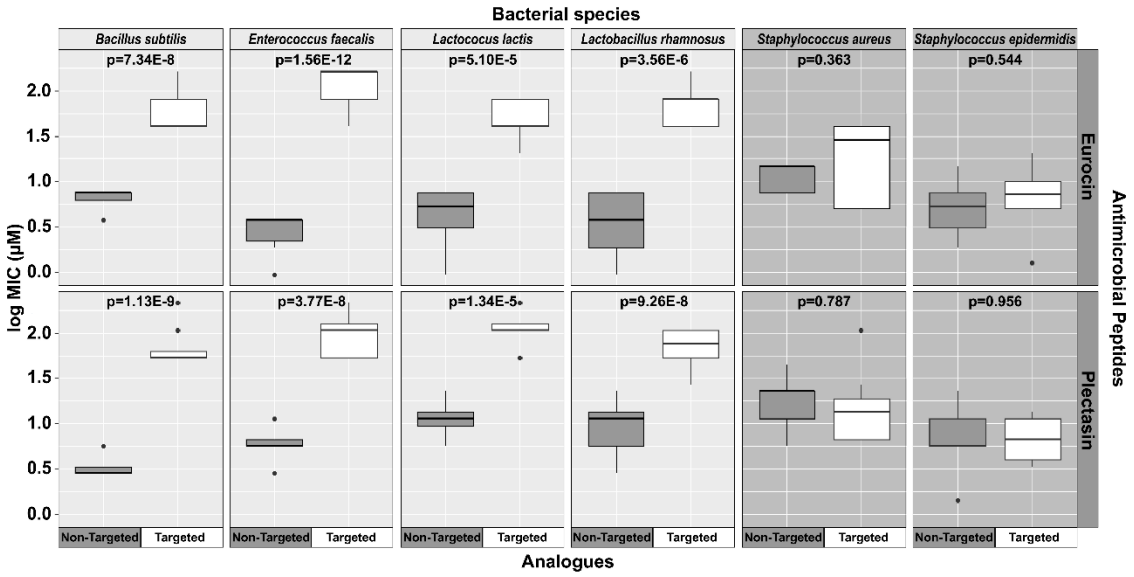

FIGURE 2.3. Log values for minimum inhibitory concentrations (MIC) in μM for non-targeted and targeted analogues of eurocin and plectasin against *Bacillus subtilis, Enterococcus faecalis, Lactococcus lactis, Lactobacillus rhamnosus, Staphylococcus aureus* and *Staphylococcus epidermidis*. The boxed regions represent 50% of the values while the bars represent 95%

Unmodified plectasin and eurocin had the expected mean MIC values of 3-6 μM, which are typical values for AMPs with sequential tri-disulfide bonds produced in *E. coli* expression systems (Li et al. 2010, Parachin et al. 2012, Li et al. 2017). In contrast, the addition of the A12C targeting peptide rendered these AMPs essentially noninhibitory to the off target bacteria, with MIC values >70 μM. In all cases, the MIC values for A12C/AMP versus AMP were significantly different for all of the off target bacteria, *E. faecalis, B. subtilis, L. lactis* and *L. rhamnosus* ($p<0.001$; ANOVA 2-tailed test). Negative controls (SUMO alone and A12C alone) showed no antimicrobial activity (data not shown) and these were run for all experiments. Eurocin and Plectasin synthesized with AgrD1

fusion peptide in our lab had inconsistent antimicrobial activity with no significant difference between *Staphylococcus* and non-*Staphylococcus* bacteria (SupplementaL Table A1). The peptide A12C had no antimicrobial activity at all.

In Vitro Cell Kinetics Study

Growth kinetics over an 8 to 9hour period more conclusively demonstrated the loss of antimicrobial activity of the A12C/AMP against the off target bacterial species. For these bacteria, A12C/AMP treatment resulted in bacterial growth that lagged only slightly behind buffer control treated cultures (Fig. 4). Unmodified AMPs were bactericidal similar to the positive control. In contrast, all peptides - both targeted and non-targeted - demonstrated a strong bactericidal effect against the target bacteria *S. epidermidis* and *S. aureus*, similar to the positive control (Figure 2.4). The relatively flatter growth curve for the *B. subtilis* control cultures reflects its growth kinetics, which is far slower than that of other bacteria. Also, fast growing bacteria like *S. epiermidis* and *S. aureus* requires a longer incubation period than 8 h for complete elimination of planktonic population.

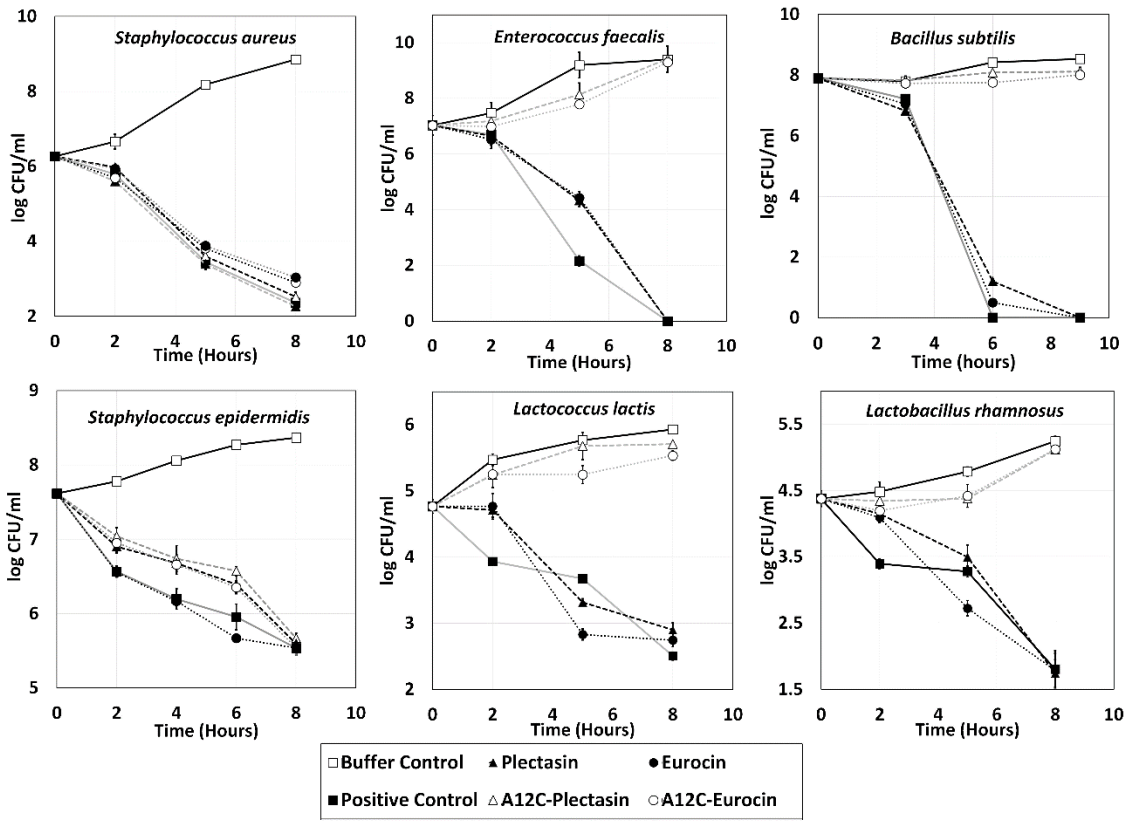

FIGURE 2.4. The cell-kinetic profile for *S. aureus*, *E. faecalis*, *B. subtilis*, *L. rhamnosus*, *L. lactis* and *S. epidermidis* (clockwise), created by plotting log CFU/ml of the bacteria grown in the presence of each peptide for 8-10 hours collected in 2-3 hour intervals

In Vitro Biofilm Inhibition Assay

Growing bacterial cultures with the peptides demonstrated the preferential inhibition of bacterial biofilm of the *Staphylococcus* strains (Figure 2.5) by the targeted AMPs over the non-*Staphylococcus* bacteria. The biofilm viability decreased with the increase in peptide concentration for all the 6 bacteria when treated with non-targeted peptides but the targeted peptides did not have similar effects on *B. subtilis* , *E. faecalis*, *L. lactis* and *L. rhamnosus* with significant ($p<0.05$ or $p<0.01$) difference in the absorbance values between targeted and non-targeted AMPs at concentrations beyond 3.125 µM.

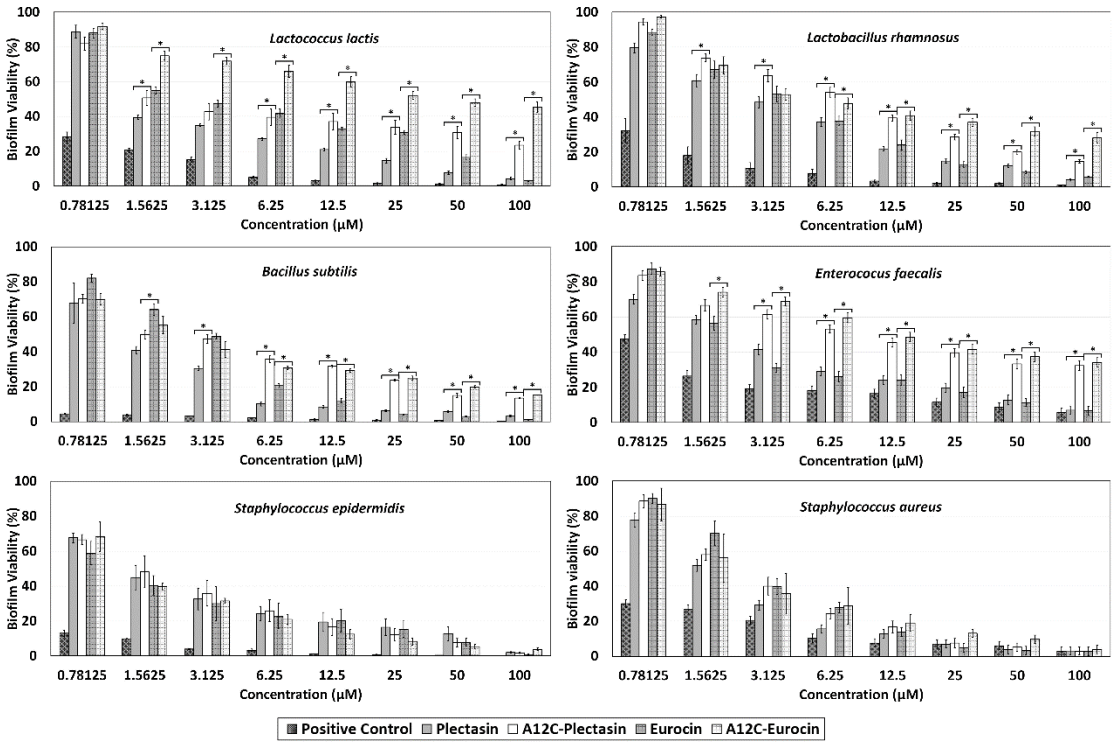

FIGURE 2.5. Biofilm inhibition activity evaluated by plotting the % viability of biofilm against the concentration of 4 AMPs on the 6 bacteria – *L. lactis, L. rhamnosus, E. faecalis, S. aureus, S. epidermidis* and *B. subtilis* (clockwise). (* = $p<0.1$, ** = $p<0.05$, $n>=3$)

Discussion

In this work, we have successfully achieved targeting of the AMPs plectasin and eurocin against two staphylococcal bacteria. Importantly, this was achieved by essentially eliminating the activity against the four off target bacteria tested. Of these four- *E. faecalis* and *B. subtilis* represent regular human gut commensal species and *L.lactis* and *L. rhamnosus* are commonly consumed probiotic bacteria that benefit human gut health. This is the expected outcome for an antimicrobial therapy that preserves the commensal/ probiotic members of the microbiome while killing the pathogenic target bacteria. This is also the outcome that was achieved against *S. aureus* by Mao *et al.* (2013) with the use of a bacterial pheromone peptide for targeting of plectasin. Other than a lower MIC for the

unmodified plectasin itself, we report the same drastic degree of reduction in the activity against the off target bacteria, *E. faecalis* and *B. subtilis*, as was reported by Mao *et al*. (2013). Thus, we have demonstrated that a biopanning-derived ligand works as efficiently as a pheromone-derived ligand, which is the class of targeting peptide used in all targeted AMPs to date. It should be noted that the pheromone-derived ligand was more specific than A12C, with activity against *S. aureus* but not *S. epidermis*, while A12C/plectasin was highly active against both species.

Three main sources of ligands exist for use as targeting peptides for AMPs. First, bacterial pheromones are species-specific peptide signals which trigger the development of competence, virulence, or other capabilities, and pheromone peptides have been determined for many pathogenic bacteria (Monnet et al. 2016). Second, biopanning is a means of screening random libraries of peptides for the ability to bind to a target sequence, such as a receptor on a bacterial cell. Usually, a bacteriophage is used to display the members of the peptide library (Wu et al. 2016). Third, bacteriophage receptor binding proteins can be used as a resource for the development of targeting peptides for AMPs. The receptor binding proteins of phages against many pathogenic bacteria have already been characterized (Dowah and Clokie 2018, Nobrega et al. 2018). In addition, screens for new phages against lesser studied bacterial pathogens can be carried out (Weber-Dąbrowska et al. 2016).

In order to block the development of resistance in bacteria, both targeting peptides and antimicrobial peptides need to be discovered and developed. We are currently working with targeting peptides derived from viruses and other sources to determine if these sequences also confer the desired differential activity to AMPs. In addition, we have

developed algorithms for the classification of highly stable small peptide structures, such as sequential tri-disulfide peptides (STPs) (Islam et al. 2015) and, at a finer grain, STPs which possess antimicrobial activity (Islam et al. 2017). Using these methods and tools, it may be possible to generate a large number of targeting peptide/AMP permutations to serve as a bank for a multi-AMP approach to treating bacterial infections in order to avoid the development of bacterial resistance.

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CHAPTER THREE

Selective inhibition of *H. pylori* in vitro using probiotic *Lactococcus lactis* bioengineered to release guided antimicrobial peptides (gAMPs)

The results were published in US Patent Application titled "Probiotic Delivery Of Guided Antimicrobial Peptides", US Patent Application No: 16997036, International Patent Application No: PCT/US20/46896, Inventors: Christopher M. Kearney, Ankan Choudhury, Patrick Ortiz

Abstract

Objectives. Targeted therapies seek to selectively eliminate a pathogen without disrupting the resident microbial community. This is especially important for a pathogen like *H. pylori*, which resides in stomach, a sensitive microbial ecosystem. Using a probiotic like *Lactococcus lactis* and bioengineering it to release a guided antimicrobial peptide (gAMP) targeted towards the pathogen offers a pathway to specifically knock out the deleterious species without disturbing the stomach microbiome.

Results. Three AMPs, alyteserin, CRAMP and laterosporulin, were genetically fused to a guiding peptide MM1, which selectively binds to Vacuolating toxin A (VacA) of *H. pylori* and cloned into an secretory vector pTKR carried by *L. lactis*. When cultured together in vitro, the *L. lactis* bioengineered with guided AMPs selectively killed the targeted *H. pylori* in comparison to the nontargeted *E. coli* or *Lactobacillus plantarum,* as determined by qPCR.

Conclusions. Probiotics bioengineered to secrete guided AMPs are a novel approach for combating pathogens without endangering the natural microbial flora. Given the wealth of AMPs and guiding ligands, both natural and synthetic, this approach can be

adapted to develop a diverse array of chimeric guided AMPs and can be cloned into probiotics to create a safe and effective alternative to conventional chemical antibiotics.

Introduction

Helicobacter pylori is the cause of one of the most prevalent infections in the world, with over 50% prevalence in many countries but often over 90% in Africa and East Asia (Salih, 2009)**.** Over 60% of gastric cancer cases can be attributed to *H. pylori* infection (Correa and Piazuelo, 2011)**,** making it one of the most widespread cancers caused by an infectious agent (Wroblewski et al., 2010)**.** However, antibiotics administered to curb *H. pylori* infection cause dysbiosis in the microbiota caused by broadly killing off-target resulting in a flattened ecosystem open to colonization by bacteria detrimental to the health of the gut (Becattini et al., 2016; Langdon et al., 2016; Zarrinpar et al., 2018)**.** Furthermore, emergence and escalation of antibiotic resistance in *H. pylori* has heightened the need for new therapeutic strategies to combat infection.

To meet the challenges associated with this infection, one proposed strategy has been the use of antimicrobial peptides (AMPs). AMP refers to a broad group of short, usually cationic peptides with bactericidal or bacteriostatic properties (Lei et al., 2019). Though resistance has been documented, it is generally thought that bacterial resistance occurs less readily against the generalized mode of action of AMPs compared to traditional antibiotic drugs, which usually act on specific molecular targets (Assoni et al., 2020; Di et al., 2020; El Shazely et al., 2020). Several specific types of AMP have been demonstrated to effectively kill *H. pylori*. Among them are the cathelicidins, including LL-37 and its murine homolog Cathelin-Related AntiMicrobial Peptide (CRAMP), which have been

demonstrated to effectively kill *Helicobacter pylori* both in vitro and in vivo (Hase et al., 2003; Zhang et al., 2016, 2013) and bacteriocins, which are small, stable AMPs released by other bacteria (Neshani et al., 2019). As more novel AMPs are discovered, a catalog of AMPs with activity against *H. pylori* has grown, showing promise as potential therapeutics.

While many of these AMPs have demonstrated effective antibacterial activity towards *H. pylori*, they also kill many other bacterial taxa. The approach to modify AMPs and make chimeric peptides with a guide peptide to "target" a specific genus or species is is appropriate. Though only a limited number of studies using guided antimicrobial peptides (gAMPs) have been conducted to date, these have shown gAMPs to be effective against a variety of bacteria (Choudhury et al., 2020; Eckert et al., 2012, 2006; Kim et al., 2020). While these studies have shown the bactericidal effects of gAMPs in an in vitro setting, the selectivity of these constructs has not been demonstrated in vivo to ascertain if the rest of the microbiota is relatively undisturbed**.** One of the reasons for this is that delivery of engineered peptides to the gastrointestinal tract, housing the most extensive and important human microbiota by far, is very difficult. as it requires a delivery mechanism that will stand up to low pH conditions and peptidases, and provide delivery at the site of the infection (Moncla et al., 2011; Svenson et al., 2008).

Employing food grade bacteria such as lactic acid bacteria can solve the problem of the peptide's survival through degradative environments such as the gastrointestinal tract (Steidler et al., 2003). Cloning the chimeric antimicrobial peptide into a secretion vector inside such lactic acid bacteria will ensure that the protein will not only survive the journey into the gastrointestinal tract (Jeong et al., 2006; Li et al., 2011). The cells will also replicate

and maintain a colony of peptide drug-releasing bacteria for an extended period (Drouault et al., 1999), unlike conventional static drug delivery systems.

In this chapter, I will demonstrate the efficacy of *L. lactis* bioengineered to secrete AMPs and gAMPs to inhibit *H. pylori* when co-cultured in vitro. In vitro tests are necessary to provide controlled conditions in order to precisely compare the toxicity of gAMPs versus AMPs against target and off-target bacteria and to determine if the choice of AMP had any effect on these outcomes. In vivo tests provide a more realistic test of these parameters, but also introduce confounding complications which can be simplified by in vitro tests.

In choosing the target protein on the pathogen, I considered how unique the protein was to *H. pylori* so as to not bind off-target bacteria. I also considered how sterically accessible the targeted protein was on the surface of the bacterium. Most importantly, I considered if published work existed demonstrating specific binding of a host protein to this pathogen target protein so that I could be certain that the host sequence would bind the pathogen. After considering several candidates, I picked Vacuolating toxin A (VacA). This virulence factor is is a major cause for *H. pylori* pathogenicity. It can be found released to the extracellular space (Cover and Blaser, 1992; Foegeding et al., 2016; Snider et al., 2016) or resident on the bacterial cell surface, which would make it an accessible target for binding to *H. pylori* (Foegeding et al., 2016; McClain and Cover, 2006; Telford et al., 1994; Voss et al., 2014). Furthermore, VacA toxin has been documented to have high affinity towards Multimerin-1(Satoh et al., 2013), a protein expressed on the surface of human platelets and is bound by the VacA toxin when it induces platelet CD62P expression76. It has been documented that VacA specifically binds to multimerin-1 from amino acid 321-

340 (Satoh et al., 2013). This peptide sequence, therefore, was used as the guide throughout this and the next chapter to target *H. pylori* when fused to the AMP.

For expressing the AMPs and gAMPs in *L. lactis* I modified the commonly available *L. lactis* expression vector pT1NX (Steidler et al., 2004, 1998; van Asseldonk et al., 1990; Waterfield et al., 1995). pT1NX contains a native *L. lactis* promoter called P1 (Madsen et al., 2005) which is induced by the low pH conditions found in the stomach and also increases expression of protein by 500-fold under growth temperatures around 40^{o}C (de Vos, 1999). pT1NX also contains the native signal peptide usp45 (van Asseldonk et al., 1990) which allows secretion of any downstream ORF. In this way, gAMP or AMP was expressed and secreted in response to low pH. I modified the pT1NX vector by adding a kanamycin resistance domain and an E. coli origin of replication to create a dual vector for use in *E. coli* as well as *L. lactis*. In this way, I was able to perform all engineering in *E. coli* and the final product was transferred to more genetically recalcitrant *L. lactis*.

Methods and Materials

Cloning Antimicrobial Peptides(AMPs) and Guided Antimicrobial Peptides (gAMPs) in L. Lactis

The ORFs of the AMPs (Table 3.1), codon-optimized for *Lactococcus lactis*, were cloned into the modified pT1NX plasmid (pT1NX-kanamycin resistant/pTKR, Figure 3.1) in between the restriction enzyme sites BamHI and SpeI by replacing the spaX protein of the original plasmid. The P1 promoter upstream of the BamHI cut-site controls the downstream expression as an inducible promoter which is upregulated by low pH. The usp45 gene immediately upstream of BamHI site is an endogenous signal peptide of *Lactococcus* species that transports the attached protein to extracellular location.

After ligation of the AMP or gAMP into pTKR vector, *E. coli* (10β, NEB) was transformed with the ligation product and the transformants plated onto kanamycin plates. The pT1NX plasmid (LMBP 3498) has erythromycin resistance but pTKR is a dual vector with an *E. coli* origin of replication and kanamycin resistance for cloning into electrocompetent *E. coli* (10β, NEB) for plasmid propagation. Extracted plasmid from the *E. coli* was then electroporated into electrocompetent *L. lactis* MG1363 (LMBP 3019) and plated on GM17 plates containing erythromycin (30^{o}C, microaerobic, overnight). After screening for the presence of the AMP/gAMP ORFs with PCR, selected colonies are propagated in liquid cultures of M17 broth with glucose (0.5% w/v) in the presence of erythromycin (5 µg/ml).

TABLE 3.1. The peptide sequences of three AMPs and their corresponding gAMPs with multimerin1 fragment fused to the N-terminus separated by a linker

AMP/ gAMP	**Peptide Sequence**
Laterosporulin	ACQCPDAISGWTHTDYQCHGLENKMYRHVYAICMNGTQVYCRTEWGSSC
Multimerin1(MM1)-laterosporulin	MQKMTDQVNYQAMKLTLLQKSGGGSACQCPDAISGWTHTDYQCHGLENKMYRHVYAICMNGTQVYCRTEWGSSC
Alyteserin	GLKDIFKAGLGSLVKGIAAHVAN
MM1-alyteserin	MQKMTDQVNYQAMKLTLLQKSGGGSGLKDIFKAGLGSLVKGIAAHVAN
CRAMP	ISRLAGLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPE
MM1-CRAMP	MQKMTDQVNYQAMKLTLLQKSGGGSISRLAGLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPE

Blue = Multimerin1, Red = linker

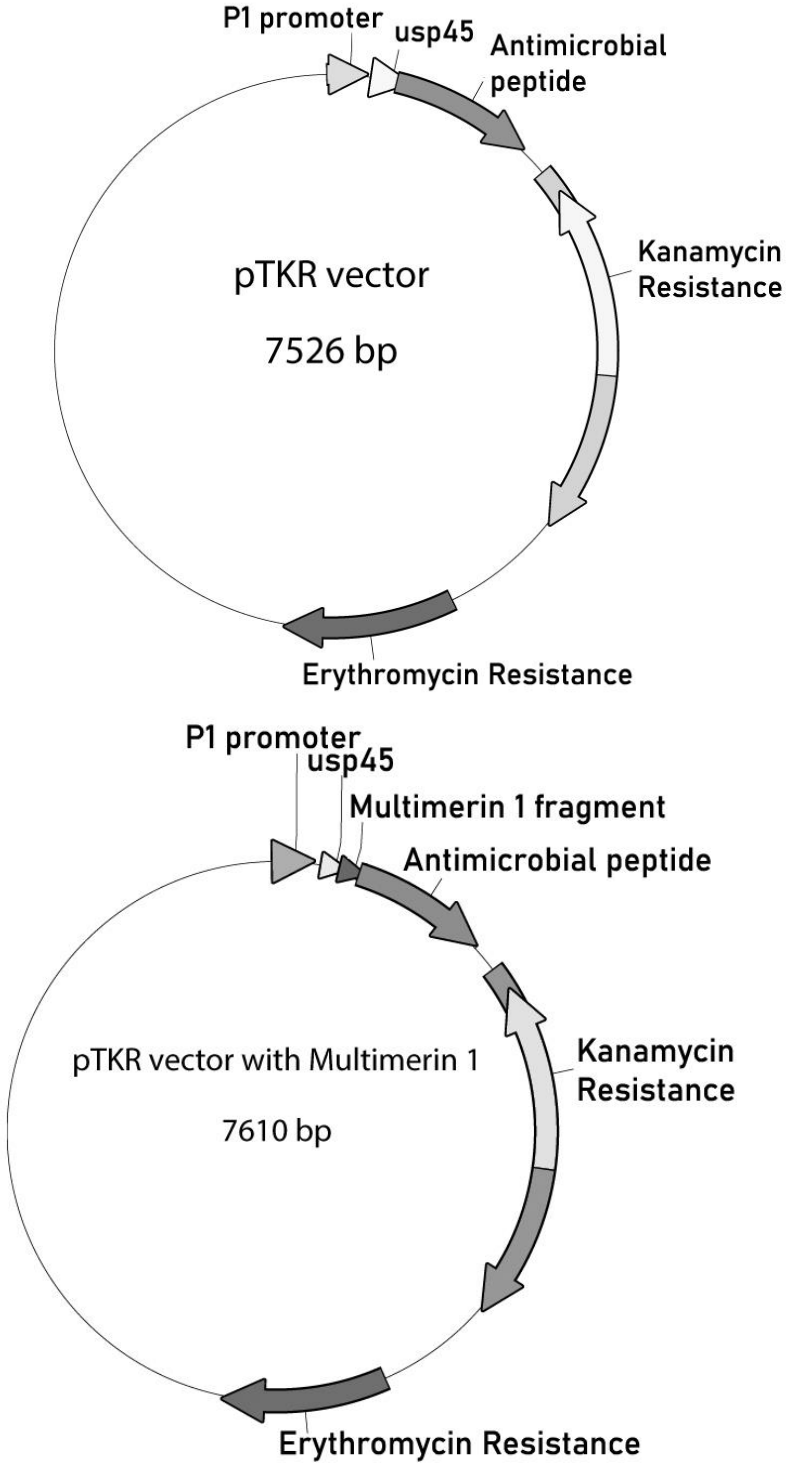

FIGURE 3.1. pTKR dual vector (engineering in *E. coli* and expression in *L. lactis*) for gAMP/AMP expression. The guided AMP sequence is placed downstream of the acid-inducible P1 promoter and the usp45 signal sequence which directs extracellular secretion. AMP control vectors lacking the guide (MM1) were also generated.

In Vitro Assay: Co-Culture Assays with Cloned L. Lactis and H. Pylori SS1

L. lactis clones engineered to express AMP or gAMP were propagated from glycerol stocks and grown in GM17 broth overnight with erythromycin (5 µg/ml) with no shaking. *H. pylori* SS1 stocks were first propagated on blood-tryptic soy agar (TSA) overnight with microaerobic condition and >5% CO_2 environment. Colonies from these plates were then transferred to TS broth with 5% newborn calf serum and grown overnight under microaerobic conditions (<10% O_2 and >5% CO_2). The *L. lactis* cultures were serially diluted in a 96-well culture plate with TS broth to make up a volume of 100 µL. To each well, 10 µL of the overnight *H. pylori* culture were added and each well volume was brought up to 200 µL with more TS broth. The plate was left to grow overnight in a microaerobic conditions. After 24 h, the well contents were transferred to a 96-well PCR plate. That PCR plate was sealed and heated for 15 min at 100^{o}C and then chilled at 4^{o}C for 5 min. This plate was then centrifuged at 2000 g for 2 min and the supernatant was used as the template for qPCR.

qPCR was carried out using primers for VacA gene to quantify *H. pylori* titer (forward: 5′-ATGGAAATACAACAAACACAC-3′; reverse: 5′-CTGCTTGA ATGCGCCAAAC-3′) and primers flanking the *L. lactis acma* gene were used to quantify *L. lactis* titer (forward: 5’ GGAGCTCGTGAAAGCTGACT 3’, reverse: 5’ GCCGGAACATTGACAACCAC 3’). Standard curves for *H. pylori* and *L. lactis* were constructed by determining C_T values from the qPCR data for different dilutions of the overnight cultures of the respective bacteria (1/10, 1/100, 1/1000, 1/10000) in the qPCR plates, and the CFUs for the dilutions were determined by plating on their respective agar plates. The same procedure was followed with the off-target bacteria where *Lactobacillus*

plantarum and *E. coli* were co-cultured with serially diluted cultures of *L. lactis* for 24 h and the titers of the off-target bacteria were determined by qPCR using primers for species-specific genes for either bacteria (DE3-T7 polymerase for *E. coli* and *recA* for *L. plantarum*). The amount of *L. lactis* added to the co-cultures of all the three assays ranged from approximately 4000 to 512000 CFU/ul.

Results

L. Lactis Expressing gAMPs Selectively Killed H. Pylori When Co-Cultured In Vitro

When co-cultured for 24 h, recombinant *L. lactis* showed a pronounced bactericidal activity against the target *H. pylori* as determined by qPCR (Figure 3.2). *H. pylori* titers were calculated from qPCR data using a concentration curve correlating C_T values with CFU/µl values of *H. pylori* determined by plating. All gAMP curves (gray symbols) appeared to indicate greater toxicity than those for the corresponding AMP (white symbols), though only for the alyteserin gAMP/AMP pair was the differential found to be statistically significant (at 64,000 CFU/µl probiotic and higher). At this probiotic concentration, co-culture with probiotic/guided alyteserin reduced *H. pylori* titers by 1,000-fold more than probiotic/empty vector, while with probiotic/unmodified alyteserin, only 5-fold difference from empty vector was seen. In contrast, these numbers for CRAMP at 100,000 CFU/µl probiotic were a fairly similar to each other, with a 30-fold and 23-fold reduction for gAMP and AMP, respectively. Thus, the choice of AMP was seen to be an important parameter for achieving increased toxicity against the target with the guide peptide.

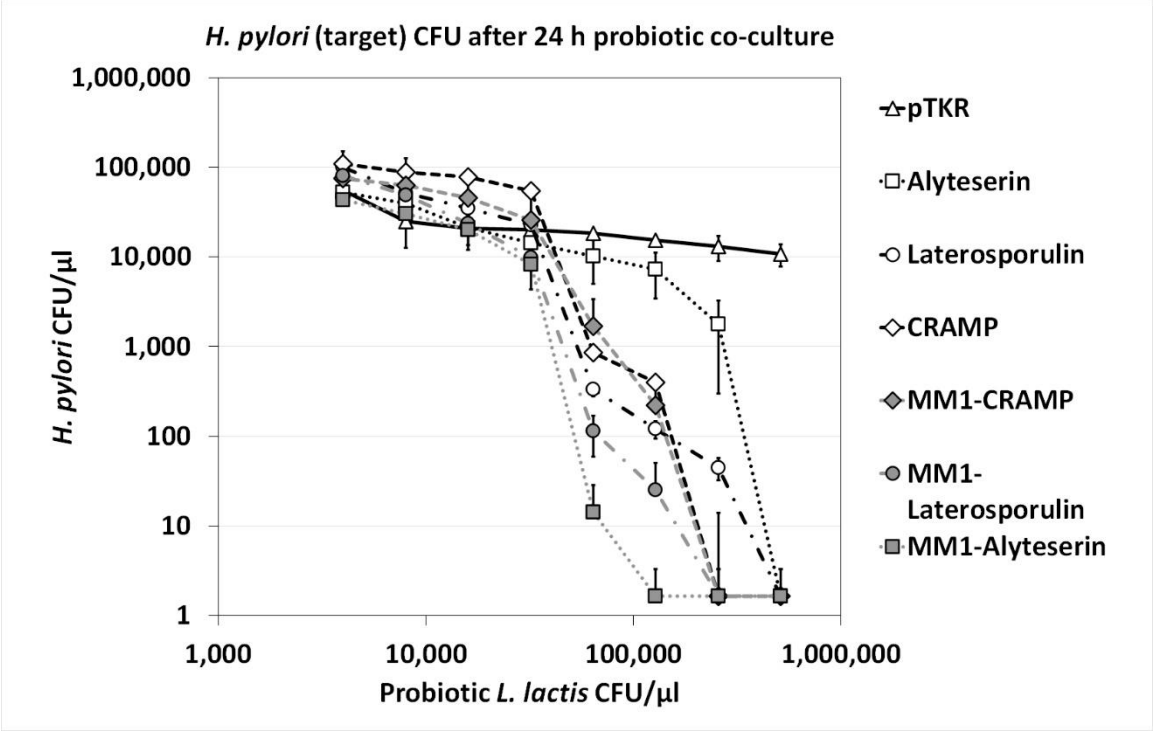

FIGURE 3.2. The inhibition of *Helicobacter pylori* growth by varying titers of probiotic *Lactococcus lactis* secreting AMPs or gAMPs, as measured at 24 h of co-culture. The guide (MM1) used in the gAMPs was derived from human Multimerin 1, an *H. pylori* receptor. pTKR, probiotic empty vector negative control.

For the off-target control bacteria, significantly less gAMP toxicity was seen (Figures 3.3 and 3.4) compared to that seen with the target bacterium (Figure 3.2), as expected. Furthermore, the guide peptide conferred an increase in toxicity against the target (Figure 3.2) but a decrease in toxicity against the non-target species (Figures 3.3 and 3.4). The gAMPs were significantly less toxic than the corresponding AMPs for both off-target species, even at the lowest probiotic titer (4800 CFU/µl). Once again, the biggest differential in toxicity between gAMP and AMP was seen with alyteserin for both off-target bacteria. It should be noted that the AMPs selected are intrinsically more toxic against Gram (-) bacteria (*H. pylori* and *E. coli)* than against Gram (+) bacteria (*L. plantarum*), accounting for the order of magnitude less toxicity for *L. plantarum*.

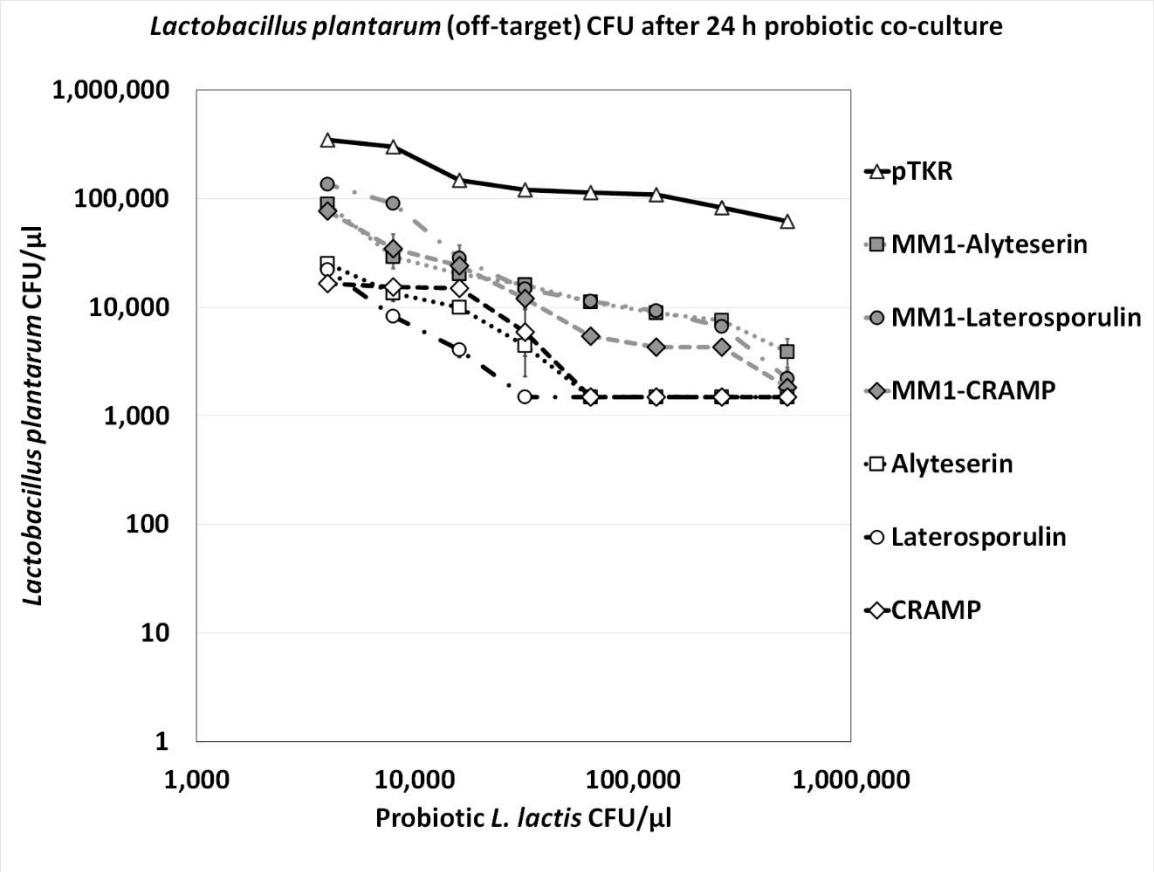

FIGURE 3.3. The inhibition of off-target *Lactobacillus plantarum* growth by varying titers of probiotic *L. lactis* secreting AMPs or gAMPs, as measured at 24 h of co-culture. MM1, Multimerin 1-derived guide peptide; pTKR, probiotic empty vector negative control.

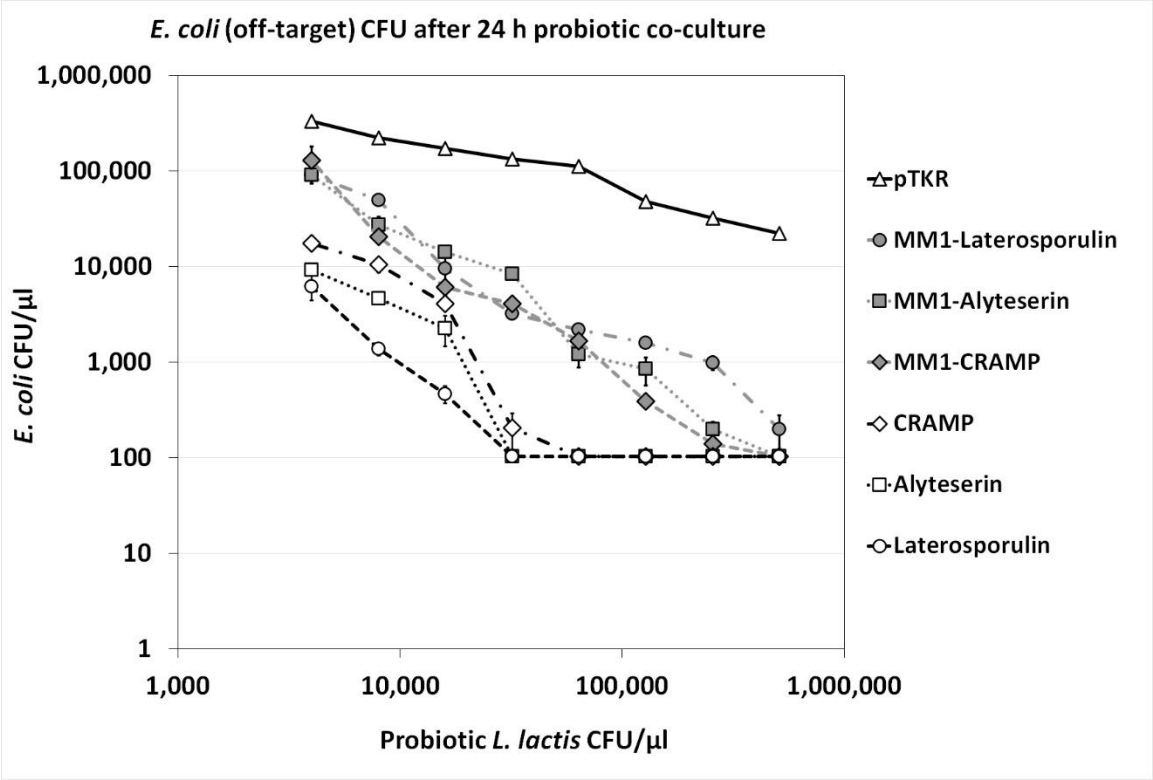

FIGURE 3.4. The inhibition of off-target *Escherichia coli* growth by varying titers of probiotic *L. lactis* secreting AMPs or gAMPs, as measured at 24 h of co-culture. MM1, Multimerin 1-derived guide peptide; pTKR, probiotic empty vector negative control.

Discussion

Though only a few previous studies have explored guided antimicrobial peptides, these have uncovered two different mechanisms whereby a differential toxicity between targeted and off-target bacteria could be effected. In one study, the differential was due to the increase of toxicity of a weakly toxic antimicrobial peptide against the target, while the low toxicity against the off-target bacteria was relatively unchanged (Eckert et al., 2004). In two other studies, a relativity toxic antimicrobial peptide was made less toxic against the off-target bacteria while retaining toxicity against the target (Mao et al., 2013; Choudhury et al., 2020). In this chapter, I have demonstrated the latter phenomenon for the AMPs, laterosporulin and CRAMP, with the gAMP form exhibiting a significantly

lessened toxicity against the off-target species, *L. plantarum* and *E. coli*, while the apparent increase in toxicity against the target species, *H. pylori*, was not statistically significant. However, for the AMP, alyteserin, a strongly significant effect was seen with the gAMP form compared to the unmodified AMP, both in lessening the toxicity against the off-target species and increasing the toxicity against the target species. This illustrates the importance of the choice of AMP in determining the effect of guide peptides on toxicity. From these data, it is apparent that any study on guide peptides should always be carried out with multiple AMPs is order to come to the proper conclusions and also to identify the best lead candidate gAMP moving forward towards commercial development.

The fragment of MM1 that we used was found to have the highest dissociation coefficient (K_D) with the VacA toxin on the surface of the *H. pylori* and may be presumed to facilitate the attachment of the AMP to the bacteria. For alyteserin, this enhanced attachment appears to be the critical factor in allowing the AMP to kill the target bacterium. AMPs as a group tend to be amphipathic and detergent-like, with many being shown to disrupt membranes and/or pass through membranes (Lei et al., 2019). It may be that alyteserin has the capability of these actions but must first be brought in close proximity to the membrane by the guide peptide. An analogy may be the affinity for heparan sulfate shared by many viral particles which brings the virus in close enough proximity to the cell surface to allow for binding to the main, specific host cell surface receptor protein or glycan (Cagno et al., 2019).

On the other hand, the impairment of the attachment of gAMP to the non-target bacterial cell, leading to decreased toxicity, would need to be explained by a different mechanism. In this case, the simplest model would be a steric hindrance of binding of the

gAMP to any cell lacking the virulence factor to which the guide peptide binds. The guide peptide would inhibit the action of an AMP normally toxic to a broad range of bacteria. However, the specific binding of the guide peptide to the virulence factor of the targeted bacterium would be sufficient to overcome this inhibition and allow the gAMP to have action against the target membrane. This concept was first detailed in the paper by Eckert et al. (2004) in which a long guide peptide was found to inactivate toxicity of the gAMP even against the target bacterium, while a shortened version of the guide peptide allowed the gAMP to specifically kill the target bacterium while still greatly reducing toxicity against off-target bacteria. Steric hindrance due to the length of the guide peptide was reasoned to be responsible for this phenomenon.

Though the forgoing discussion compared the results to other gAMP studies, this is the first study using gAMPs expressed from probiotic bacteria rather than being applied as a purified peptide. The advantages of probiotic delivery to the gut include the survival of the AMP through the oral route, past areas of low pH and peptidases. Additionally, the AMP can be produced from replicating probiotic bacteria *in situ* over a long period of time rather than being dependent upon a static dose of purified peptide. Commercially, the difference in cost of goods is considerable when comparing food grade probiotics and purified peptide drugs.

Several publications demonstrating the effectiveness of bacteriocins delivered using probiotics have been published by the Kaznessis Lab at the University of Minnesota (Borrero et al., 2015; Geldart et al., 2016; Forkus et al., 2017; Geldart et al., 2018). The use of a bacteriocin expressed in a probiotic has also been reported by the Chang Lab in Singapore (Saeidi et al., 2011). However, no one to date has published guided antimicrobial

peptides from probiotics. Antimicrobial peptides have little target specificity and suffer the same drawbacks of broad-spectrum antibiotics in this regard. Bacteriocins have excellent specificity but are effective only against a strain of the same bacterium from which they are isolated, making their development against a specific desired target species a difficult journey of exploration. In contrast, potential guide peptide candidates abound and can be derived from receptor binding sites of bacteriophages, virulence factor receptors (this chapter), bacterial pheromones (Eckert et al., 2006), or synthetically via biopanning (Yacoby et al., 2006).

The choice of VacA toxin as a potential target for this study was driven by the fact that it is commonly found on the bacterial cell surface (Foegeding et al., 2016; McClain and Cover, 2006; Telford et al., 1994; Voss et al., 2014), even though it is also considered as a secretory protein (Cover and Blaser, 1992; Foegeding et al., 2016; Snider et al., 2016). VacA is in fact critical for pathogen binding to the host cell and is transferred to host cell via contact dependent mechanisms (Ilver et al., 2004; Keenan et al., 2000; McClain and Cover, 2006). VacA binds to host cells and is internalized, causing severe "vacuolation" characterized by the accumulation of large vesicles that possess hallmarks of both late endosomes and early lysosomes (Foegeding et al., 2016; Palframan et al., 2012). The development of "vacuoles" has been attributed to the formation of VacA anion-selective channels in membranes. Apart from its vacuolating effects, it has recently become clear that VacA also directly affects mitochondrial function (Foo et al., 2010). The VacA toxin binds to stomach lining cells by associating with the lipid rafts on the cell membrane which causes it to be internalized by the cell and promote vacuole formation (Fiocca et al., 1999).

Once internalized, the p34 subunit of VacA toxin also forms an anionic pore into the mitochondrial membrane and interfere with its function (Domańska et al., 2010).

The function of VacA as such as strong virulence factor provides a second reason for its choice as a target for guide peptide development. In the development of resistance against this targeted therapy, the *H. pylori* will have to do so by jettisoning the important pathogenicity determinant VacA or at least majorly modify its structure. This provides an opportunity to steer the bacterium away from being pathogenic to escape antimicrobial activity from gAMPs built to specifically bind with VacA, in a way directing its evolution into a more benign strain. This is in fact the topic of current research in the Kearney Lab.

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CHAPTER FOUR

In situ treatment of *H. pylori* infection in mice stomach with the probiotic *Lactococcus lactis* bioengineered to release guided antimicrobial peptides (gAMPs)

The results were published in US Patent Application titled Probiotic Delivery Of Guided Antimicrobial Peptides, US Patent Application No: 16997036, International Patent Application No: PCT/US20/46896, Inventors: Christopher M. Kearney, Ankan Choudhury, Patrick Ortiz

Abstract

Objectives. The ability to selectively reduce infection by the pathogen, *Helicobacter pylori*, in mouse stomach without disrupting the resident microbial community was examined. The probiotic, *Lactococcus lactis*, was bioengineered to release a guided antimicrobial peptide (AMP) targeted against *H. pylori* and administered by oral gavage.

Results. Three AMPs, alyteserin, CRAMP and laterosporulin, were genetically fused to the guide peptide MM1, which selectively binds to the *H. pylori* virulence factor, vacuolating toxin A (VacA). Each guided antimicrobial peptide was cloned into the secretory vector pTKR carried by *L. lactis*. The engineered probiotics were fed therapeutically to mice previously infected for 5 days with *H. pylori*, or provided as a prophylactic for 5 days before infection of with *H. pylori*. Samples were collected using a novel reverse oral gavage method and analyzed using qPCR and Illumina 16S sequencing over a 10-day period. Guided antimicrobial peptides (gAMPs) provided therapeutically reduced *H. pylori* counts by 1860-fold compared to untreated infected mice and, as prophylactics, effectively inhibited challenge by *H. pylori*. gAMPs also resulted in a larger

number of OTUs than did AMPs or antibiotics in the stomach microbiota when used as therapy following *H. pylori* infection. However, gAMPs, AMPs and antibiotics all alleviated the tremendous microbial dysbiosis created by *H. pylori* infection of the stomach. A correlated network model was developed to measure this dysbiosis more precisely in response to different treatments.

Conclusions. Probiotics bioengineered to secrete guided AMPs powerfully protected the mouse stomach as a prophylactic and nearly eliminated *H. pylori* therapeutically. Given the wealth of AMPs and guiding ligands, both natural and synthetic, this approach can be adapted to develop a diverse array of chimeric guided AMPs and can be cloned into probiotics to create a safe and effective alternative to conventional chemical antibiotics.

Introduction

Current treatment regimens for *H. pylori* infections often include triple and quadruple antibiotic therapies to match the growing challenge of antibiotic resistance. Such regimens include combinations of amoxicillin, tetracycline, bismuth, metronidazole, clarithromycin, and other antibiotics and adjuvants. Alongside such vigorous employment of antibiotics, quadruple, quintuple, and sextuple antibiotic-resistant strains of *H. pylori* have arisen (Boyanova et al., 2019). Concurrently, typical broad spectrum antibiotics, which function as rRNA inhibitors, β-lactams, and nucleic acid inhibitors, create microbial dysbiosis by killing off-target bacteria (Becattini et al., 2016; Langdon et al., 2016; Zarrinpar et al., 2018**).** Antibiotic-associated dysbiosis often leads to intestinal inflammatory diseases such as colitis (Strati et al., 2021), worsens neuro-immune

mechanisms and viscerosensory functionalities (Aguilera et al., 2015) and often makes way for a bloom of pathogens in a microbiota free of competing commensals (Vangay et al., 2015). Treatment of *H. pylori* for the prevention of gastric cancer requires treatment over the long term since it is the inflammation brought on by *H. pylori* that, over time, induces gastric cancer (Wroblewski et al., 2010). The dysbiosis induced by antibiotics makes antibiotic treatment unsuited for the long-term treatment necessary to prevent gastric cancer. A selective therapy that does little harm to the microbiota at large is clearly needed for the long-term approach.

To develop a therapy selectively active against *H. pylori*, I used antimicrobial peptides (AMP) conjugated to a guide peptide which enables specific attachment to the target pathogen. I have demonstrated the efficacy of such guided antimicrobial peptides (gAMPs) in Chapter Two by using a guide peptide, discovered in previous literature through biopanning experiments, to add specificity to the general bactericidal activity of two AMPs and in killing *Staphylococcus* bacteria. In Chapter Three, I used three AMPs (CRAMP, laterosporulin and alyteserin) (Baindara et al., 2016; Conlon et al., 2010, 2009; Hase et al., 2003; Neshani et al., 2019; Singh et al., 2015; Zhang et al., 2016, 2013) active against Gram (-) bacteria and conjugated a fragment of a human platelet protein multimerin 1 to it. This fragment of multimerin 1 (AA 321-340) was found to specifically bind to VacA toxin (Satoh et al., 2013), a toxin released by *H. pylori* which often remains on the surface of the bacterium (Foegeding et al., 2016; Ilver et al., 2004; Voss et al., 2014). Using this fragment MM1 I have created chimeric gAMPs that were cloned into *L. lactis* using a modified pT1NX vector (pTKR) that releases the expressed gAMP using the secretory signal usp45 and triggered by a low pH promoter P1 (Steidler et al., 2004, 1998; van

Asseldonk et al., 1990; Waterfield et al., 1995). In Chapter Three, I also demonstrated how the probiotic, *Lactococcus lactis,* expressing gAMPs, efficiently killed *H. pylori*, but showed a diminished activity towards non-target bacteria such as *Lactobacillus plantarum* and *E. coli* and an increased activity against the target bacterium when compared to unguided AMPs.

In the present chapter, I detail the results of in vivo tests in mouse stomach of this probiotic/gAMP technology. I administered *H. pylori*-infected C57BL/6J mice with the engineered *L. lactis* expressing the gAMP. To validate this system as a prophylactic, I administered the mice with bioengineered *L. lactis* before being infecting with *H. pylori*. The stomach samples of these mice were collected using a novel reverse oral gavage method and were sequenced using an Illumina MiSeq with primers targeted towards the 16S V4 region (Caporaso et al., 2012, 2010). Metagenomic analysis of the sequencing data was performed using QIIME2 (Bolyen et al., 2019) and CCREPE (Schwager et al., n.d.), for the correlation network analysis on differential taxa abundance.

Materials and Methods

Administering L. Lactis and H. Pylori in Mice by Oral Gavage and Sample Collection

Cultures of probiotic and *H. pylori* were grown out and fed to mice by oral gavage. Briefly, the *L. lactis* cultures were propagated overnight GM17 broth with erythromycin (5 µg/ml) and no shaking. The overnight cultures were spun down at 4000 g for 15 min at 4^{o} C. The pellets were resuspended in sterile PBS. *H. pylori* SS1 stocks were grown overnight on Blood-TS agar under microaerobic condition and >5% CO_2 environment and then scraped by a sterile loop and resuspended in sterile PBS. Both the bacterial suspensions

were fed to the mice using 1.5 oral gavage needle not exceeding half their stomach volume (~250 µL). The CFU of the resuspension being fed were determined by diluting the resuspension 1/1000 and 1/10000 times and plating on appropriate plates. For both *L. lactis* and *H. pylori*, the inoculum sizes were kept ~50,000 CFU/µl.

Samples were taken using a reverse oral gavage method invented for this study. Pre- and post-inoculation samples from the mouse stomach were collected by flushing the mouse stomach with excess PBS (~250 µL). The mice were fed the PBS using a gavage needle that reaches well into the stomach. Immediately after, without losing the suction and removing the needle out of the mouse esophagus, the plunger is moved up and down twice without drawing any substantial volume of fluid out. The presence of a negative pressure during pulling the plunger is preferable. The plunger is then completely pulled out which will draw out around 50-75 µl of stomach fluid. The collected fluid is then put in tubes/vials for storage. It is expected that the fluid should be slightly viscous and may or may not have suspended food fragment and /or mucus.

Four different schemes of bacterial inoculation were designed to cover each of the experimental types: Probiotic Therapy, Antibiotic Therapy, Probiotic Prophylactic, and the Null (no treatment) Control.

For the Probiotic Therapy, stomach samples were collected on Day 0 before *H. pylori* inoculation. Over the next three days, resuspended *H. pylori* were fed by oral gavage once daily. On Day 5, stomach samples were collected to test for *H. pylori* presence followed immediately by the probiotic therapy, which consisted of a single oral gavage feeding of resuspended *L. lactis* carrying either AMP, gAMP or the control empty pTKR vector. Follow-up stomach samples were collected on Days 8 and 10.

The Antibiotic Therapy was performed identically to the Probiotic Therapy, with the substitution of an antibiotic cocktail (amoxycillin:tetracycline :: 4.5:4.5 mg/25 g of mice) fed to the mice by oral gavage in place of the probiotic on Day 5.

For the Probiotic Prophylactic, stomach samples were collected on Day 0 followed immediately by *L. lactis* inoculation carrying one of the three pTKR vectors as for the Probiotic Therapy. On Day 3, stomach samples were taken by reverse oral gavage, followed immediately by a challenge inoculation with *H. pylori* by oral gavage, with daily *H. pylori* challenge inoculations for a total of three consecutive days. Stomach samples were collected on Day 8 and 10 to test for *H. pylori* presence.

For the Null Control mice, stomach samples were collected on Day 0 before *H. pylori* inoculation; followed by daily *H. pylori* inoculations for a total of three consecutive days. Stomach samples were then collected on Day 5, 8 and 10 to test for *H. pylori* presence.

Six mice were used per AMP and gAMP both for both the Probiotic Therapeutic and Probiotic Prophylactic treatments. Six mice each were also used to constitute the Null Control group, the Antibiotic Therapy group, and the empty vector (pTKR) group for both the probiotic therapeutic and prophylactic experiments.

PCR Determination of Presence of Bioengineered L. Lactis in Mouse Stomach

The stomach samples of mice at Day 10, 5 days after feeding them cloned *L. lactis*, were subjected to a PCR test. The samples were heated at 100^{o}C for 15 min and chilled at 4^{o}C for 5 min and then used as the template for PCR (NEB Taq Polymerase, 95^{o}C denaturation for 5 min; 30 cycles of 95^{o}C denaturation for 30 s, 60^{o}C annealing for 15 s, 68^{o}C extension for 30 s; final extension for 2 minutes) using primers specific to the pTKR

vector (forward: 5’ – GCCTGAGCGAGACGAAATAC – 3’, reverse: 5’ – TTATGCCTCTTCCGACCATC – 3’). The PCR products were ran on a 1% agarose gel to ascertain the size of the products.

Assay for Determining H. Pylori Titer in Mice Stomach by qPCR

The stomach samples collected were heated at 100^{o}C for 15 min and chilled at 4^{o}C for 5 min. The supernatants were collected and plated in a 96-well plate and qPCR was performed with primers for VacA gene (forward: 5′-ATGGAAATACAACAAACACAC-3′, reverse: 5′-CTGCTTGAATGCGCCAAAC-3′) to quantify *H. pylori*. Standard curves for *H. pylori* against the C_T values were constructed by including different dilutions of the overnight cultures of *H. pylori* (1/10, 1/100, 1/1000, 1/10000) in the qPCR and plating those dilutions on respective plates to determine the corresponding CFU/µl values. The CFU/µl values for each sample were determined by plotting the C_T values against the standard curve previously built.

Analysis of Mouse Stomach Microbiome Affected by L. Lactis and H. Pylori Inoculation

The stomach samples collected were heated at 100^{o}C for 15 min and chilled at 4^{o}C for 5 min. The supernatants were collected and plated in 96-well plate for upstream processing for Illumina sequencing. The samples were amplified with 16S V4 primers (forward: 5’-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG GTG YCAGCMGCCGCGGTAA-3’, reverse:5’-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GCC GYCAATTYMTTTRAGTTT-3’) (Caporaso et al., 2012, 2010) and then with Illumina index primers with subsequent clean-up and purification. The samples were pooled into a library, spiked with PhiX phage DNA and sequenced using the

Illumina MiSeq v3 kit. The data was demultiplexed, denoised and analyzed for taxonomic abundance using QIIME2 (Bolyen et al., 2019). The alpha and beta diversity analysis were also performed using QIIME2. The taxonomic abundance data (at genus level) was analyzed using the CCREPE package in R (Schwager et al., n.d.) with the microbial community of the mouse stomach at Day 0 compared against the community from the samples taken at Day 5 after three consecutive days of *H. pylori* inoculation, to determine the correlation between the taxa which went up or down in relative abundance upon addition of *H. pylori*.

Results

The Probiotic And Its Vector, pTKR, Were Still Present In The Mouse Stomach Five Days After Inoculation

The samples from the mice stomach at Day 10 of the Probiotic Therapy, five days after feeding *L. lactis* cloned with laterosporulin and MM1-laterosporulin, showed positive results when subjected to PCR with primers specific to the pTKR vector, demonstrating that the bioengineered *L. lactis* remained for the duration of the therapy in the stomach with the plasmid still at a detectable amount (Figure 4.1)

Mouse stomach samples collected by the reverse oral gavage method were analyzed by qPCR to estimate the *H. pylori* load changing with time (Figure 4.2). The loads reached their maxima at Day 5 after inoculation with *H. pylori* and then were strongly reduced back down to baseline levels following treatment with the *L. lactis* probiotic expressing either AMPs or gAMPs, with no significant difference in efficacy between AMP and gAMP groups or between any of the three AMPs used.

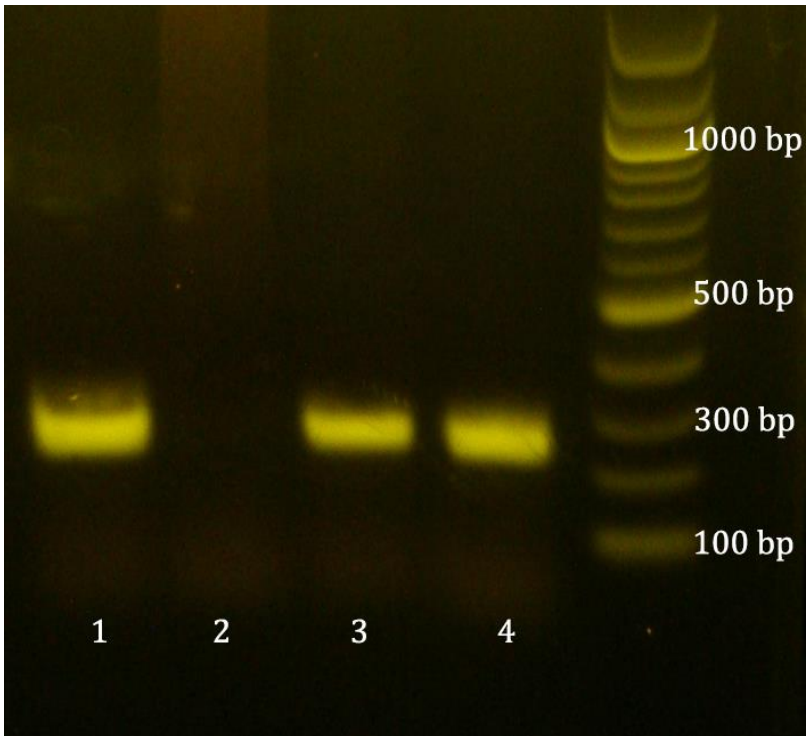

FIGURE 4.1. 1% Agarose gel electrophoresis of stomach samples subjected to PCR with primers specific to the pTKR *L. lactis* vector. Lane 1), positive control (200 ng of pTKR plasmid), 2) negative control (mice stomach sample with no *L. lactis* inoculation, 3) stomach sample at Day 10 from mice fed with laterosporulin-*L. lactis*, 4) stomach sample at Day 10 from mice fed with MM1-laterosporulin-*L.lactis*

L. lactis probiotic expressing AMP or gAMP as a therapy against H. pylori infection of mouse stomach

The *H. pylori* titers of the empty vector group and null control (no treatment) group continued to increase unabated between Days 5 and 10. In comparing the average *H. pylori* titer at Day 10 of the six AMP and gAMP treatments versus the titers of the negative controls, the five days of AMP/gAMP therapy led to a reduction by 520-fold and 1100-fold in comparison to the empty vector and null control groups, respectively. When the gAMP therapy is considered in isolation, these numbers are a 860-fold and 1860-fold difference with negative controls, while the AMP therapy was recorded as a 370-fold and 800-fold difference with negative controls. The titer of *H. pylori* for the gAMP-*L. lactis*

were lower than the corresponding AMP-*L. lactis* but as stated, the values were not significantly different, echoing the findings of the in vitro assay in previous chapter for laterosporulin and CRAMP. The antibiotic therapy did result in a reduction of *H. pylori*, but this was significantly less than the reduction seen by any of the AMP or gAMP treatments. No significant decrease in *H. pylori* was observed in the empty vector treatment over the null control, indicating that the probiotic *L. lactis* by itself did not provide any therapeutic effect.

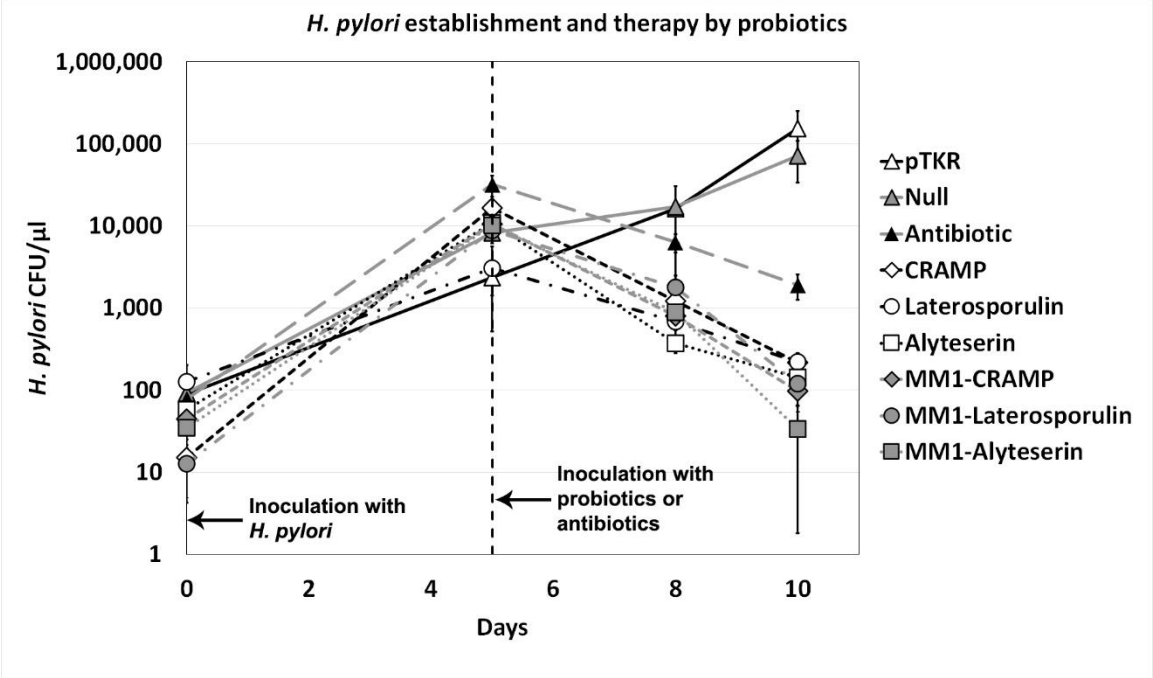

FIGURE 4.2. Probiotic therapy of *H. pylori* infection of mouse stomach. The CFU/µl of *H. pylori* found in the mice stomach, estimated from the corresponding C_T values determined by qPCR, is plotted against days after inoculation with *H. pylori*. The dotted line at Day 5 represented the point of feeding *L. lactis* probiotic expressing AMP or gAMP to mice.

L. lactis Probiotic Expressing AMP/gAMP as a Prophylactic Against H. pylori Challenge

Treatment with probiotics expressing AMP or gAMPs for three days preceding challenge infection with *H. pylori* led to lower infection levels than found in mice with no

prophylactic probiotic treatment or mice pretreated with probiotics containing on an empty vector (Figure 4.3). Specifically, the average *H. pylori* titer of AMP and gAMP treated mice at Day 10 was 50-fold less than the infected mice with no treatment and 5-fold less than infected mice with the empty vector prophylactic control. This difference was significant as was the difference between the null and empty vector controls, pointing to a slight prophylactic effect due to the probiotic alone. No significant difference in final *H. pylori* titers was seen among the six AMP and gAMP treatments.

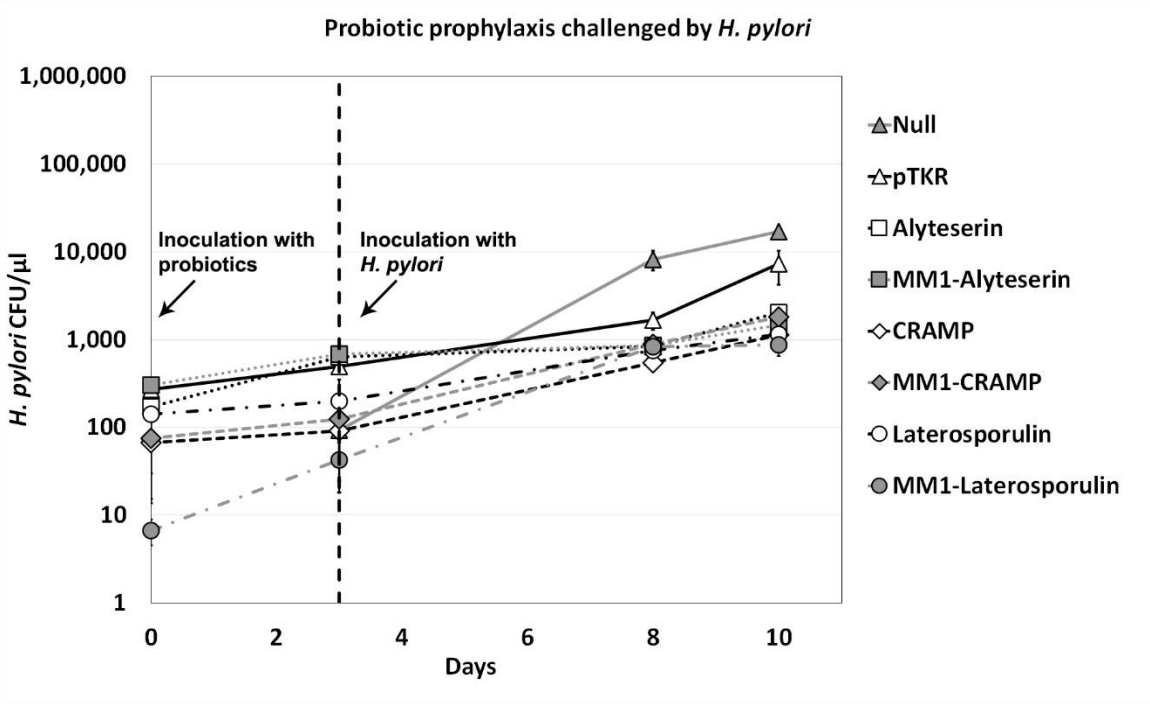

FIGURE 4.3. Probiotic prophylaxis to protect mouse stomach from *H. pylori* challenge infection. The CFU/µl of *H. pylori* found in the mice stomach, estimated from the corresponding C_t values determined by qPCR, plotted against days after inoculation with *H. pylori*. The dotted line at Day 3 represented the point of feeding *H. pylori* to mice.

L. lactis Expressing gAMP Reversed Dysbiosis in Mice Stomach and Restored Microbiome Diversity

Microbiome analysis through 16S rRNA gene sequencing of the 350 collected mice stomach samples revealed that *H. pylori* infection caused significant dysbiosis which was reversed by the subsequent feeding of recombinant *L. lactis*. The dysbiosis was conspicuous on the samples taken 5 days after infection with *H. pylori* and was marked by a drastic drop in the diversity of species identified from the samples. In those samples a multitude of species/genera went down from over 100 to fewer than 10 across all infected mice (Figure 4.4). The microbiome diversity was restored after therapy with gAMP-*L. lactis*. Probiotic therapy with gAMPs resulted in significantly higher taxonomic diversity than with AMPs after three and five days of therapy (Days 8 and 10 in Figure 4.4). In fact, OTU counts for AMP treatments were not significantly different from empty vector OTU counts (Table 4.1). Antibiotic treatment resulted in significantly higher species richness than no treatment at all, but significantly less than any of the probiotic treatments.

TABLE 4.1. The p-values of the t-test performed between observed OTU values (at genus level) for each treatment group from the Day 10 data presented in Figure 4.4.

Treatment	gAMP	AMP	Antibiotic	Empty
Null	4.04362E-45	7.75921E-29	4.88147E-20	2.9924E-12
Empty	0.000118242	0.296878437	4.98665E-40	
Antibiotic	7.1317E-129	7.2617E-108		
AMP	8.24156E-05			

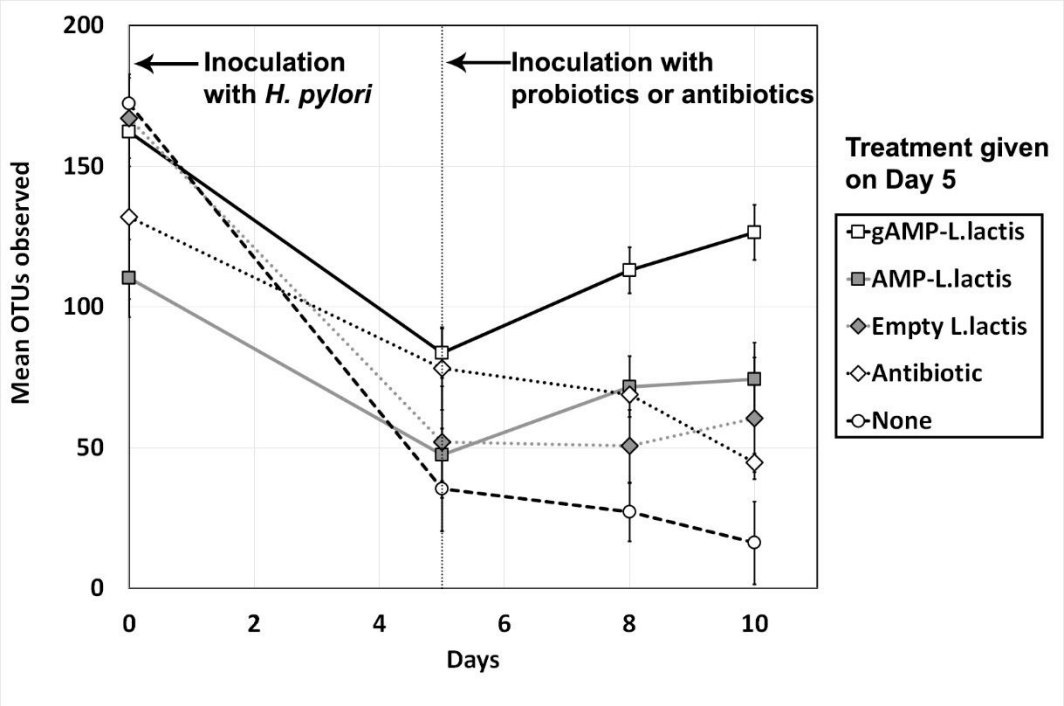

FIGURE 4.4. Decline of taxonomic diversity due to *H. pylori* infection and effect of recombinant probiotic therapy. The mean observed operational taxonomic unit (OTU) numbers at genus level for each therapeutic treatment groups are displayed for each stomach sample collection time point. Each group of mice was first fed *H. pylori* for 3 consecutive days beginning with Day 0. At Day 5, each group was given their respective probiotic or control treatment.

For the prophylactic experiments, all probiotic pre-treatment groups preserved the taxonomic diversity of the stomach after challenge with *H. pylori*. In contrast, the null control group, with no probiotic pre-treatment, experienced a plummeting effect in the OTU levels (Figure 4.5). As in previous experiments, the gAMP probiotic group had the best response, and led to an OTU count that surpassed even the Day 0 count. On the other hand, the AMP probiotic treatment yielded an OTU count significantly less than even the Empty Vector probiotic treatment.

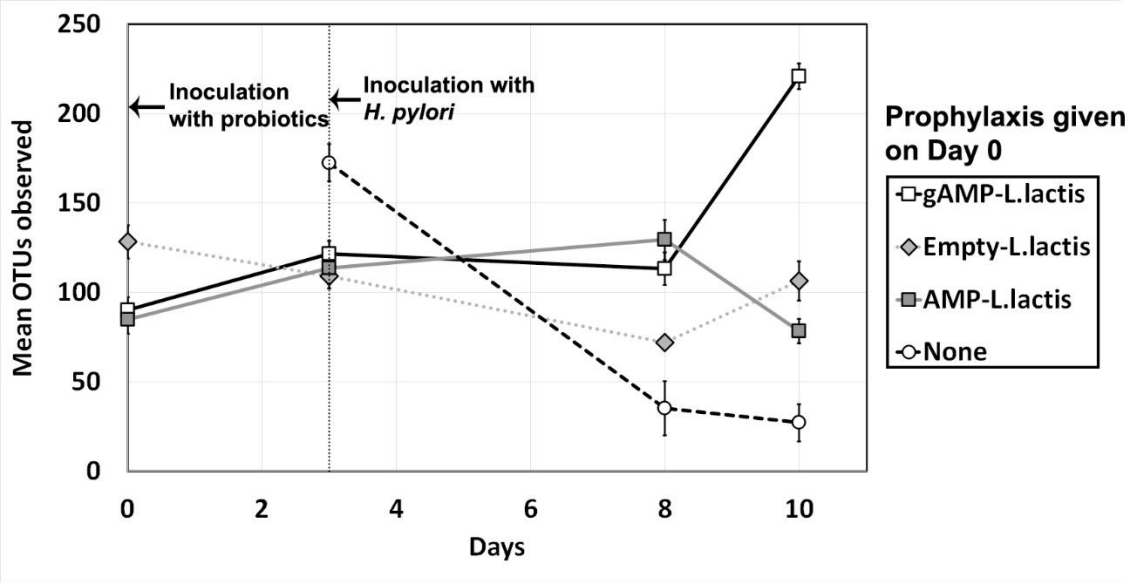

FIGURE 4.5. Prophylactic probiotic protection from decline in taxonomic richness from *H. pylori* challenge. The mean observed operational taxonomic unit (OTU) numbers at genus level for each therapeutic treatment groups are displayed for each stomach sample collection time point. Each group of mice was first fed their respective probiotic prophylactic at Day 0. At Day 3, each group was challenged with *H. pylori* by oral gavage.

Generating a Microbial Dysbiosis Index (MDI) to Measure Taxonomic Recovery from H. pylori Infection After Recombinant Probiotic Treatment

From the sequencing dataset of the samples displayed in Figure 4.2, the relative abundance of all sequenced taxa at genus level were used to establish a healthy microbiota snapshot (Day 0 for all mouse groups) and a dysbiotic microbiota snapshot (5th day of *H. pylori* infection (Day 5) for all mouse groups). The compositional data between the samples presented correlations between features (here, relative abundance of different taxa) due to the nonindependence of values that must sum to a fixed total. The CCREPE package was used to abrogate the correlation and determine the significance of a similarity measure for each feature pair using permutation/renormalization and bootstrapping (Faust et al., 2012; Schwager et al., n.d.). This generated an N-dimensional checkerboard with similarity scores, p-values and false discovery rate q-values corrected for the effects of compositionality. The top features with best p-values and q-values (<0.12) were selected

to create a correlation network with the features (Figure 4.6) based on their similarity scores (between 1 and -1 based on their correlation across the samples of Day 0 and Day 5). This revealed eight genera being positively correlated with each other and two other genera being negatively correlated with the other eight, based on their relative abundance across Day 0 samples and Day 5 samples. On analyzing the change in relative abundances of these 10 genera between the samples of Day 0 and Day 5, all of the eight positively correlated genera had a higher relative abundance in Day 0 compared to Day 5 and the other two had significantly higher abundance in Day 5 compared to Day 0 (Figure 4.7).

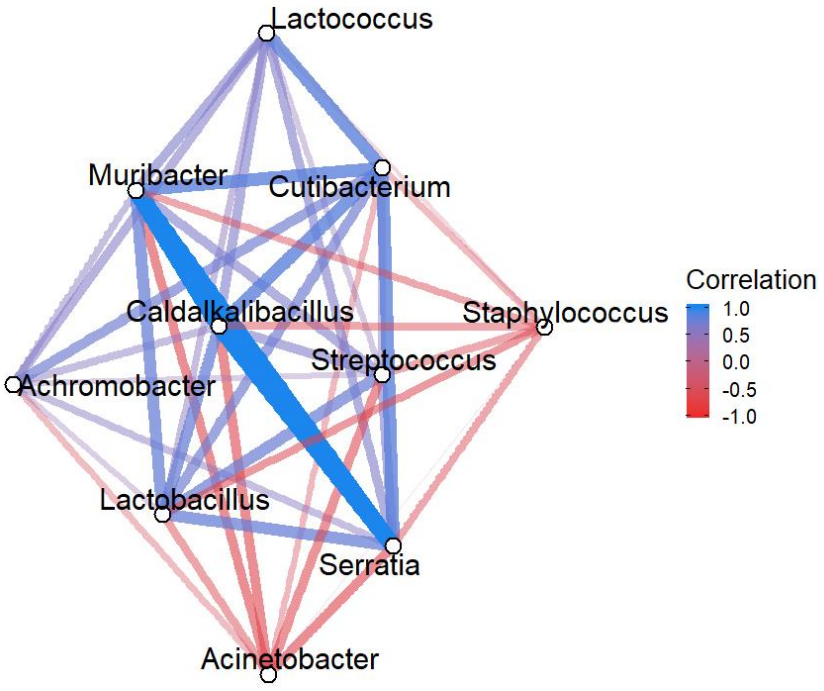

FIGURE 4.6. The top 10 genera showing significant similarity scores in change of relative abundance from Day 0 to Day 5 using the sequencing dataset associated with the samples displayed in Figure 4.2. The eight genera (*Lactococcus*, *Muribacter*, *Cutibacterium*, *Caldalkaibacillus*, *Streptococcus*, *Achromobacter*, *Lactobacillus* and *Serratia*) had the most positive correlation with each other whereas *Staphylococcus* and *Acinetobacter* were negatively correlated with all the other eight

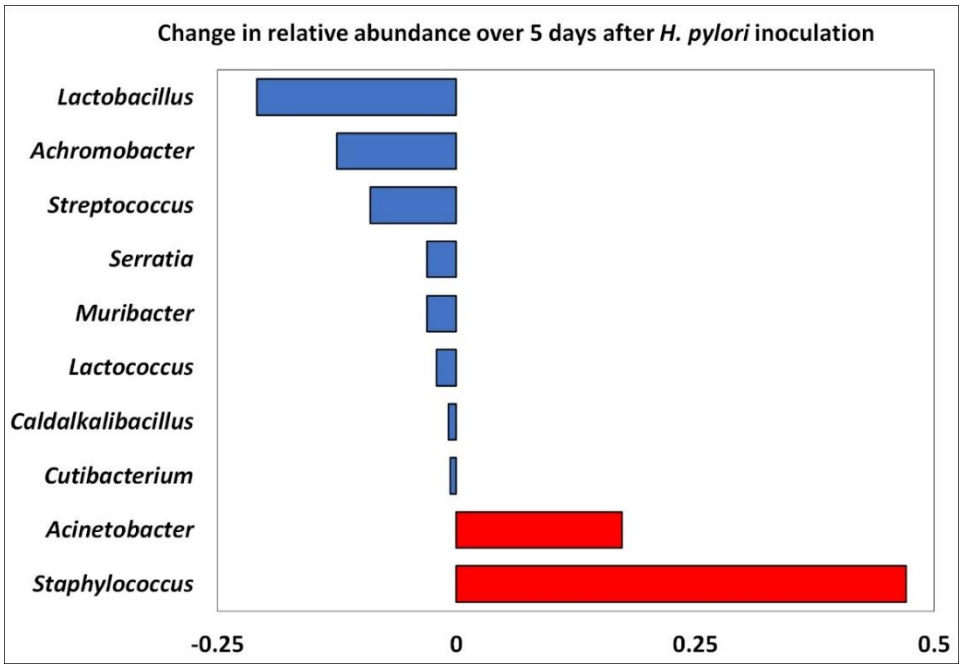

FIGURE 4.7. Decrease (blue) or increase (red) in relative abundance of different bacterial genera in mouse stomach samples starting from uninfected (Day 0) to 5-day infected with *H. pylori* (Day 5). Genera that were found to be most similar to each other (blue) in Figure 4.6 in terms of relative abundance changes are shown here to have a decrease in relative abundance from Day 0 to Day 5. The two genera most dissimilar from these eight (red) in Figure 4.6 are shown here to have increased in relative abundance from Day 0 to Day 5.

The differential abundance of the 10 genera was used to create a Microbial Dysbiosis Index (MDI) using the formula:

$$\mathbf{MDI} = \log_{10}\left(\frac{\sum \textbf{Relative abundance of bacteria that increased in abundance}}{\sum \textbf{Relative abundance of bacteria that decreased in abundance}}\right)$$

A positive MDI predicts a dysbiotic condition in which the two bacteria more abundant in the Day 5 (*H. pylori* infected) samples are overrepresented and the eight bacteria that were positively correlated in the Day 0 samples, which are characteristic of the healthy microbiome, are underrepresented.

Using the formula, the MDI for all the samples were calculated to observe the clustering of the samples based on the dysbiosis and the relation dysbiosis has with taxonomic richness (genus count) for each sample

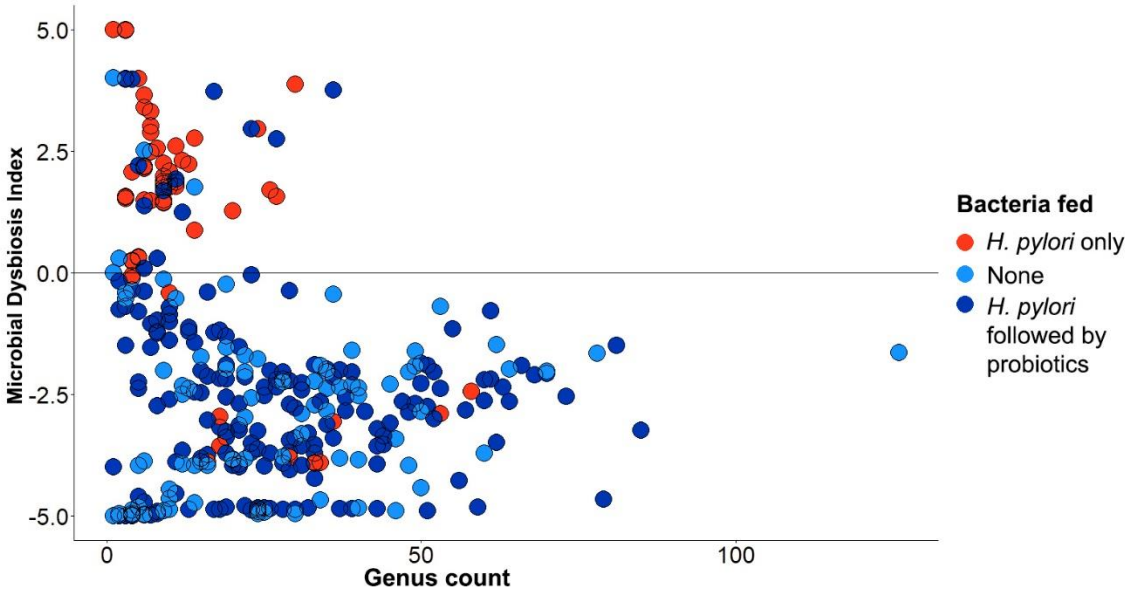

FIGURE 4.8. The relationship between dysbiosis and taxonomic richness, compiled from sequencing data from mouse stomach samples used in the experiments of Figure 4.2 and 4.3. The dots are colored representing whether the samples were from a mouse infected with *H. pylori* and untreated (Red) versus infected mice treated with probiotic or antibiotic (dark blue) or mice both untreated and uninfected (light blue).

The model was validated by checking the prediction it made about all the 350 samples collected in both therapeutic and prophylactic groups across all days on being dysbiotic (MDI>0) or non-dysbiotic (MDI>0). A training subset (30%, selected randomly) was created for the model using Random Forest variable algorithm (AUC=0.891) and used to predict whether the test samples were really 'dysbiotic' (here, infected with only *H. pylori*) or not as learnt from their MDI score.

The samples were then divided into Therapeutic (Figure 4.9) and Prophylactic (Figure 4.10) groups and examined for the affect of each treatment the dysbiosis in the mouse stomach microbiota. For the Therapeutic group, the greatest number of dysbiotic samples were found among the mice infected with *H. pylori* and left untreated, with the second greatest number being *H. pylori* infected and treated with probiotic/empty vector.

As expected from consideration of Figure, 4.8, samples in Figure 4.9 with negative MDI scores had greater taxonomic richness (larger diameter circles) across all groups.

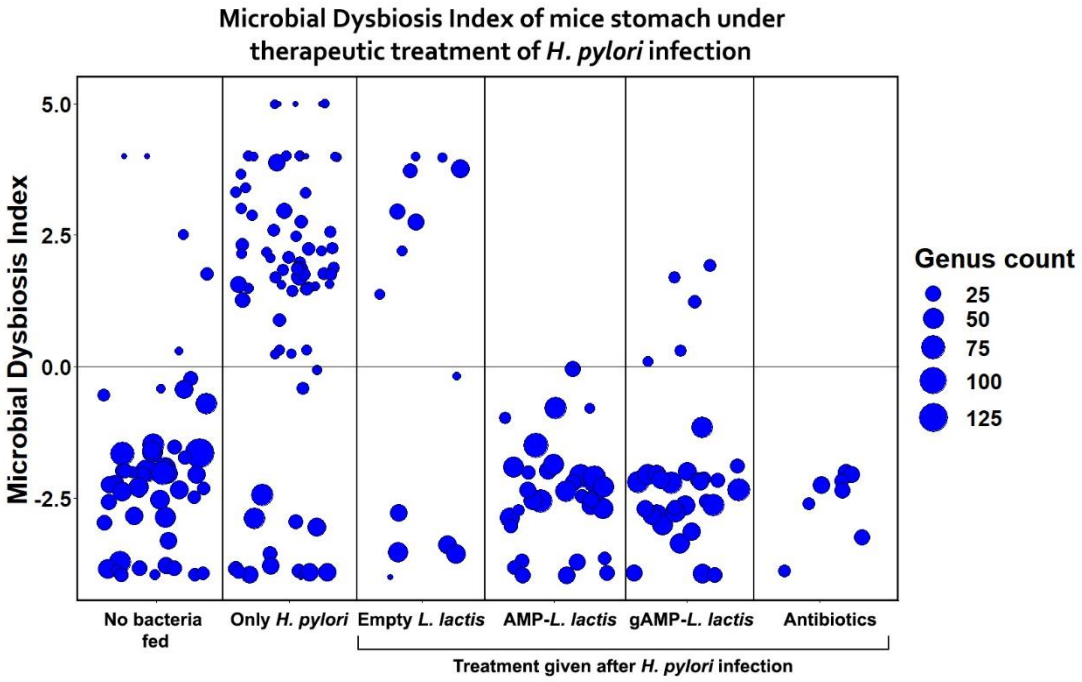

FIGURE 4.9. The samples from the Therapeutic group plotted according to their MDI scores (y-axis) and taxonomic diversity (circle diameter).

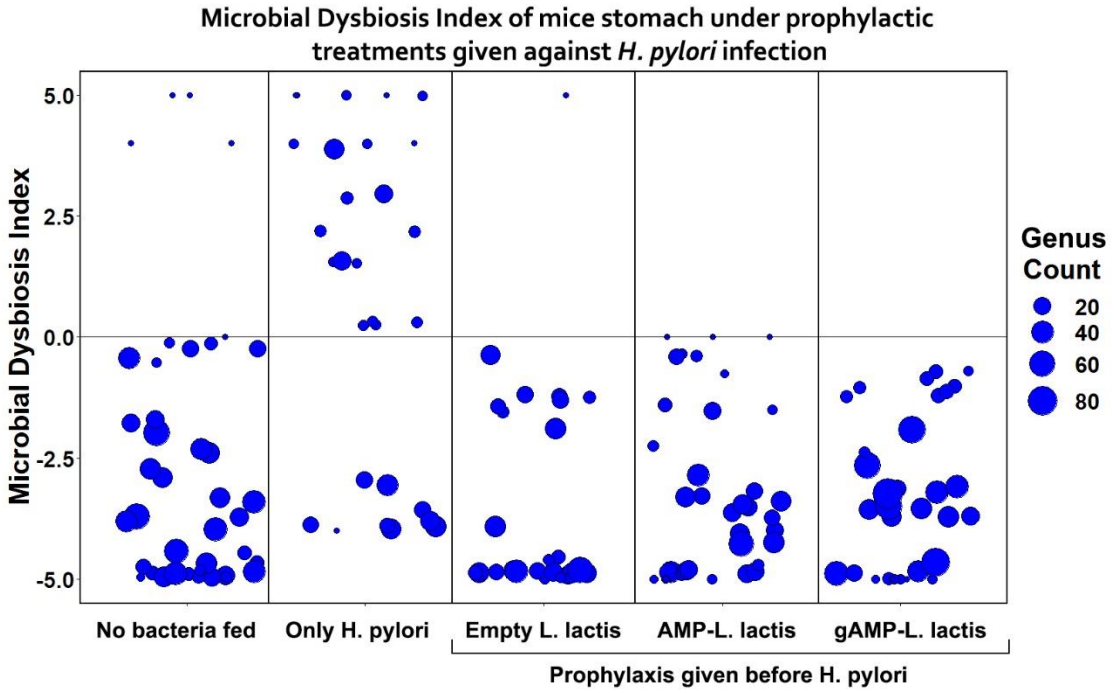

FIGURE 4.10. The samples from the Prophylactic group plotted according to their MDI scores (y-axis) and taxonomic diversity (circle diameter).

For the prophylactic groups, the MDI scores were negative for every sample belonging to mice that were pre-treated with *L. lactis* probiotic, with only the *H. pylori*/no treatment samples having mostly positive MDI scores. These trends were also seen when the mean MDI scores of each treatment group in the Therapeutic and Prophylactic schemes were plotted against the day the samples were taken. For the Therapeutic schemes (Figure 4.11), the MDI scores were negative for all treatments in Day 0, with all of them becoming positive on Day 5 after 3 consecutive days of administering *H. pylori*. On Day 8 and Day 10, for the AMP-*L. lactis*, gAMP-*L. lactis* and antibiotic treated groups, the scores returned to being negative while those of the Empty-*L.lactis* and null control group remained positive.

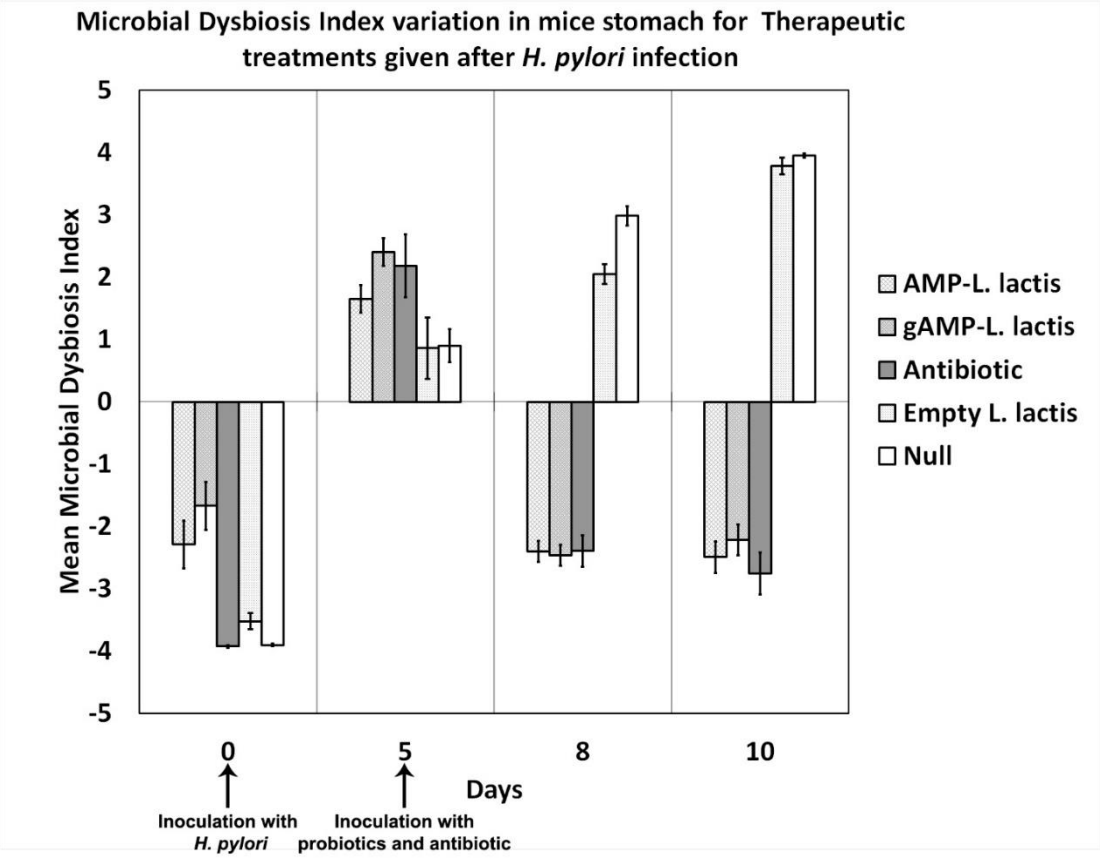

FIGURE 4.11. Mean MDI scores of mice stomach microbiota for each therapeutic treatment group with *H. pylori* administration Days 0, 1, and 2 and therapeutics given at Day 5.

For the Prophylactic schemes (Figure 4.12), all the prophylactic treatment groups, including the Empty Vector control, had negative MDI scores at Day 0 and remained negative after *H. pylori* challenge (Day 3, 8 and 10). The null control (prophylaxis) group had negative MDI scores on Day 3, which turned positive on Day 8 and 10 after administering *H. pylori* for 3 consecutive days.

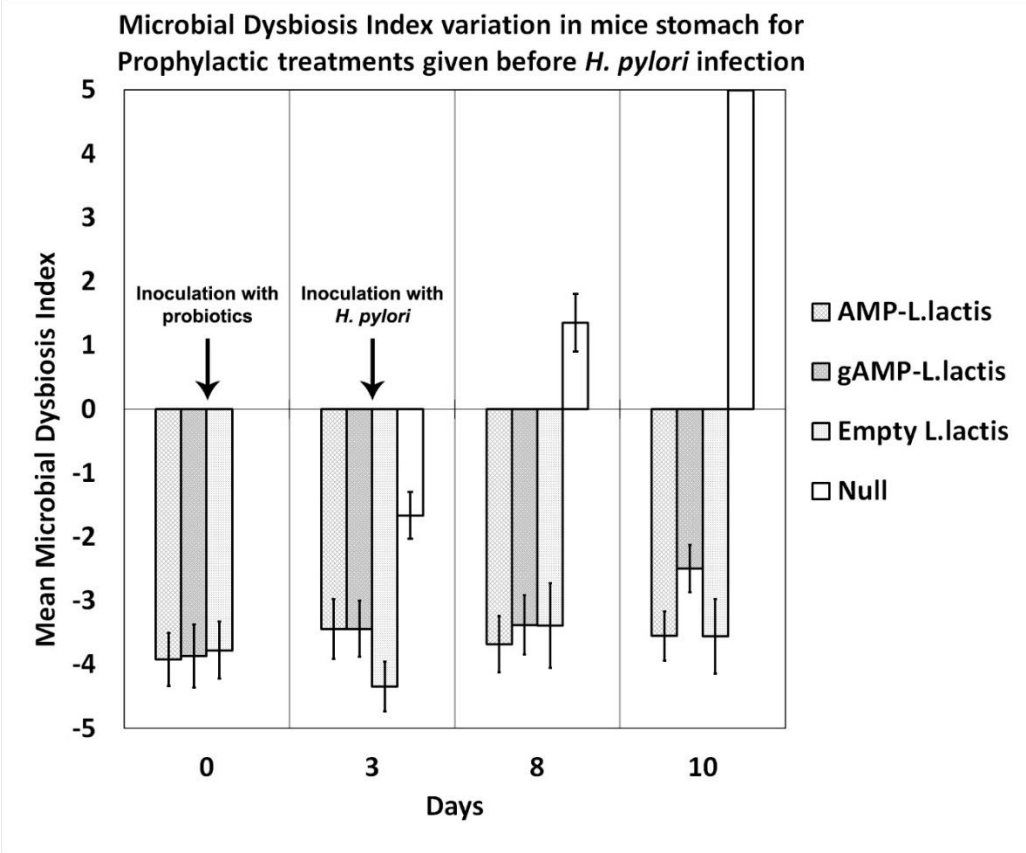

FIGURE 4.12. Mean MDI scores of the mice stomach microbiota for each prophylactic treatment group with probiotic prophylactic given at Day 0 and H. pylori administered on Days 3, 4, and 5.

Discussion

All in vivo assays demonstrated that *L. lactis* probiotic expressing AMP or gAMP was effective at clearing *H. pylori* infections of the stomach as a therapy or inhibiting its establishment as a prophylactic. No statistically significant difference against *H. pylori* infection was seen between the gAMP and AMP versions or among any of the three AMPs tested, in contrast to the in vitro co-culture results for alyteserin presented in Chapter Three. The growth of *H. pylori* in the mouse stomach in the presence of probiotic carrying the empty pTKR vector was not significantly different from the Null (no probiotic) control for either the therapy or prophylactic experiments, demonstrating that the toxicity against *H. pylori* was due to the secretion of gAMP or AMP rather than to the probiotic bacterium itself. Studies prior to this have seen similar effect of *L.lactis* releasing antimicrobial peptides on a mix of both gram positive and gram negative bacteria like *E. faecalis, S. aureus, Pseudomonas aureginosa,* (Tanhaeian et al., 2020), *Salmonella typhimurium, E. coli* (Tanhaeian et al., 2020, Volzing et al., 2013). In those studies too, the probiotic *L. lactis* by itself and/or cloned with the empty vector did not produce appreciable toxicity against the desired pathogen either in planktonic or biofilm form. There were no in vivo studies involving the use of bioengineered probiotic *L. lactis* for us to compare, so the antimicrobial action of the cloned probiotics in mouse stomach, as demonstrated in our experiment, is a remarkable proof of our principle.

The difference made by the guide peptide was seen in the effect on the native stomach microbiota. This difference in the impact of the AMP-*L. lactis* and the gAMP-*L. lactis* on the native microbiota was determined by the 16S rRNA sequencing using Illumina MiSeq to see what changes they caused in the stomach microbiome. For therapeutic

treatment, probiotics expressing gAMPs promoted stronger recovery of the taxonomic diversity seen in the stomach microbiota before *H. pylori* infection. For prophylactic treatment, gAMPs not only retained but increased the pre-*H. pylori* taxonomic diversity of the stomach microbiota. In contrast, the probiotic AMP treatments resulted in taxonomic diversity that was not significantly different from the empty vector controls. The therapeutic effect of bioengineered *L. lactis* in reducing and even reversing chemotherapy induced dysbiosis in mice has been previously documented (Carvalho et al., 2018) and it mirrors the approach our recombinant *L. lactis* took to treat *H. pylori* induced dysbiosis in the mice. In the same study, it was shown that the recombinant *L. lactis* releasing Pancreatitis-Associated Protein 1 (PAP) had a better result in restoring gut microbiome diversity and reduce the proliferation of a key dysbiotic family *Enterobacteriaceae* when compared to native *L. lactis* after a 5-fluorouracil (5-FU) induced dysbiosis (Carvalho et al., 2018). Similar results were seen in using Hsp65 releasing *L. lactis* to alter dysbiotic microbiota composition in arthritis-induced mice and especially increasing the abundance of key beneficial genus like the *Lactobacillus* (Gusmao-Silva et al., 2020), something we have also prominently observed in our study. In either study, the recombinant *L. lactis* had a better therapeutic impact on the dysbiosis over either native bacterium or cloned with empty vector, as in our experiment. On the other hand, the native strain of *L. lactis* were seen to be on par with its bioengineered counterpart (that releases PAP) in preventing intestinal dysbiosis and acts equally protective against 5-FU induced epithelial mucositis in mice when used as a prophylaxis (Carvalho et al., 2017). This is again reflected in our findings as both AMP- *L. lactis* and empty vector-*L. lactis* had protective action against *H. pylori* induced dysbiosis like the gAMP-*L. lactis* even though empty vector-*L. lactis* had

poor therapeutic activity and a prognosis similar to the null control group. But in both therapeutic and prophylactic groups, the microbiome diversity (as seen from OTU counts) achieved by the gAMP-*L. lactis* were much higher than any other treatment/prophylaxis group, again strengthening our hypothesis.

The bioengineered *L. lactis* also had a differential impact in the microbiota qualitatively. We could see that on Day 5 of our therapeutic scheme experiment, the mice that have been fed *H. pylori* for 3 days had a marked change in their microbiome diversity in which almost all the genera were either wiped out or reduced substantially except for *Staphylococcus* and *Acinetobacter*. According to prior literature, both these genera are linked to diseased condition in mice and hence the growth in their relative abundance is a major sign of dysbiosis (Zavros et al., 2002, Misawa et al., 2015). Even though the sequencing did not capture the corresponding rise in *H. pylori* level, mainly because the technique is not ideal for quantitative analysis and hence we used the qPCR, this remarkable drop in the abundance of other species may be induced by the conditions created by *H. pylori* infection like increasing stomach fluid pH, erosion of gastric mucosa which serves as a substrate for many gastric flora etc. *Staphylococcus* prefers alkaline environment to acidic and *Acinetobacter* has similar gastric colonization pattern as *H. pylori*, and often these two genera are found as concurrent bacterial flora in samples from patients with *H. pylori* induced hypochlorhydria, dyspepsia and gastritis. *Acinetobacter* causes gastritis and hypergastrinemia, like *H. pylori*, and their coexistence maybe due to their similar nature in sculpting gastric environment like increasing pH, creating inflammation and vacuolation of gastric tissue to enhance their survivability (Zavros et al., 2002). Thus, the rise of these two genera can be seen as an after effect of *H. pylori*

colonization in the mice stomachs. These two genera were also represented in the top 10 genera represented in the 350 samples we sequenced from the mice stomach. Among them were *Lactobacillus, Streptococcus, Muribacter, Cutibacterium etc*, few genera often associated with healthy mice gastric and gut microbiome which often maintains mice gastric pH, lactate levels, metabolite homeostasis etc (Dargahi et al., 2020; Granland et al., 2020; Rocha Martin et al., 2019; Wang et al., 2018). Here we can see that the samples from the mice fed gAMP-*L. lactis* and AMP-*L. lactis* had a significantly better recovery of the relative abundance for these genera than empty *L. lactis*.

The MDI equation predicted the dysbiotic (infected with only *H. pylori*) states of the mice stomach samples with 90% accuracy and hence could be used as a credible yardstick for the purpose. Plotting the MDI score distribution, we saw that the therapeutic administration of AMP and gAMP-*L. lactis* had equivalent response in modulating the score and hence recovering from the dysbiosis. This result perhaps look different from the quantitative diversity outcome we saw from raw OTU count in the stomach samples because of the nature of the MDI equation. Since there were only 2 key marker genera that increased in abundance during dysbiosis, the change in their abundance had an exaggerated influence on the MDI score. And since there were 8 different genera in the denominator of the equation, the MDI score were less sensitive towards the changes in their abundance. Also, the MDI score entirely depends on the changes in abundance of the 10 most significantly and concurrently linked genera across the samples, it does not consider the net diversity or OTU or genera count exhibited by the samples. Therefore, the MDI scores of the samples treated with AMP-*L. lactis*, gAMP-*L. lactis* and even empty vector-*L. lactis* may appear similar due to the similar abundance level of mostly the two genera –

Staphylococcus and *Acinetobater* and not the overall diversity of microbiome in that sample which is far superior for the gAMP-*L. lactis* group. The MDI score should be treated only as a narrowly applicable measure of the *H. pylori* induced dysbiosis created by rapid proliferation of the two aforementioned genera and not the overall microbial health of the mouse stomach.

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CHAPTER FIVE

Conclusions

Microbial infection has been inflicted upon humanity for millennia and before the early 20th century we had no viable means of combating these infections. Common bacterial infections, such as whooping cough, scarlet fever, tetanus fever, meningitis, cholera, septicemia, puerperal fever had an alarming mortality rate in the 18th century and 19th century, which went as high as 80% for cholera or 60% for tuberculosis (Erdem et al., 2011; Runcie, 2015). From the sulfonamides of the 1930s, to the advent of penicillin in the 1940s and the antibiotic development boom of the 1950s and 60s (Powers, 2004), the introduction of antibiotics has led to generations that have forgotten the high death rates of the past due to bacterial diseases. However, for every new antibiotic introduced, resistant strains are seen clinically within just a few years (Ventola, 2015). This has been managed by the introduction of newly discovered antibiotics, but no new classes of antibiotics have been discovered since the 1970s, with only variants within the presently known classes being brought into production (Conly and Johnston, 2005). The development of resistance to antibiotics is well-exemplified by the standard *H. pylori* treatment, which now requires three antibiotics and an antibiotic adjuvant to deal with resistant strains found in the clinic. The long-term outlook for the control of bacterial disease using antibiotics is not optimistic, with some foreseeing a "post-antibiotics era" in the near future.

A second problem with antibiotics is their broad-spectrum activity. Though this is appreciated by physicians since there is no need to identify the genus of pathogen causing

the patient's disease, there are adverse health consequences with this approach. Broad-spectrum antibiotics typically end up arbitrarily killing many members of the native microbiota ("Duration of antibiotic therapy and resistance | NPS MedicineWise," n.d.; Gerber et al., 2017), many of which are beneficial and some of which are essential key species in the microbial ecosystem and often determine the survivability and quality of life for the host organism (Langdon et al., 2016). Wanton killing of non-targeted bacterial species thins out the community and often screens for mutant species of deleterious bacteria or opportunistic bacteria (Langdon et al., 2016) that have the rare mutation that makes them resistant to the antibiotic that has been administered. These mutant strains that have resistance against the antibiotic used often harbor such mutations at the expense of other genes that enhance any bacteria's survivability in a regular ecosystem, and hence are often poorer fit to the niche (Ferri et al., 2017). Furthermore, these resistance genes can be horizontally transmitted to other bacterial species, expanding the population of deleterious bacteria and creating a long-term bank of genetic resistance within the patient's microbiota (Gyles and Boerlin, 2014).

Since the indiscriminate use of antibiotics has forced many conventional antibiotics into obsolescence, many researchers have abandoned the idea of using small molecule drugs to combat microbes and instead look towards other approaches that evolution has provided to prevent infections. Among these innate antimicrobial arsenals, antimicrobial peptides are commonly found in a wide variety of taxa and operate in a multitude of simple but effective mechanisms that bacteria often find hard to develop resistance against compared to conventional antibiotics. In this dissertation, my work was to find out a way of making these naturally-provided antimicrobial peptides feasible to use, making them

target towards a certain pathogen by using a guiding moiety, ensuring the survivability of other species of bacteria in the native microbiota and making it possible to deliver peptide molecules in the gut of organisms by circumventing gastric degradation by cloning them into a probiotic bacterium that would release the peptides directly in the stomach.

It should be noted that guided antimicrobial peptides have been a technology in search of an application, since there are roadblocks to all forms of delivery to the patient. At present, peptide/protein drugs provided by injection are reserved for only the most essential, such as insulin, or expensive applications, such as cancer immunotherapy (Boohaker et al., 2012) . This being said, antibody drugs are the largest source of income for the large pharmacological companies due to the high price that they command (Dolgin, 2018) . However, to compete against inexpensive and widely commercialized antibiotics, antimicrobial peptides would have to come in at a low price point and receive broad FDA approval for use as an injectable. Antibiotics are also commonly available in pill form. Delivery to the bloodstream via oral intake is not possible for a peptide drug, since molecules as large as peptides cannot be effectively passed from the gut to the bloodstream (Verma et al., 2021) . Topical use would be more appropriate then, but, often, as in the case of *Staphylococcus aureus* infection of the skin, some form of injection is also required (Vingsbo Lundberg and Frimodt-Møller, 2013). Thus, delivery to the gastrointestinal tract for infections local to these organs is the only remaining viable application for gAMPs. However, the degradation of peptide drugs in the gastrointestinal tract has been a roadblock for the use of orally-delivered peptides drugs (Verma et al., 2021).

In this dissertation, I hope to show that the obstacles to the use of antimicrobial peptides to treat bacterial diseases of the gastrointestinal tract may now be largely

overcome using the results of this dissertation and current work in progress in the Kearney Lab. The broad-spectrum activity of antimicrobial peptides, potentially resulting in a disruption of the microbial community similar to that seen with antibiotics, has been overcome by the use of guide peptides. The problem of degradation of peptides by the gastrointestinal tract has been overcome by delivery using probiotics. The final problem of the development of resistance is the current focus of the Kearney Lab, and I will discuss this solution briefly at the end of this chapter.

In Chapter Two, I demonstrated that guide peptides other than the type previously published in the literature can be utilized to create effective guided antimicrobial peptides, broadening the pathway to new guide peptide discovery. In the literature, the guide peptides used were largely derived from bacterial pheromones that are responsible for the recruitment of bacteria of a single species to form protective biofilms (Eckert et al., 2006; Mao et al., 2013). In the work of Chapter Two, I have demonstrated that guide peptides randomly developed via biopanning can serve as successful guide peptides as well. I successfully modified the targeting of the AMPs plectasin and eurocin against two staphylococcal bacteria by essentially eliminating the activity against the four off-target bacteria tested. Of these four off-target species, *E. faecalis* and *B. subtilis* are an important part of human gut commensal fauna and *L. lactis* and *L. rhamnosus* are commonly consumed probiotic bacteria used to improve gut health. A similar outcome was achieved against *S. aureus* by Mao *et al.* (2013) with the use of a bacterial pheromone peptide for targeting of plectasin. In our research we not only achieved a lower MIC for the unmodified plectasin itself, but also exhibited a much stronger degree of reduction in the activity against the off-target bacteria, *E. faecalis* and *B. subtilis* than was reported by Mao *et al*.

(2013). Thus, we have demonstrated that a guide peptide, that was derived through biopanning, works as efficiently as a pheromone-derived guide peptide, which has been the only class of guide peptide used by researchers to create gAMPs to date. It should also be noted that the pheromone guide was more specific than the biopanning-derived guide, with activity against *S. aureus* but not *S. epidermis*, while biopanning-derived guide was highly active against both species. Thus, guide peptides can have different breadths of coverage.

In order to block the development of resistance in bacteria, both targeting peptides and antimicrobial peptides need to be discovered and developed. We have further worked with targeting peptides derived from viruses and other sources to determine if these sequences also confer the desired differential activity to AMPs. In addition, we have developed algorithms for the classification of highly stable small peptide structures, such as sequential tri-disulfide peptides (STPs) (Islam et al. 2015) and, at a finer grain, STPs which possess antimicrobial activity (Islam et al. 2017). Using these methods and tools, it may be possible to generate a large number of targeting peptide/AMP permutations to serve as a bank for a multi-AMP approach to treating bacterial infections in order to avoid the development of bacterial resistance.

This led me to the development of the multimerin 1 fragment (MM1) guide peptide, which are detailed in Chapter Three. This guide peptide exhibited a higher specificity towards its target, *H. pylori*, as expected from a peptide derived from a receptor protein which itself is the target of the VacA 1 toxin, which specifically evolved to bind to this sequence on this receptor (Satoh et al., 2013, Foegeding et al., 2016; Ilver et al., 2004; Voss et al., 2014). In Chapter Three, I went further than testing with purified peptides and instead

investigated the possibility of using probiotic bacteria to express the guided antimicrobial peptide (gAMP), in anticipation of the in vivo tests in mouse stomach. Co-culturing *H. pylori* with the probiotic *L. lactis* expressing a gAMP consisting of MM1 and alyteserin showed strong kill of *H. pylori*, far stronger than probiotic expressing the guide-less AMP. On the other hand, though this trend was apparent with the other two AMPs tested, the difference in toxicity between gAMP and AMP was not statistically significant. With off-target bacteria, no variation was seen between the three AMPs tested. The MM1 guide significantly reduced the toxicity against off-target bacteria for all three AMPs. A valuable lesson learned from the results of Chapter Three is that a drug discovery phase for gAMPs needs to include a search for the right AMP partner as well as the right guide partner.

The conferring of specificity on an actuator molecule by a guide peptide is not without precedent. Antibodies are commonly used as guides for drug targeting in cancer therapy technologies to activate the activity of a toxin against a particular cancer cell type (Kreitman, 2001, Attarwala, 2010). Conventional cancer immunotoxins consists of a MAb chemically conjugated to a toxin which is mutated or chemically modified to minimize binding to normal cells, similar to our purpose of binding a guide peptide to an already established toxin for bacteria (Kreitman, 2001, Allahyari et al., 2017). This has also been utilized in building drug-carrying nanoparticles (Tietjen et al., 2018, Jeong et al., 2018) or RNAi therapy (Abdelaal and Kasinski, 2021) or even chemical drug molecules (Chen et al., 2017), where guide peptides are used to shield off-target cells from the toxin/ active molecules and reduce their activity to such off-target cells or receptors without affecting their actions against the desired target cell or receptor.

In Chapter Four, we reproduced the same therapy developed in the previous chapter and applied it in vivo to see its efficacy in a more complex physiological setup. The therapy did unexpectedly well in reducing the load of *H. pylori* in mouse stomach and moreover, it averted microbial dysbiosis by preserving and enhancing the biodiversity of their native microbiome. *L. lactis* cloned with both AMP and gAMP were effective against *H. pylori* in mice stomach, reducing the load by as much as 800 and 1860-fold respectively at the end of the experiment when compared to the null control and by as 370 and 860-fold when compared to the empty vector group. This shows that both gAMP and AMP delivered by probiotics to the complex ecology of the mouse stomach were exceedingly effective against *H. pylori* as was seen in the more simplified in vitro co-culture studies in the previous chapter. These results exceed other antimicrobial peptide studies such as Volzing et al (2013) which had reduction levels at around 20-fold compared to the control after 14 hrs post-inoculation. But since that study was in vitro, it does not approximate the conditions of an animal subject, we can look at the most important pioneering work on antimicrobial peptides, Mygind et al (2005) and their exploration of plectasin. In that study, the intraperitoneal injection of plectasin in mice saw a reduction of *S. pneumoniae* CFUs by a 1000-fold and similar levels of reduction of infection in their lungs. So compared to an antimicrobial peptide injected directly into subjects, my approach of using bioengineered probiotics to deliver them orally performed substantially well. Also our approach avoids directly injecting peptides which often has immunogenic reactions associated with it. And since the reduction of *H. pylori* was significant compared to both the null control and *L. lactis* cloned with empty vector which proves that the action of eliminating *H. pylori* was

done exclusively by the AMP/gAMPs being released and not just by the presence of the probiotic *L. lactis* bacteria.

The bioengineered probiotic delivering gAMP not only acted against *H. pylori* infection but also preserved and even boosted the diversity of the stomach native microbiota.. *H. pylori* infection led to a remarkable drop in the abundance of bacterial species possibly due to increasing of the stomach fluid pH, or the erosion of gastric mucosa which serves as a substrate for many gastric flora. The genera experiencing the greatest decrease were *Lactobacillus*, *Streptococcus*, *Muribacter*, and *Cutibacterium*, genera which are often associated with healthy mice gastric and gut microbiome (Dargahi et al., 2020; Granland et al., 2020; Rocha Martin et al., 2019; Wang et al., 2018). The relative abundance of these key bacterial species before and after *H. pylori* infection was used to create a Microbial Dysbiosis Index, which predicted the state of dysbiosis of the mice stomach samples with 90% accuracy. The MDI was modeled after similar indices used in previous microbiome studies that measure dysbiosis created by inflammatory diseases that creates an imbalance in the native microbiota (Gevers et al., 2014; Vázquez-Baeza et al., 2016). Such dysbiosis can also be attributed to antibiotic use (Aguilera et al., 2015; Strati et al., 2021; Vangay et al., 2015) and as well as the cause or a precursor of debilitating infections that completely change the native microbial landscape (Bien et al., 2013). The therapy described in this dissertation would be able to combat an overwhelming bacterial infection and also improve dysbiosis created by such infection and could be seen as a strong candidate for therapies in the post-antibiotic era. Not only did the MDI score distribution demonstrate that probiotic delivery of either AMP or gAMP lead to a recovery from *H. pylori*-induced dysbiosis, but the use of gAMP probiotics greatly increased the taxonomic

diversity of the entire microbiota, as seen by OTU analysis. This reflects the in vitro results of Chapter Three, in which probiotics expressing gAMP or AMP had a much attenuated effect on off-target bacteria while specifically killing *H. pylori*.

The delivery of the peptide is another roadblock that needs to be addressed before gAMPs can be used as standard drugs. Encapsulation methods like liposome or amphiphilic nanoparticles often circumvent gastric, enteric, or systemic degradation of peptides but parenteral administration of any peptide often carries the risk of anaphylaxis. As an alternative, probiotic delivery has the advantage of avoiding the expense of heterologous protein production and purification. Probiotics bioengineered to release peptides such as interleukins in the gut have been already explored and adopted as a viable healthcare approach for diseases like Crohn's disease, ulcerative colitis etc both as a therapeutic (Steidler et al., 2003, 2000, 1998; Vandenbroucke et al., 2010) and as a prophylactic (Bermúdez-Humarán et al., 2003; Foligne et al., 2007). *L. lactis* also has the distinction of being the first genetically engineered microorganism that has been approved by FDA for delivering therapeutic agents and has become a platform of choice for companies and research groups. Currently it is being used in at least two preclinical studies for combating Crohn's diseases through delivering interleukin-10 (Braat et al., 2006) and chemotherapy induced oral mucositis by delivering human trefoil factor 1 (Limaye et al., 2013). Pharmaceutical companies like Precigen has also been exploring *L .lactis* as a platform for therapeutic treatment of Type 1 diabetes, which is currently in Phase 2a clinical trial under the name AG019 ActoBiotics™ (ActoBio, 2021), and also as a treatment for Celiac disease, currently under preclinical trial. Even, so, the use of probiotics in delivering antimicrobial peptides to the gut has been rarely seen (Borrero et al., 2015; Volzing et al.,

2013). The research expounded in this dissertation now demonstrates that it would be feasible to not only create gAMPs that show specific killing potential towards the targeted pathogen in vitro but also cloning them into probiotics like *L. lactis* would ensure they would survive the journey into the stomach and show antimicrobial activity in situ. This is essential to achieve recovery from infectious agents in the stomach or gut without harming the rich and active and fragile commensal microbial ecosystem in those regions which are not only beneficial but almost essential for the well being of our gut health.

A possible pitfall with the probiotic delivery approach is consistency of dosage in such therapeutic regimen. Once the probiotic has been delivered to the target organ, there is no control over the degree of colonization or payload gene expression. In order to avoid under or over release of such peptides from the probiotics, a genetic kill switch could be employed that will ensure the cessation of expression of such peptides once certain levels are reached or even stop the propagation of the bacteria itself so as to maintain a desired level in the gut. Imaging techniques using IR spectroscopy on luciferin illumination or similar approaches could be used to monitor the level of the bacteria growth and gene expression in the stomach or gut in order to optimize dosage or the time point for terminating the current regimen. Such synthetic biology techniques would add another degree of safety and control to the delivery of gAMPs via probiotics.

The ability to selectively eliminate a targeted pathogen while preserving the native microbiota can bring positive health outcomes not seen in current antimicrobial treatment strategies. Targeted elimination of pathogens such as *H. pylori*, *Clostridium. difficile* or *Fusobacterium nucleatum* could eliminate inflammation long term if the native microbiota were also preserved, unlike the use of broad-spectrum antibiotics. Nonintrusive probiotic

treatments might avoid the more expensive treatment of the consequences of gut inflammation, such as inflammatory bowel diseases (Monaghan et al., 2017) or colon cancer (Kostic et al., 2013). Identification of key species that acts as the lynch pin of the native microflora is essential for this and can pave a way for future researchers to engineer complex microbiomes without resorting to using often inconsistent therapy routes like fecal microbiota transplant (Merrick et al., 2020). A gAMP delivery technology based on a safe and highly tested and curated probiotic platform like *L. lactis* could be a more patient-acceptable option for microbiome engineering. The market for probiotic supplements and other products already exists and *L. lactis* strains have already been approved by the FDA for clinical use as a delivery platform of therapeutic molecules. Also, the ease of incorporating such probiotics into easily consumable forms like yogurts or aqueous suspensions or plain gelatin capsules may increase consumer acceptance. Since such formulations can be easily upscaled in production and distribution for minimum expenditure, there would be the opportunity for deployment in developing countries of Asia and Africa, where gut and stomach infections like *H. pylori* are much of a bigger menace than the Western world. In the western world, bioengineered probiotics have already been experiencing sound market acceptability including the recent bioengineered *B. subtilis* formulation being pioneered by the Zbiotics company, a probiotic that synthesizes aldehyde dehydrogenase enzyme in the gut to breakdown the acetaldehyde bioproduct after alcohol consumption, reducing the metabolic load for the liver and the morning-after "hangover" associated with alcohol overuse.

We now return to the final major detriment of antibiotics, which is the development of resistant pathogen strains, making the antibiotic obsolescent. Though not addressed in

this dissertation, the Kearney Lab is exploring the possibility of using this natural evolution of pathogens towards resistance to sculpt pathogen populations towards an avirulent state. In essence, the nature of the gAMP therapy may actually turn bacterial resistance into a useful tool. Since gAMP therapy involves targeting the vacA toxin with the MM1 fragment, the only way for the pathogen to develop resistance would be to modify the sequence of that very toxin. Since VacA is a prime pathogenicity factor for *H. pylori*, any evolutionary change that modifies VacA sequence or expression would be expected to cause a loss of virulence. Research is currently underway to study the evolution of *H. pylori* under the exposure of sublethal gAMP doses to see whether the functionality or expression of vacA toxin is attenuated. Treatment with gAMP probiotics would be well-suited to the prevention of inflammation and gastric cancer if a long-term evolutionary pathway away from virulence can be established for *H. pylori*. This would also demonstrate a path forward for designing long-term control via gAMPs for other pathogens.

APPENDIX

APPENDIX

Supplemental Material: Repurposing a drug targeting peptide for targeting antimicrobial peptides against *Staphylococcus*

Untitled
Associated Datafile: 2017_01_31_Plec (100 - 2000 amu)
Trypsin:/K-\P /R-\P

Frag#	Res#	Sequence	Theor (Bo)	[M+H]	[M+2H]	[M+3H]
T6	39-40	(K) CY (-)	284.08	285.09	143.05	95.70
T2	21-23	(K) SIK (G)	346.22	347.23	174.12	116.42
T3	24-26	(K) GYK (G)	366.19	367.20	184.10	123.07
T4	27-32	(K) GGYCAK (G)	597.26	598.27	299.64	200.09
T5	33-38	(K) GGFVCK (C)	609.29	610.30	305.66	204.11
T2-3	21-26	(K) SIKGYK (G)	694.40	695.41	348.21	232.47
T5-6	33-40	(K) GGFVCKCY (-)	875.37	876.37	438.69	292.80
T3-4	24-32	(K) GYKGGYCAK (G)	945.44	946.45	473.73	316.15
T4-5	27-38	(K) GGYCAKGGFVCK (C)	1188.54	1189.55	595.28	397.19
T2-4	21-32	(K) SIKGYKGGYCAK (G)	1273.65	1274.66	637.83	425.56
T4-6	27-40	(K) GGYCAKGGFVCKCY (-)	1454.61	1455.62	728.32	485.88
T3-5	24-38	(K) GYKGGYCAKGGFVCK (C)	1536.72	1537.73	769.37	513.25
T3-6	24-40	(K) GYKGGYCAKGGFVCKCY (-)	1804.15	1805.16	903.08	602.39
T2-5	21-38	(K) SIKGYKGGYCAKGGFVC K (C)	1866.24	1867.25	934.13	623.09
■T1	1-20	(-) GFGCNGPWDEDDMQCHN HCK (S)	2293.49	2294.50	1147.75	765.50
T1-2	1-23	(-) GFGCNGPWDEDDMQCHN HCKSIK (G)	2621.90	2622.91	1311.96	874.97
T1-3	1-26	(-) GFGCNGPWDEDDMQCHN HCKSIKGYK (G)	2970.30	2971.31	1486.16	991.11
T1-4	1-32	(-) GFGCNGPWDEDDMQCHN HCKSIKGYKGGYCAK (G)	3549.98	3550.99	1776.00	1184.33

Figure A.1. Theoretical peptide peaks generated by auto-digest simulation with Trypsin in MassLynx v4.1 (up to 3 missed cleavage) matched with MaxEnt3 processed MS^E spectrum data (channel 1) of plectasin, (Peak Mass match freedom: ±0.25 amu)

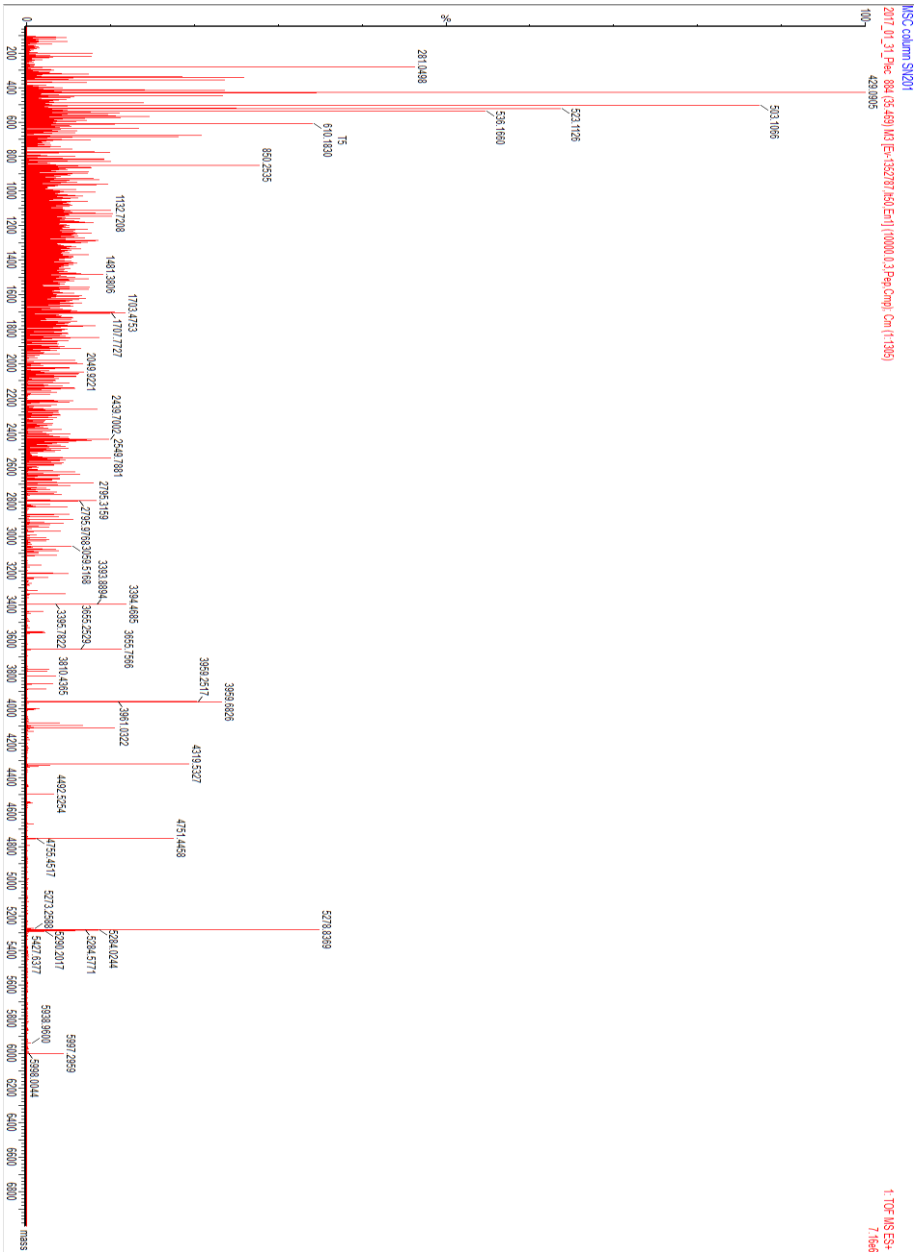

Figure A.2. MaxEnt3 processed MS^E spectrum data of plectasin from LC/MS (channel 1)

Untitled
Associated Datafile: 2017_01_31_Plec (100 - 2000 amu)
Trypsin:/K-\P /R-\P

Frag#	Res#	Sequence	Theor(Bo)	[M+H]	[M+2H]	[M+3H]
T6	39-40	(K)CY(-)	284.08	285.09	143.05	95.70
T2	21-23	(K)SIK(G)	346.22	347.23	174.12	116.42
T3	24-26	(K)GYK(G)	366.19	367.20	184.10	123.07
T4	27-32	(K)GGYCAK(G)	597.26	598.27	299.64	200.09
T5	33-38	(K)GGFVCK(C)	609.29	610.30	305.66	204.11
T2-3	21-26	(K)SIKGYK(G)	694.40	695.41	348.21	232.47
T5-6	33-40	(K)GGFVCKCY(-)	875.37	876.37	438.69	292.80
T3-4	24-32	(K)GYKGGYCAK(G)	945.44	946.45	473.73	316.15
T4-5	27-38	(K)GGYCAKGGFVCK(C)	1188.54	1189.55	595.28	397.19
■ T1	1-20	(-)GFGCNGPWDEDDMQCHN HCK(S)	2293.49	2294.50	1147.75	765.50
T1-2	1-23	(-)GFGCNGPWDEDDMQCHN HCKSIK(G)	2621.90	2622.91	1311.96	874.97

Figure A.3. Theoretical peptide peaks generated by auto-digest simulation with Trypsin in MassLynx v4.1 (up to 1 missed cleavage) matched with Centered MS^E spectrum data (channel 1) of plectasin, (Peak Mass match freedom: ±0.05 amu)

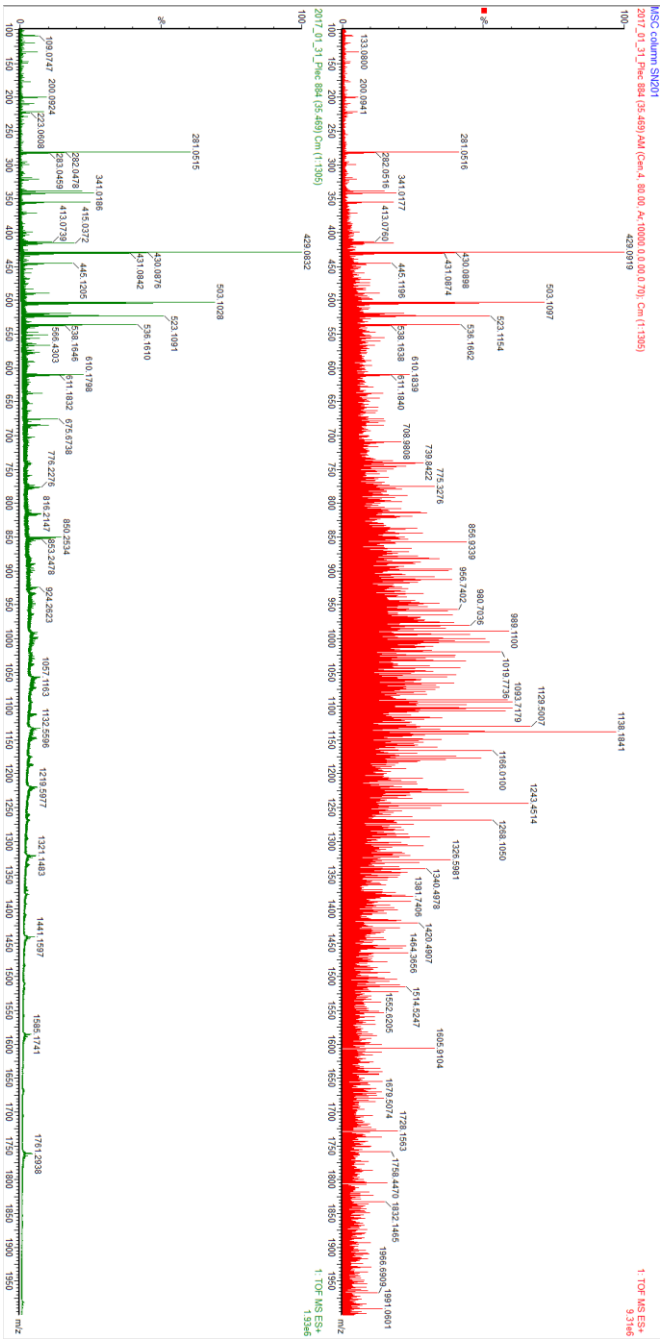

Figure A.4. Centered (red) and non-centered (green) MS^E spectrum data of plectasin from LC/MS (channel 1)

Untitled
Associated Datafile: 2017_01_31_A12C_Plec (100 - 2000 amu)
Trypsin:/K-\P /R-\P

Frag#	Res#	Sequence	Theor (Bo)	[M+H]	[M+2H]	[M+3H]
T7	57-58	(K) CY (-)	284.08	285.09	143.05	95.70
T3	39-41	(K) SIK (G)	346.22	347.23	174.12	116.42
T4	42-44	(K) GYK (G)	366.19	367.20	184.10	123.07
T5	45-50	(K) GGYCAK (G)	597.26	598.27	299.64	200.09
T6	51-56	(K) GGFVCK (C)	609.29	610.30	305.66	204.11
T3-4	39-44	(K) SIKGYK (G)	694.40	695.41	348.21	232.47
T6-7	51-58	(K) GGFVCKCY (-)	875.37	876.37	438.69	292.80
T4-5	42-50	(K) GYKGGYCAK (G)	945.44	946.45	473.73	316.15
■T1	1-10	(-) GVHMVAGPGR (E)	979.50	980.51	490.76	327.51
T5-6	45-56	(K) GGYCAKGGFVCK (C)	1188.54	1189.55	595.28	397.19
T3-5	39-50	(K) SIKGYKGGYCAK (G)	1273.65	1274.66	637.83	425.56
T5-7	45-58	(K) GGYCAKGGFVCKCY (-)	1454.61	1455.62	728.32	485.88
T4-6	42-56	(K) GYKGGYCAKGGFVCK (C)	1536.72	1537.73	769.37	513.25
T4-7	42-58	(K) GYKGGYCAKGGFVCKCY (-)	1804.15	1805.16	903.08	602.39
T3-6	39-56	(K) SIKGYKGGYCAKGGFVCK (C)	1866.24	1867.25	934.13	623.09
T2	11-38	(R) EPTGGGHMGFGCNGPWDEDDMQCHNHCK (S)	3060.32	3061.33	1531.17	1021.11
T2-3	11-41	(R) EPTGGGHMGFGCNGPWDEDDMQCHNHCKSIK (G)	3388.73	3389.74	1695.37	1130.59
T2-4	11-44	(R) EPTGGGHMGFGCNGPWDEDDMQCHNHCKSIKGYK (G)	3737.13	3738.14	1869.58	1246.72
T1-2	1-38	(-) GVHMVAGPGREPTGGGHMGFGCNGPWDEDDMQCHNHCK (S)	4022.46	4023.47	2012.24	1341.83
T2-5	11-50	(R) EPTGGGHMGFGCNGPWDEDDMQCHNHCKSIKGYKGGYCAK (G)	4316.81	4317.82	2159.41	1439.95
T1-3	1-41	(-) GVHMVAGPGREPTGGGHMGFGCNGPWDEDDMQCHNHCKSIK (G)	4350.88	4351.88	2176.45	1451.30
T1-4	1-44	(-) GVHMVAGPGREPTGGGHMGFGCNGPWDEDDMQCHNHCKSIKGYK (G)	4699.28	4700.29	2350.65	1567.43

Figure A.5. Theoretical peptide peaks generated by auto-digest simulation with Trypsin in MassLynx v4.1 (up to 3 missed cleavages) matched with MaxEnt3 processed MS^E spectrum data (channel 1) of A12C-plectasin, (Peak Mass match freedom: ±0.25 amu)

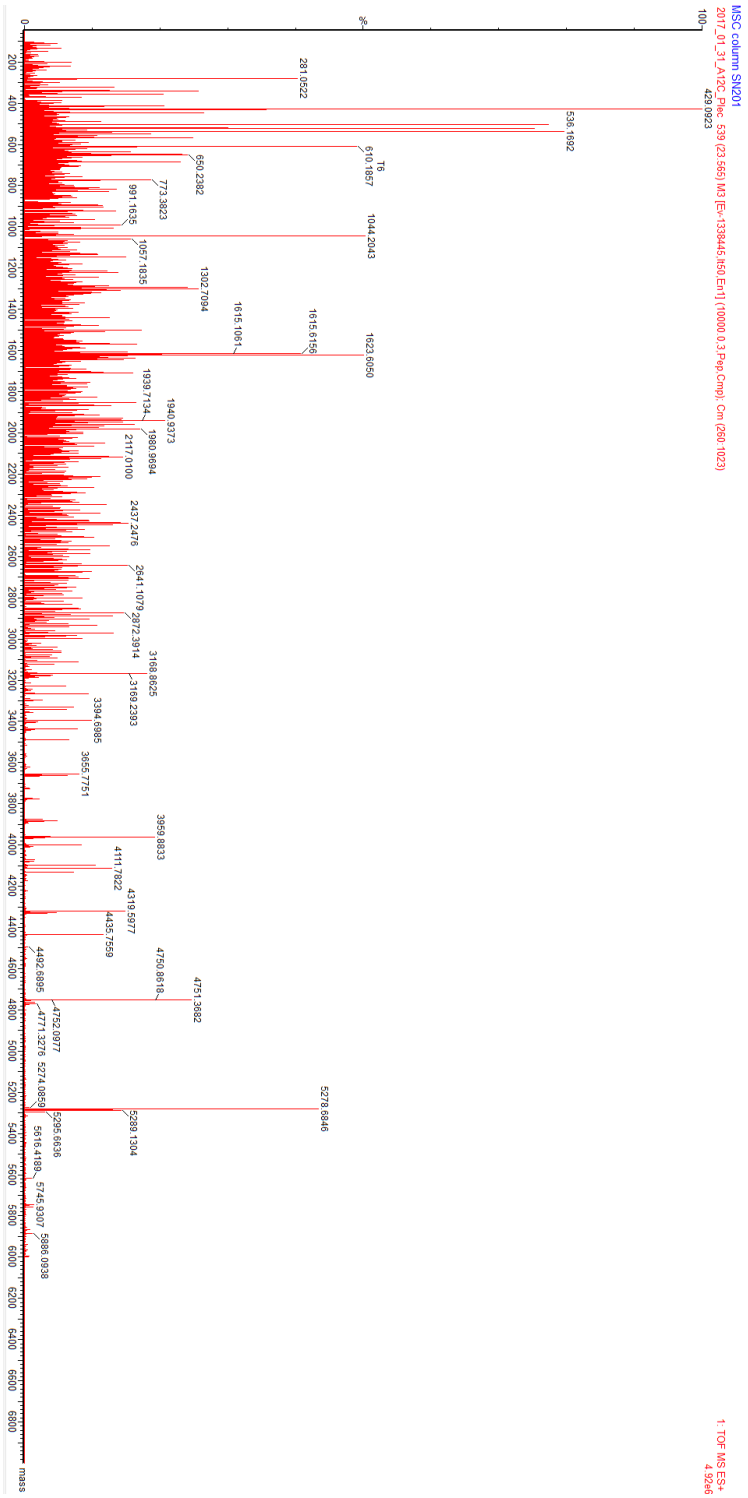

Figure A.6. MaxEnt3 processed MS^E spectrum data of A12C-plectasin from LC/MS (channel 1)

Untitled
Associated Datafile: 2017_01_31_A12C_Plec (100 - 2000 amu)
Trypsin:/K-\P /R-\P

Frag#	Res#	Sequence	Theor(Bo)	[M+H]	[M+2H]	[M+3H]
T7	57-58	(K)CY(-)	284.08	285.09	143.05	95.70
T3	39-41	(K)SIK(G)	346.22	347.23	174.12	116.42
T4	42-44	(K)GYK(G)	366.19	367.20	184.10	123.07
T5	45-50	(K)GGYCAK(G)	597.26	598.27	299.64	200.09
T6	51-56	(K)GGFVCK(C)	609.29	610.30	305.66	204.11
T3-4	39-44	(K)SIKGYK(G)	694.40	695.41	348.21	232.47
T6-7	51-58	(K)GGFVCKCY(-)	875.37	876.37	438.69	292.80
T4-5	42-50	(K)GYKGGYCAK(G)	945.44	946.45	473.73	316.15
■T1	1-10	(-)GVHMVAGPGR(E)	979.50	980.51	490.76	327.51
T5-6	45-56	(K)GGYCAKGGFVCK(C)	1188.54	1189.55	595.28	397.19
T2	11-38	(R)EPTGGGHMGFGCNGPWDEDDMQCHNHCK(S)	3060.32	3061.33	1531.17	1021.11
T2-3	11-41	(R)EPTGGGHMGFGCNGPWDEDDMQCHNHCKSIK(G)	3388.73	3389.74	1695.37	1130.59
T1-2	1-38	(-)GVHMVAGPGREPTGGGHMGFGCNGPWDEDDMQCHNHCK(S)	4022.46	4023.47	2012.24	1341.83

Figure A.7. Theoretical peptide peaks generated by auto-digest simulation with Trypsin in MassLynx v4.1 (up to 1 missed cleavage) matched with Centered MS^E spectrum data (channel 1) of A12C-plectasin, (Peak Mass match freedom: ±0.05 amu)

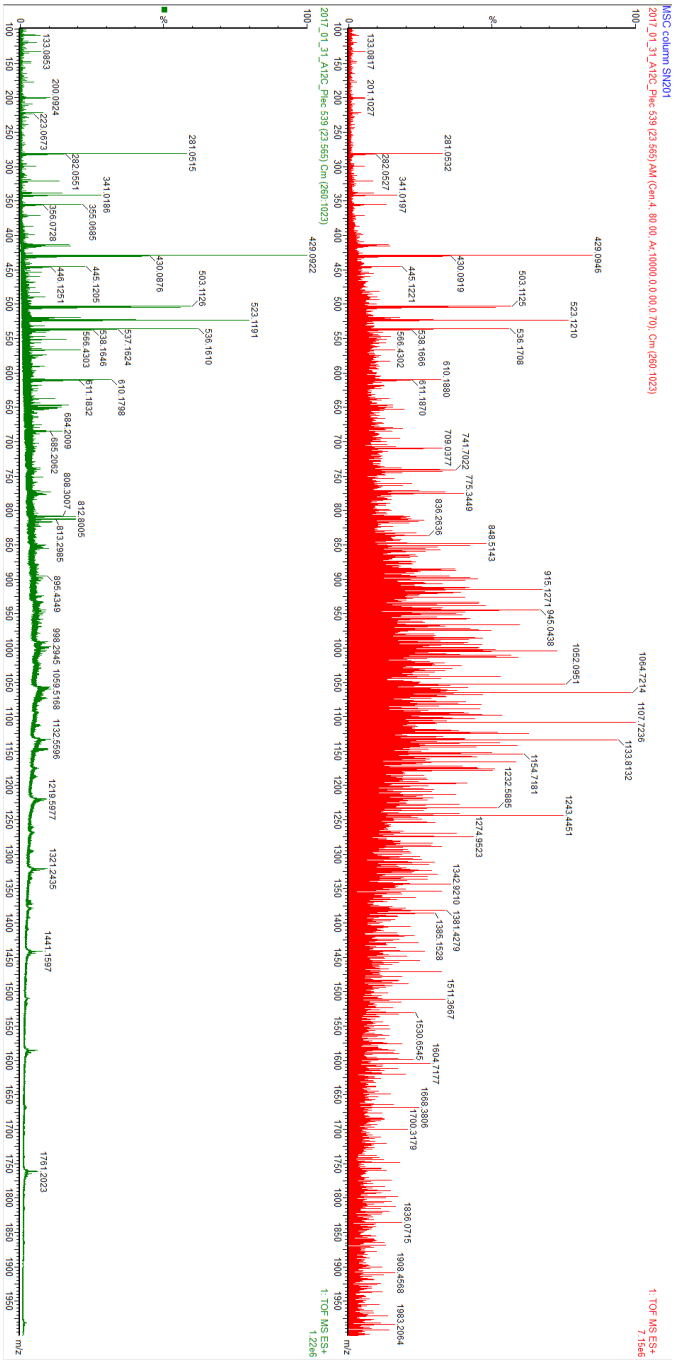

Figure A.8. Centered (red) and non-centered (green) MS^E spectrum data of A12C-plectasin from LC/MS (channel 1)

Untitled
Associated Datafile: 2017_01_31_Euro (100 - 2000 amu)
Trypsin:/K-\P /R-\P

Frag#	Res#	Sequence	Theor(Bo)	[M+H]	[M+2H]	[M+3H]
T2	17-22	(R)ALGGGR(T)	529.30	530.31	265.66	177.44
T1	1-16	(-)GFGCPGDAYQCSEHCR(A)	1728.64	1729.65	865.33	577.22
T3	23-42	(R)TGGYCAGPWYLGHPTCT CSF(-)	2121.41	2122.41	1061.71	708.14
T1-2	1-22	(-)GFGCPGDAYQCSEHCRA LGGGR(T)	2241.48	2242.49	1121.75	748.17
T2-3	17-42	(R)ALGGGRTGGYCAGPWYL GHPTCTCSF(-)	2632.99	2634.00	1317.50	878.67
T1-3	1-42	(-)GFGCPGDAYQCSEHCRA LGGGRTGGYCAGPWYLGHPT CTCSF(-)	4344.87	4345.88	2173.44	1449.30

Figure A.9. Theoretical peptide peaks generated by auto-digest simulation with Trypsin in MassLynx v4.1 (up to 3 missed cleavages) matched with MaxEnt3 processed MS^E spectrum data (channel 1) of eurocin, (Peak Mass match freedom: ±0.25 amu)

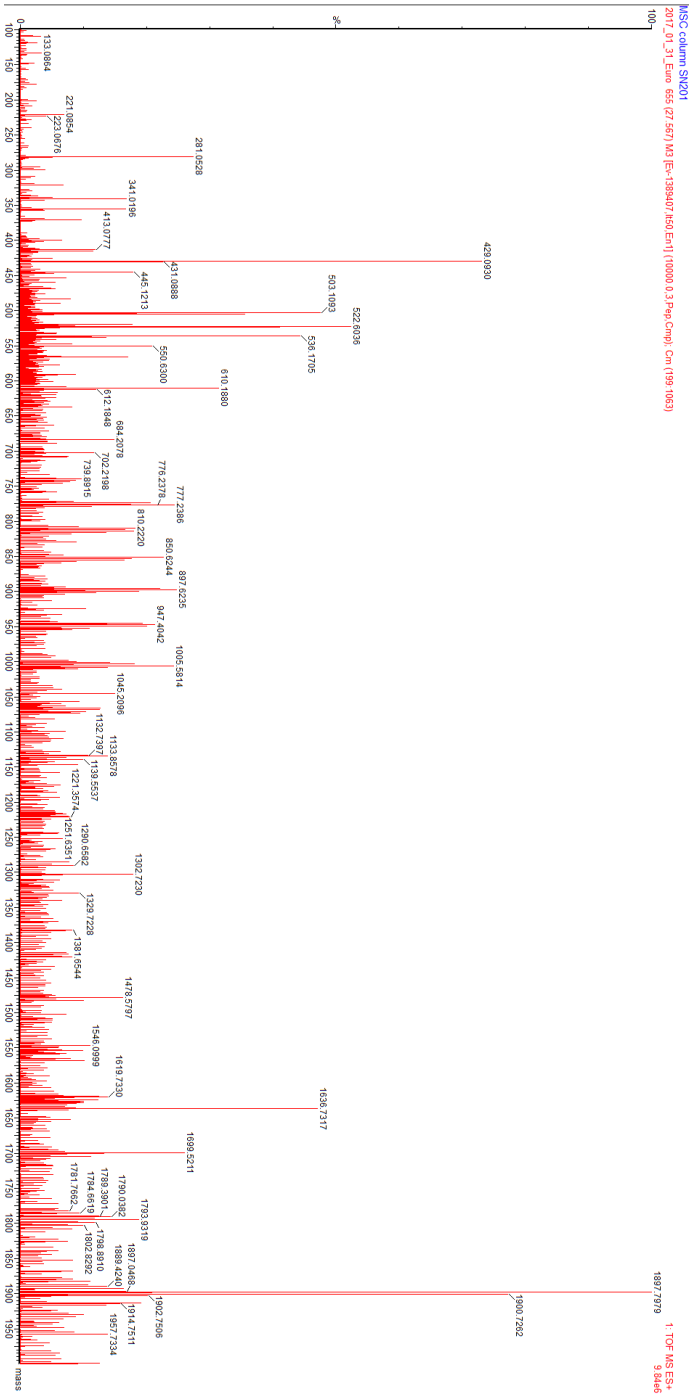

Figure A.10. MaxEnt3 processed MS^E spectrum data of eurocin from LC/MS (channel 1)

Untitled
Associated Datafile: 2017_01_31_Euro (100 - 2000 amu)
Trypsin:/K-\P /R-\P

Frag#	Res#	Sequence	Theor(Bo)	[M+H]	[M+2H]	[M+3H]
T2	17-22	(R)ALGGGR(T)	529.30	530.31	265.66	177.44
T1	1-16	(-)GFGCPGDAYQCSEHCR(A)	1728.64	1729.65	865.33	577.22
T3	23-42	(R)TGGYCAGPWYLGHPTCT CSF(-)	2121.41	2122.41	1061.71	708.14
T1-2	1-22	(-)GFGCPGDAYQCSEHCRA LGGGR(T)	2241.48	2242.49	1121.75	748.17
T2-3	17-42	(R)ALGGGRTGGYCAGPWYL GHPTCTCSF(-)	2632.99	2634.00	1317.50	878.67
T1-3	1-42	(-)GFGCPGDAYQCSEHCRA LGGGRTGGYCAGPWYLGHPT CTCSF(-)	4344.87	4345.88	2173.44	1449.30

Figure A.11. Theoretical peptide peaks generated by auto-digest simulation with Trypsin in MassLynx v4.1 (up to 2 missed cleavages) matched with Centered MS^E spectrum data (channel 1) of eurocin, (Peak Mass match freedom: ±0.15 amu)

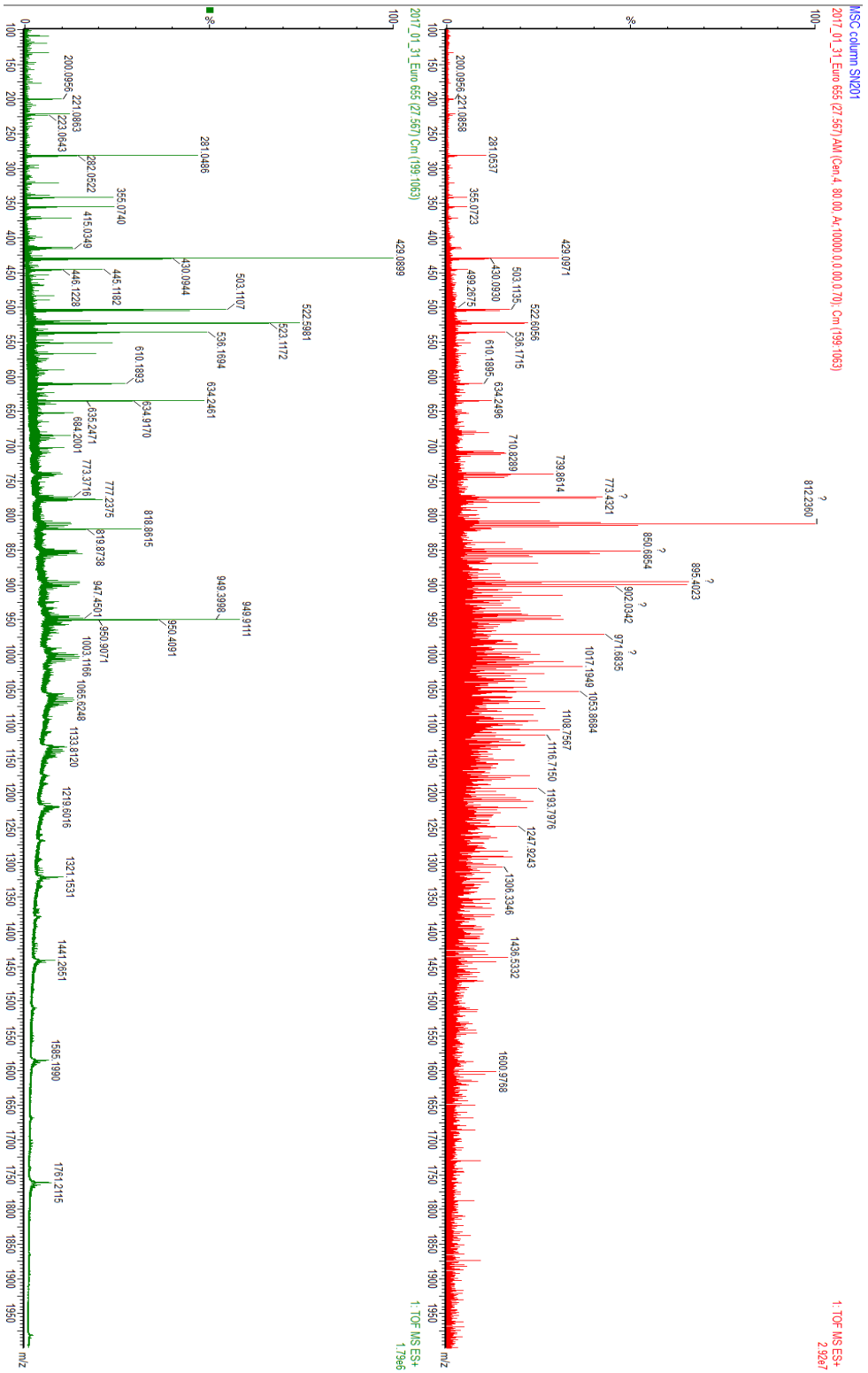

Figure A.12. Centered (red) and non-centered (green) MS^E spectrum data of eurocin from LC/MS (channel 1)

Untitled
Associated Datafile: 2017_01_31_A12C_Euro (100 - 2000 amu)
Trypsin:/K-\P /R-\P

Frag#	Res#	Sequence	Theor(Bo)	[M+H]	[M+2H]	[M+3H]
T3	35-40	(R)ALGGGR(T)	529.30	530.31	265.66	177.44
T1	1-10	(-)GVHMVAGPGR(E)	979.50	980.51	490.76	327.51
T4	41-60	(R)TGGYCAGPWYLGHPTCTCSF(-)	2121.41	2122.41	1061.71	708.14
T2	11-34	(R)EPTGGGHMGFGCPGDAYQCSEHCR(A)	2496.73	2497.74	1249.37	833.25
T3-4	35-60	(R)ALGGGRTGGYCAGPWYLGHPTCTCSF(-)	2632.99	2634.00	1317.50	878.67
T2-3	11-40	(R)EPTGGGHMGFGCPGDAYQCSEHCRALGGGR(T)	3008.31	3009.32	1505.16	1003.78
T1-2	1-34	(-)GVHMVAGPGREPTGGGHMGFGCPGDAYQCSEHCR(A)	3458.87	3459.88	1730.44	1153.97
T1-3	1-40	(-)GVHMVAGPGREPTGGGHMGFGCPGDAYQCSEHCRALGGGR(T)	3970.45	3971.46	1986.24	1324.49
T2-4	11-60	(R)EPTGGGHMGFGCPGDAYQCSEHCRALGGGRTGGYCAGPWYLGHPTCTCSF(-)	5111.70	5112.71	2556.86	1704.91
T1-4	1-60	(-)GVHMVAGPGREPTGGGHMGFGCPGDAYQCSEHCRALGGGRTGGYCAGPWYLGHPTCTCSF(-)	6073.85	6074.85	3037.93	2025.62

Figure A.13. Theoretical peptide peaks generated by auto-digest simulation with Trypsin in MassLynx v4.1 (up to 3 missed cleavages) matched with MaxEnt3 processed MS^E spectrum data (channel 1) of A12C-eurocin, (Peak Mass match freedom: ±0.25 amu)

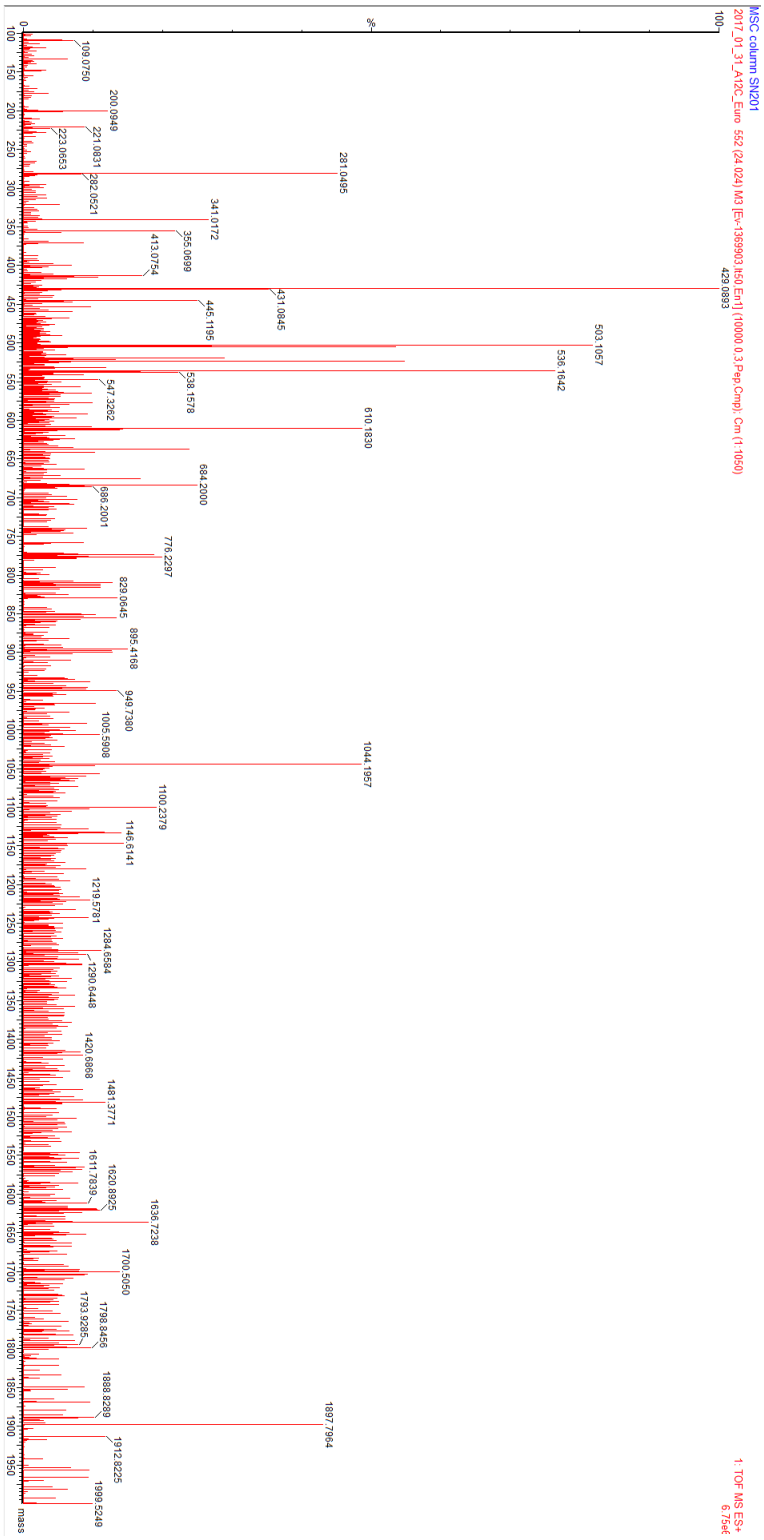

Figure A.14. MaxEnt3 processed MS^E spectrum data of A12C-eurocin from LC/MS (channel 1)

Untitled
Associated Datafile: 2017_01_31_A12C_Euro (100 - 2000 amu)
Trypsin:/K-\P /R-\P

Frag#	Res#	Sequence	Theor(Bo)	[M+H]	[M+2H]	[M+3H]
T3	35-40	(R)ALGGGR(T)	529.30	530.31	265.66	177.44
T1	1-10	(-)GVHMVAGPGR(E)	979.50	980.51	490.76	327.51
T4	41-60	(R)TGGYCAGPWYLGHPTCTCSF(-)	2121.41	2122.41	1061.71	708.14
T2	11-34	(R)EPTGGGHMGFGCPGDAYQCSEHCR(A)	2496.73	2497.74	1249.37	833.25
T3-4	35-60	(R)ALGGGRTGGYCAGPWYLGHPTCTCSF(-)	2632.99	2634.00	1317.50	878.67
T2-3	11-40	(R)EPTGGGHMGFGCPGDAYQCSEHCRALGGGR(T)	3008.31	3009.32	1505.16	1003.78
T1-2	1-34	(-)GVHMVAGPGREPTGGGHMGFGCPGDAYQCSEHCR(A)	3458.87	3459.88	1730.44	1153.97
T1-3	1-40	(-)GVHMVAGPGREPTGGGHMGFGCPGDAYQCSEHCRALGGGR(T)	3970.45	3971.46	1986.24	1324.49
T2-4	11-60	(R)EPTGGGHMGFGCPGDAYQCSEHCRALGGGRTGGYCAGPWYLGHPTCTCSF(-)	5111.70	5112.71	2556.86	1704.91

Figure A.15. Theoretical peptide peaks generated by auto-digest simulation with Trypsin in MassLynx v4.1 (up to 2 missed cleavages) matched with Centered MS^E spectrum data (channel 1) of A12C-eurocin, (Peak Mass match freedom: ±0.10 amu)

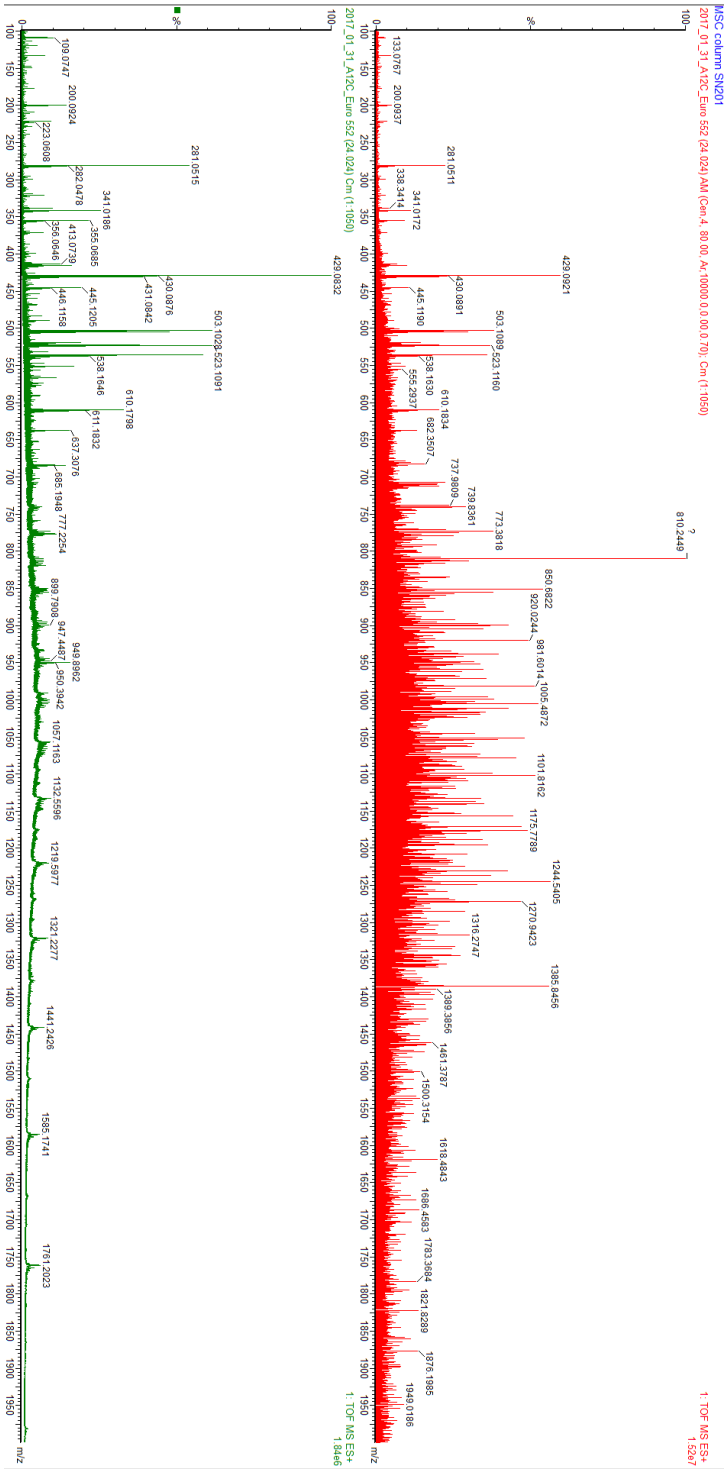

Figure A.16: Centered (red) and non-centered (green) MS^E spectrum data of A12C-eurocin from LC/MS (channel 1)

Table A.1. MIC and log MIC values of the 4 AMPs against the 4 bacteria

Bacteria	Mean (SD) of MIC (µM) (n≥6)				Mean (SD) of log MIC (µM) (n≥6)			
	Plectasin	**A12C-Plectasin**	**p-value**	**AgrD1-Plectasin [p-value]**	**Plectasin**	**A12C-Plectasin**	**p-value**	**AgrD1-Plectasin [p-value]**
Bacillus subtilis	3.49 (1.21)	79.65 (53.1)	0.0020	12.79 (15.71) [0.7856]	0.52 (0.489)	1.83 (0.21)	1.133E-09	1.21 (0.11) [0.1048]
Enterococcus faecalis	6.62 (2.76)	112.83 (61.91)	0.0005	11.69 (12.01) [0.8332]	0.784 (0.18)	1.988 (0.24)	3.769E-08	1.08 (0.17) [0.8755]
Lactococcus lactis	11.50 (6.89)	76.33 (30.95)	0.0004	-	0.97 (0.29)	1.84 (0.21)	1.341E-05	-
Lactobacillus rhamnosus	12.55 (6.08)	126.11 (52.68)	0.0003	-	1.05 (0.21)	2.06 (0.18)	9.257E-08	-
Staphylococcus aureus	19.21 (10.87)	25.60 (13.55)	0.6238	16.12 (7.31) [0.4374]	1.24 (0.25)	1.16 (0.40)	0.7873	1.21 (0.89) [0.7237]
Staphylococcus epidermidis	8.96 (6.32)	7.74 (4.14)	0.7167	15.30 (11.81) [0.1889]	0.83 (0.35)	0.82 (0.24)	0.9558	1.18 (0.65) [0.3544]
Bacteria	**Mean (SD) of MIC (µM) (n≥6)**				**Mean (SD) of log MIC (µM) (n≥6)**			
	Eurocin	**A12C-Eurocin**	**P-value**	**AgrD1-Eurocin [p-value]**	**Eurocin**	**A12C-Eurocin**	**P-value**	**AgrD1-Eurocin [p-value]**
Bacillus subtilis	6.44 (1.59)	65.16 (39.78)	0.0016	15.06 (18.45) [0.3268]	0.79 (0.13)	1.75 (0.21)	7.341E-08	1.18 (0.27) [0.5777]
Enterococcus faecalis	3.03 (1.01)	124.31 (45.54)	2.512E-07	12.56 (11.02) [0.6802]	0.44 (0.2)	2.05 (0.20)	1.563E-12	1.10 (0.42) [0.7923]
Lactococcus lactis	4.26 (2.56)	75.19 (37.17)	0.0007	-	0.53 (0.32)	1.83 (0.20)	5.101E-07	-
Lactobacillus rhamnosus	4.95 (2.56)	52.63 (22.28)	0.0004	-	0.60 (0.32)	1.68 (0.20)	3.562E-06	-
Staphylococcus aureus	11.964 (3.56)	24.06 (16.59)	0.0744	23.18 (23.77) [0.6138]	1.05 (0.14)	1.212 (0.42)	0.3632	1.37 (0.38) [0.8332]
Staphylococcus epidermidis	5.98 (3.98)	8.56 (5.98)	0.3882	18.19 (12.40) [0.4811]	0.67 (0.29)	0.80 (0.37)	0.5442	1.26 (0.39) [0.6830]

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