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A New All-Natural Wound Treatment Gel Shows Strong Inhibitory Activity Against Staphylococcus aureus and Other Wound Pathogens

Tasha K. Nelson
East Tennessee State University

A New All-Natural Wound Treatment Gel Shows Strong Inhibitory Activity Against *Staphylococcus aureus* and Other Wound Pathogens

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by

Tasha K Nelson

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Dr. Sean Fox, Chair

Dr. Ranjan Chakraborty

Dr. W. Andrew Clark

Dr. Rebecca Pyles

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ABSTRACT

A New All-Natural Wound Treatment Gel Shows Strong Inhibitory Activity

Against *Staphylococcus aureus* and Other Wound Pathogens

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Skin related injuries are some of the most dangerous forms of wounds. In addition to treating the wound itself, health care providers must be cautious of microbial infections. In this study, we evaluate a novel all-natural antimicrobial gel compound (AMG) designed to kill planktonic bacteria, penetrate bacterial biofilms, and accelerate wound healing. In -vitro experiments demonstrate that AMG is effective in inhibiting planktonic growth and biofilm development of eight common pathogens. LIVE/DEAD staining and confocal microscopy reveal that planktonic growth and three-dimensional structure of biofilms were significantly reduced. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was used to investigate a small panel of genes (PrsA, Sprx) and showed potential targets for future study. A physiologically relevant wound model was created for treating S. aureus infections by using AMG alone or in combination with a common topical antibiotic, Mupirocin. AMG is a safe and effective treatment option for skin related infection.

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CHAPTER 1. INTRODUCTION

Antibiotic Resistance in Human Medicine

Antibiotic resistance has evolved rapidly over the last 50 years and is a major threat to human health and medical treatments. More than 2.8 million antibiotic- resistant infections occur in the U.S. each year (1) and approximately 700,000 people globally lose their lives to resistant infections (2). This exponentially growing bacterial resistance is predicted to become the greatest challenge faced by health care by the year 2050 (2).

Bacterial species accumulate new genetic mutations or acquire new genetic elements when exposed to antimicrobial compounds. These genetic changes allow bacteria to survive antimicrobial treatments and host immune responses. The evolution of resistant pathogens is of great concern in human medicine and it is well understood that the continued use of antibiotics in human medicine, including clinical therapies, contribute to the increase in resistant microbes through selective pressures (3). Organism-specific, as well as population-based studies, have indicated that various mechanisms contribute to the increase in resistance. In certain cases, resistant variants have become the dominate species even in the absence of antibiotic selective pressures because bacterial species share genetic material through horizontal gene transfer by sharing plasmids encoding resistance genes to other bacteria. This plasmid transfer can occur between similar bacteria or among different species of bacteria.

The mechanisms that bacterial species have evolved to resist antimicrobial attack have been extensively studied and some are well characterized. These include enzymes that degrade antibiotic products, changes in structural conformation of surface proteins that are antibiotic targets, changes in bacterial membrane permeability, and regulation of efflux pumps that remove antibiotic products from the cytosol of the cell (4).

With the continued usage of antibiotic compounds, increasingly resistant strains will undoubtedly arise along with multidrug resistance. One of the most alarming forms of resistance that plagues human medicine is Methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA is common in hospital settings where patients have open wounds, surgical procedures breaking the patient's physical barriers, hospital device implants including intravenous catheters, and overall weakened immune responses. MRSA can cause serious illnesses such as pneumonia and sepsis. This particularly difficult pathogen is spread through direct contact with an infection site, although some patients can be directly colonized with MRSA and show little or no symptoms. According to the Center for Disease Control, 2 of every 100 people unknowingly carry MRSA in their nasal tract. The extreme plasticity of *S. aureus* has allowed the bacterium to acquire new features in terms of antibiotic resistance (5).

Drug resistance is not only limited to bacteria, as another pathogen whose commensal nature can become dangerous is *Candida albicans.* This fungus lives as a normal member of the human microbiome but can be the culprit of a variety of problems ranging from mild skin infections to deadly systemic infection (6).

Antibiotic Resistance in Veterinary Medicine

The population of companion animals has drastically increased during the last decades and continues to grow (3). The increased attention towards animal health and hygienic conditions has increased animal welfare as a whole. Since antibiotic discovery, there has been a steady rise in their usage in all applicable fields including animal husbandry. Antibiotics have been used as a growth promotion strategy resulting in reduction in natural microbiota in the intestine, reduced immune stimulation, and an increase in biosynthesis of vitamins in the intestine (3). Using

sublethal doses of antibiotics puts selective pressure on bacterial resistance allowing for the evolution of more resistant strains or species to arise. Veterinary Medicine faces unique challenges when treating patients with resistant microbial infections. Not only are veterinarians concerned with eliminating microbial infections but must also address the safety concerns of using a product that may be ingested by patients.

A commonly treated infection in canines is pyotraumatic dermatitis, commonly known as "hot spots". This is a superficial area of skin that has been disrupted due to licking, chewing, and gnawing on an area. As the skin barrier begins to deteriorate, common skin bacteria will grow in the moist environment. As the area becomes more inflamed, infection will begin, and a purulent exudate can be visualized. This condition is often very painful for veterinary patients and the need to clean and chew the area only worsens the growing infection.

One of the most common bacteria on the surface of canine patients' skin is *Staphylococcus pseudintermedius*. This member of the *Staphylococcus* genus is considered a zoonotic agent between canines and humans and one of the earliest species to be described (7). This opportunistic pathogen is also commonly responsible for ear, skin, and post-surgical site infections in canine veterinary patients. Although the occurrence of *S. pseudintermedius* in humans is lower than that reported in animals, reported cases are increasing. Currently, *S. pseudointermedius* in human medicine is not well studied and is often mistakenly reported as *S. aureus* (7).

With the increasing occurrence of *S. pseudintermedius* infections, antibacterial compounds are becoming decreasingly effective. Multi-drug resistant clones of *S. pseudintermedius* complicate the treatment of canine hot spots. These resistant forms are even

more difficult to treat when patients slow wound healing by continually disrupting topical compounds applied to the area.

Biofilms

One mechanism of pathogen resistance is the development of biofilms. Biofilms are sessile microbial communities that can embed themselves under a self-produced extracellular polymeric matrix of polysaccharides, proteins, lipids, and extracellular genetic material (8). Biofilm-producing pathogens have evolved this protective feature as a unique way to hide from host immune defenses, antibiotics, disinfectants, and topical antimicrobial products. The formation of biofilms has been described in four main steps, including cell attachment, microcolony formation, maturation, and dispersion (9).

During the first phase of biofilm development, attachment, planktonic cells attach themselves to surfaces by secreting an extracellular polymeric matrix. This matrix consists of extracellular polysaccharides, proteins, lipids, and genetic material. The second phase of biofilm formation is irreversible attachment of planktonic cells followed by maturation of the biofilm. A mature biofilm can consist of either one bacterial species alone, numerous types of bacterial species together, or even eukaryotic and bacterial microorganisms living in symbiosis.

Mixed species biofilms are predominant in most environments, but single bacteria biofilms exist in a variety of infectious wounds and on the surface of medical equipment and implants. Following maturation, biofilms enter the fourth step in development, dispersion, which often is triggered by environmental signals such as nutrient depletion. Cells transition back to their planktonic phase in the form of vesicles. Planktonic bacteria are able to break free from their protective biofilm in order to colonize neighboring nutrient dense areas.

It is thought that the genetic expression of bacteria found inside biofilms differs from that of planktonic cells. These changes are directly correlated with phenotypic characteristics developed by biofilm bacteria and also directly correlated with environmental changes experienced by the bacterium (10). One of the physiological changes that planktonic cells exhibit during biofilm attachment and maturity is the increase in expression of exopolysaccharides that may help to protect the biofilm.

Thermal Injury

Burns are among the most common and most dangerous forms of injury. These include thermal, chemical, and electrical burns. Severe burns demand specific and immediate treatment to lower the risk of secondary infection and sepsis. Thermal injuries contaminated with pathogenic bacteria can delay wound healing and cause wound breakdown if left untreated. These fatal complications contribute to approximately 5,000 patient deaths per year in human medicine (11).

Due to the commonality and severity of burns and other related skin infections, topical antimicrobial compounds that assist in collagen formation and pain management can be beneficial. The skin serves as the body's largest protective organ and is vital to maintaining normal body temperature and fluid homeostasis. Thermal injury to this vital organ leaves the host susceptible to hypovolemia and hypothermia. Survival is directly correlated with the size of the burn and the risk of secondary bacterial infection and sepsis. The most common bacterial species to be found in severe burn wounds that cause invasive infections are *Staphylococcus aureus*, *Methicillin-resistant S. aureus*, *Enterococcus* species, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumonia*, *Serratia marcescens*, *Proteus* species, and the fungi *Candida albicans* (11).

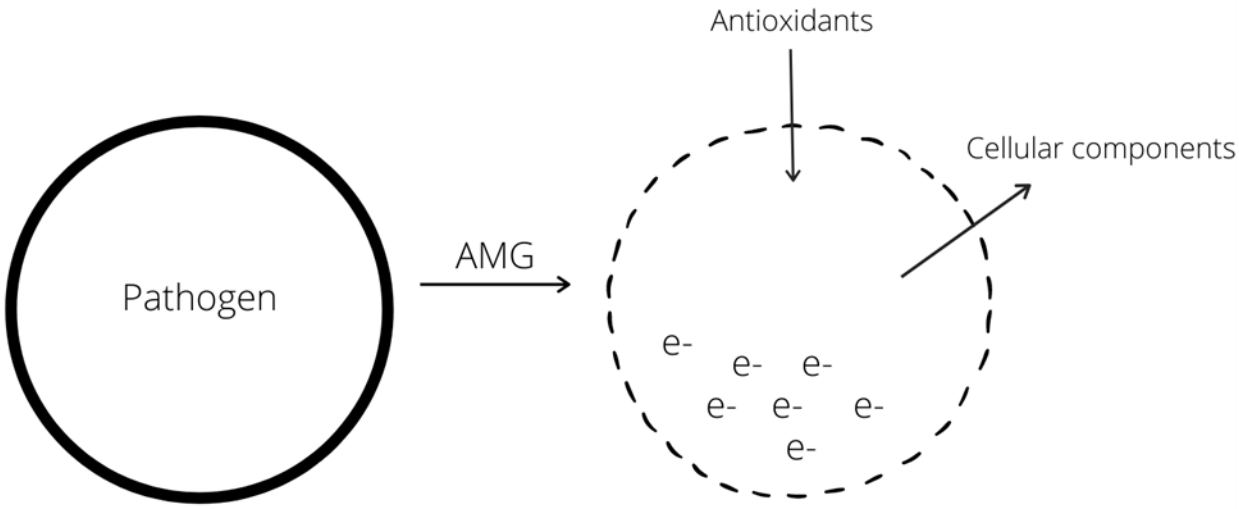
Current Treatments of Staphylococcal Infections

Staphylococcal infections are common in clinical settings. By the time penicillin was discovered in the 1940's, antibiotic resistant strains of *Staphylococcus* were already detected. Currently, a majority of *Staphylococcal* strains are resistant to the antibiotic penicillin. To overcome this resistance, newer beta-lactam antibiotics were developed and used in treatment, however, *Staphylococcus* evolved resistance and there is ever increasing incidence of methicillin-resistant strains. Nosocomial acquired Staphylococcal strains typically carry multi-drug resistant (mrMRSA) genetic elements and those infections must always be treated using combinational therapies. Strategies currently used in the treatment of methicillin- resistant *S. aureus* involve treatment with antibiotics such as vancomycin, bacitracin, rifampin, mupirocin, and retapamulin (12). Mupirocin (pseudomonic acid A) was first isolated from *Pseudomonas fluorescens* and proved its topical value in the treatment of *S. aureus* infections. However, with increased usage of mupirocin, resistant strains have emerged (13).

New Alternative Treatment for Skin Wound Infections

With the increase in not only drug resistance, but also multidrug resistant bacteria, new strategies and therapeutics are needed to combat skin wound infections. The purpose of this study is to quantify the effectiveness of an all-natural antimicrobial gel (AMG). AMG is a nutritional based compound comprised of Vitamin E TPGS, ascorbyl palmitate, zinc aspartate, and lavender oil. Vitamin E TPGS is a form of Vitamin E that distributes water across a wound surface and creates an amphoteric molecule that easily crosses lipid bilayers such as the skin and mucous membranes. The bioavailable form of zinc and vitamin E of AMG stimulates wound healing and creates a moisturizing layer over the wound. Ascorbyl palmitate, vitamin E TPGS,

and zinc also help to reduce free radicals that slow wound healing as well as promote skin and collagen formation. Additionally, lavender oil has an analgesic effect on burns and wounds. Previous work with AMG has shown decreased healing times, reduced scar formation, and decreased bacterial load of wounds. We hypothesize that AMG's detergent-like activity works to dissolve the cell membrane of pathogens creating pores or holes (Scheme 1). These openings allow for antioxidants to flood in, and essential components to leave the cell. These stresses result in lysis of the cellular membrane. Characterization of AMG involved testing the compound at various concentrations, investigating the cellular process that the compound impacts, and demonstrating the potential applications using an artificial wound model. Delineating the effects and mode-of-action that AMG has on microorganisms will provide a new, natural, and potentially novel treatment for skin wound infections that does not possess the selective pressures that cause drug-resistant species to form.



Scheme 1: Proposed Mechanism of AMG action. AMG is believed to target bacterial membranes by creating pores. This allows for antioxidants produced by AMG to flood the cytoplasm creating an oxidizing environment while also allowing vital cellular components to leak outside the cell.

CHAPTER 2. METHODS

Microorganisms, Strains, and Media

For all experiments, microorganism cultures, found in Table 1, were made from the inoculation of isolated colonies into nutrient rich Luria Broth (LB) medium and incubated for 24 hours at 37°C with shaking (250rpm). To standardize the starting inoculum for experiments, optical density of the 24-hour culture was determined using standard spectroscopy at 600nm and all strains were adjusted to an $OD_{600} = 0.01$ (roughly equivalent to ~$1x10^6$ cells/ml). For determining planktonic and biofilm growth by colony forming units (CFU), LB agar plates and Mannitol Salt Agar (MSA) plates were used. For the growth of S. aureus in a wound-like media, M199 was used to represent a more physiologically relevant environment.

Table 1: Microorganisms Used in this Study

Microorganism	**Designation**
Candida albicans	SC5314
Escherichia coli	ATCC 25922
Klebsiella pneumonia	ATCC 13883
Proteus vulgaris	ATCC 25923
Pseudomonas aeruginosa	ATCC 10145
Serratia marcescens	ATCC 13880
Staphylococcus aureus	ATCC 13315
Staphylococcus aureus (Contains pCM11 for GFP)	AH133 (Miller et al., 2014)
Staphylococcus pseudintermedius	Clinical isolate

Growth Inhibition Kinetics by Microtiter Plate Assay

To measure the inhibition of microbial growth by AMG on microbial species, overnight cultures (LB broth, 37°C, 250rpm, 24 hours) of *S. aureus, S. pseudintermedius*, *E. coli*, *P. aeruginosa*, *K. pneumonia*, *S. marcescens*, *P. vulgaris*, and *C. albicans* were standardized to ~1x10^6 cells/ml and inoculated into 96-well plates containing fresh LB or wells with increasing concentrations (1-10%) of AMG + fresh LB. Using a Synergy HTX multimode plate reader, OD_{600} readings were taken every 2 hours for 24 hours. Plates were held at 20°C with shaking and the results were obtained for three independent trials.

Growth Inhibition Kinetics by Fluorescent Assay

To measure the inhibition of *S. aureus,* a constitutively expressed GFP labeled strain (AH133) (14) was used to measure florescence. An overnight culture of AH133 (LB broth, 37°C, 250rpm, 24 hours) was standardized to ~1x10^6 cells/ml and inoculated into 96-well plates containing fresh LB or wells with increasing concentrations (1-10%) of AMG + fresh LB. Florescent readings were taken by a Synergy HTX multimode plate reader at excitation 360/340: emission 528/20 every 2 hours for 24 hours. Plates were held at 20°C with shaking and the results were obtained for three independent trials.

Growth Inhibition Kinetics of Planktonic Cultures by Colony Forming Units

To measure the inhibition of planktonic cultures, an overnight culture of *S. aureus* (LB broth, 37°C, 250rpm, 24 hours) was standardized to ~1x10^6 cells/ml and inoculated into LB broth or 10% AMG + LB and incubated at 37°C for 24 hours with shaking. Following incubation,

control and AMG samples were serially diluted, aliquots spread on LB agar plates, and plates were incubated for 24 hours and colony forming units (CFU) enumerated.

Growth Inhibition of Biofilms

A crystal violet staining method was used to measure biomass of prepared biofilms. Overnight cultures (LB broth, 37°C, 250rpm, 24 hours) of *S. aureus, S. pseudintermedius*, *E. coli*, *P. aeruginosa*, *K. pneumonia*, *S. marcescens*, *P. vulgaris*, and *C. albicans* were standardized to ~$1x10^6$ cells/ml.

For Biofilm Attachment Assays

Microorganisms were inoculated into 96-well plates containing fresh LB or 10% AMG + fresh LB and incubated statically for 24 hours. After incubation, the remaining liquid was carefully removed from the wells so as not to disturb the biofilms and wells were washed gently with 1X PBS buffered solution to remove loosely adherent cells. The wells were then stained with 0.01% Crystal Violet and incubated for 5 minutes at room temperature. The remaining crystal violet was removed, washed gently with 1X PBS, and plates were placed at 37°C until dry. A 33% glacial acetic acid solution was added to all wells to lyse cells open and solubilize the remaining Crystal Violet. Using a Synergy HTX multimode plate reader, OD_{550} readings were taken to determine the absorbance amount of the Crystal Violet.

For Biofilm Maturation Assays

Microorganisms were inoculated into 96-well plates containing fresh LB and incubated statically for 24 hours. After incubation, the used LB broth was aspirated off and either fresh LB

or 10% AMG + fresh LB was added to the wells and incubated for an additional 24 hours. After the second incubation, the remaining liquid was carefully removed from the wells so as not to disturb the biofilms and wells were washed gently with 1X PBS buffered solution to remove loosely adherent cells. The wells were then stained with 0.01% Crystal Violet and incubated for 5 minutes at room temperature. The remaining crystal violet was removed, washed gently with 1X PBS, and plates were placed at 37°C until dry. A 33% glacial acetic acid solution was added to all wells to lyse cells open and solubilize the remaining Crystal Violet. Using a Synergy HTX multimode plate reader, OD_{550} readings were taken to determine the absorbance amount of the Crystal Violet.

Confocal Imaging of Planktonic Cultures

To examine the microscopic effects of AMG on planktonic cultures, an overnight culture of *S. aureus* (LB broth, 37°C, 250rpm, 24 hours) was standardized to ~$1x10^6$ cells/ml and inoculated into LB broth or 10% AMG + LB and incubated at 37°C for 24 hours with shaking. Following incubation, samples were centrifuged and washed with 1X PBS. Equal ratio of SYTO9 and PI (LIVE/DEAD stain ThermoFisher) were added to the samples and incubated for 15 minutes at 37°C. Images of 10µl aliquots were obtained at 40X magnification using the Leica TC2 SP2 Laser Scanning Spectral confocal microscope.

Confocal Imaging of Biofilm Attachment

To examine the microscopic effects of AMG on biofilm attachment, glass bottom petri dishes were inoculated with 5µl of AH133 in 2ml of LB or 2ml of 10% AMG. Samples were incubated statically at 37°C for 24 hours. Following incubation, all liquid was removed from the

dishes and biofilms were washed with 1X PBS to remove non-adherent cells and then submerged in 1X PBS solution. Biofilms were then imaged at 40x magnification using the Leica TC2 SP2 Laser Scanning Spectral confocal microscope.

Confocal Imaging of Mature Biofilms

To examine the microscopic effects of AMG on mature biofilm architecture, glass bottom petri dishes were inoculated with 5µl of AH133 in 2mL of LB. Samples were incubated statically at 37°C for 24 hours. Following incubation, all liquid was removed from the dishes and control dishes were treated with 2mL of LB broth and experimental dishes were treated with 2ml of 10% AMG + LB broth. Biofilms were incubated for an additional 24 hours at 37°C. After the second incubation, biofilms were washed with 1X PBS, submerged in 1X PBS, and imaged at 40x magnification using the Leica TC2 SP2 Laser Scanning Spectral confocal microscope.

Primer Design

To create gene specific primers, the NCBI website was utilized to obtain sequences for the *Staphylococcus aureus* species. Sequences were obtained for ATCC 25923 for each of the two genes used in this study. The NCBI website was utilized again to create gene specific primers. Those sequences were transferred to The Bio-Web for primer clean up. Primers were analyzed to ensure no secondary structure or self-binding. The clean sequences were then transferred to Integrated DNA Technologies (IDT) website to compare the potential primers to other sequences within the genome. Primer parameters were set to a melting point within 4°C of one another, guanine/cystine content between 50-65%, and length of 15-25 base pairs. Primers used in this study were aliquoted and frozen for future use (Table 2).

Table 2: Primers Used in this Study

Target Gene	Primer sequence
SprX	Forward 5'- CACATGCATCAACTATTTACATCTATCC - 3'
	Reverse 5'- GCACCCCGTAAACTATTATAC - 3'
PrsA	Forward 5'- AGTTAATGATAAGAAGATTGACGAACAA - 3'
	Reverse 5' - 5'GAAGGGCCTTTTCAAATTTATCTTT – 3'

RNA Isolation

To obtain total RNA, an overnight culture of *S. aureus* (LB broth, 37℃, 250rpm, 24 hours) was used to inoculate 100µl into LB broth or 1% AMG + LB broth or 1% Mupirocin + LB broth and incubated at 37°C with shaking for 2 hours. After incubation, RNA isolation was carried out on all three samples using the Promega SV Total RNA Isolation system following the manufactures protocol to obtain pure total RNA samples for cDNA synthesis and amplification.

cDNA synthesis

cDNA synthesis was carried out using both Invitrogen SuperScript III One-Step TR-PCR System with Platinum *Taq* High Fidelity DNA polymerase, and Invitrogen SuperScript II Two Step-System. The high-fidelity DNA polymerase used in the One-step system is designed for sensitive end point detection and analysis of RNA molecules by reverse transcription PCR. Using this one step formulation, cDNA synthesis and PCR amplification can happen in the same PCR tube which reduces the likelihood of contamination or error in performing PCR. After RNA isolation, the following was loaded into a PCR tube: 1µg pure RNA, 25µl 2X Master Mix (containing 0.4mM of each dNTPs, 2.4 mM $MgSO_4$), 1µl Super Script III high fidelity enzyme mix, 1µl Forward gene specific primer, and 1µl Reverse primer. This mix is then volumized to

50µl with sterile RNase free water (Table 3). This mix was then loaded into a thermocycler for PCR. Protocols were dependent on enzyme used (Table 4, Table 5).

Table 3: Components of Sample Reactions for RT-PCR Amplifications Using Invitrogen SuperScript III One- step Enzyme

Substrate	Amount
Pure RNA	1 µg
2x Master Mix	25 µl
Enzyme	1µl
Forward Primer	1µl
Reverse Primer	1µl

Table 4:cDNA synthesis and amplification protocol for Invitrogen SuperScript III One-Step System

Step	Temperature	Duration
1	50°C	2:00
2	94°C	2:00
3	94°C	0:15
4	57°C	0:30 (Go to step 3 40X)
5	68°C	7:00 (Final elongation)

Table 5: cDNA Synthesis Thermocycler Protocol using SuperScript II RT Two-step system

Step	Components	Temperature	Duration
1	1µl Gene specific Primers, 1ng Total RNA, Sterile water Volumized to 12 µl	65°C	5:00
2	4 µl 5X First Strand Buffer, 2 µl 0.1MDTT	42°C	2:00
3	1 µl Super Script II RT	42°C	50:00
4		70°C	15:00

Table 6: PCR protocol for Super Script II Two-Step

Step	Components	Temperature	Duration
1	5 µl 10X PCR Buffer, 1.5 µl 50mM $MgCl_2$, 1 µl Forward primer, 1 µl Reverse primer, 0.4 µl Taq DNA polymerase, 2 µl cDNA product, volumize to 50 µl distilled sterile water	95°C	2:00
2		95°C	00:30
3		55°C	00:30
4		68°C	1:00 go to step 2 x40 cycles

LIVE/DEAD Viability Assay

Cell viability of *S. aureus* was investigated using Invitrogen LIVE/DEAD BacLight Bacterial Viability Kit. An overnight culture of *S. aureus* (LB broth, 37°C, 250rpm, 24 hours) was standardized to ~$1x10^6$ cells/ml and inoculated into LB broth or 10% AMG + LB and incubated at 37°C for 24 hours with shaking. To create a linear control, an aliquot of S. *aureus* was heat killed by treating the cells in an 80°C water bath for 20 minutes. The dead cells are mixed in different ratios with live cells (Table 7). Adjusting the concentration of live and dead bacteria within the sample create a positive linear line that allows for comparison to our own experimental values. Cells from the linear line as well as control and experimental cells were

treated with equal ratios of SYTO9 and for 15 minutes at 37°C. Samples were then loaded into a sterile 96-well plate and analyzed using a Synergy multi-node reader and green and red florescence is read at 488nm excitation and 530nm and 630nm emission respectively. Percent live cells were determined by using the formula % LIVE = (100 x (SYTO9/PI)) / (1 + (SYTO9/PI)) and by comparison to the standard linear line (15).

Table 7: Ratio of LIVE/DEAD Cell Staining for Control Comparison.

RATIO LIVE:DEAD	DEAD CELLS (ML)	LIVE CELLS (ML)
	0	2.0
10:90	0.2	1.8
50:50	1.0	1.0
90:10	1.8	0.2
100:0	2.0	0

In-Vitro Gauze Model

In order to visualize AMG's effects on *S. aureus* lawns, LB agar plates were treated with 2x2 sterile gauze seated with either water, or 10% AMG solution. Plates were incubated at 37°C for 24 hours. Gauze was then removed, and plates were imaged. Additionally, AMG infused into gauze pads were applied to bacterial lawns of *S. aureus* containing the GFP plasmid grown on physiologically relevant M199 agar plates. Following incubation for 24 hours the gauze was removed, and these wounds were imaged using UV light.

In-Vitro Artificial Wound Model

Artificial wound media was prepared using 21.9% MI99, 43.2% Tris Base, 34.7% Bactoagar, and dH_2O. M199, Tris Base and dH_2O was combined and filter sterilized. The pH of this mixture was adjusted to 7.5. Then, an agar solution was prepared and autoclaved. Once cooled to room temperature the agar and MI99 solution were combined slowly, stirred into a homogenous mixture, and poured into petri dishes. Polystyrene fashioned molds were sterilized by UV light for 30 minutes and placed gently in the media while cooling. The conformation of the polystyrene molds allowed the agar to settle unevenly creating a wound like surface.

In-Vitro Artificial Wound Attachment

AH133 (*S. aureus*) was inoculated into 5ml of LB broth and incubated at 37°C for 24 hours. Each wound model was then treated with 1 x 10^6 cells of the bacterial stock. These samples were allowed to dry and then treated with 0.25g AMG, 0.25g 2% Mupirocin, or a mixture of 0.125g AMG and 0.125g 2% Mupirocin. All samples were incubated at 37°C for 24 hours. After incubation, plates were removed for quantification and visualized using a UV light.

In-Vitro Mature Artificial Wound Models

To demonstrate AMG's ability to treat mature wounds, AH133 (*S.* aureus) was inoculated into 5ml of LB broth and incubated at 37°C for 24 hours. Each model was then treated with 1 x 10^6 cells of the bacterial stock. These samples were incubated at 37°C for 24 hours. After incubation, plates were removed and treated with 0.25g AMG, 0.25g 2% Mupirocin, or a mixture of 0.125g AMG and 0.125g 2% Mupirocin. Following the second incubation the plates were imaged using UV light and quantitated. Artificial wounds were irrigated generously with 1ml of

PBS solution to remove attached bacteria on the wound. These washings were vortexed to disperse the biofilm and serially diluted 10-fold in PBS, after which 100µl aliquots of each dilution were plated on LB agar, incubated for 24 hours at 37°C, and the number of CFU/ml were determined using the formula CFU/ml = (CFU count X dilution factor) X 100. Duplicates of all plate test conditions were created at the same time to ensure that one set could be imaged, and the sister set could be quantitated. This was done to ensure that the UV light did not disrupt any cellular processes of AH133 before quantification.

CHAPTER 3. RESULTS

AMG Inhibits the Planktonic Growth of Microorganisms

AMG inhibits planktonic growth of both Gram-positive and Gram-negative pathogens commonly associated with burn wounds. Using a microtiter assay, varying concentrations of AMG (1-10%) were tested against eight of the most common microorganisms associated with burn wound infections. Optical density (OD) readings were taken over 24 hours and compared to a control sample. It is important to note that AMG in solution has a dark yellow color and a thick consistency. Control readings of AMG alone were taken for comparison. For this reason, AMG treated samples appear to begin at a higher OD reading than control samples that do not contain AMG.

Planktonic growth of eight pathogens were inhibited by AMG beginning at 2% concentration and were continually inhibited through 10% AMG (Fig 1, Supplemental Fig A). As seen in Figure 1, all bacteria tested had decreased optical density growth in 10% AMG verses matched controls of LB over 24 hours. In all bacterial species, the suppressive effects of AMG on bacteria is immediate and sustained throughout the 24 hours. Only *S. marcescens* had any increase in growth in 10% AMG over the 24 hours and the growth was minimal compared to the LB control. AMG inhibited growth of *S. aureus* by 81%, *E. coli* by 72%, *P. aeruginosa* by 74%, *K. pneumoniae* by 72%, *S. marcescens* by 45%, and *P. vulgaris* by 68%.

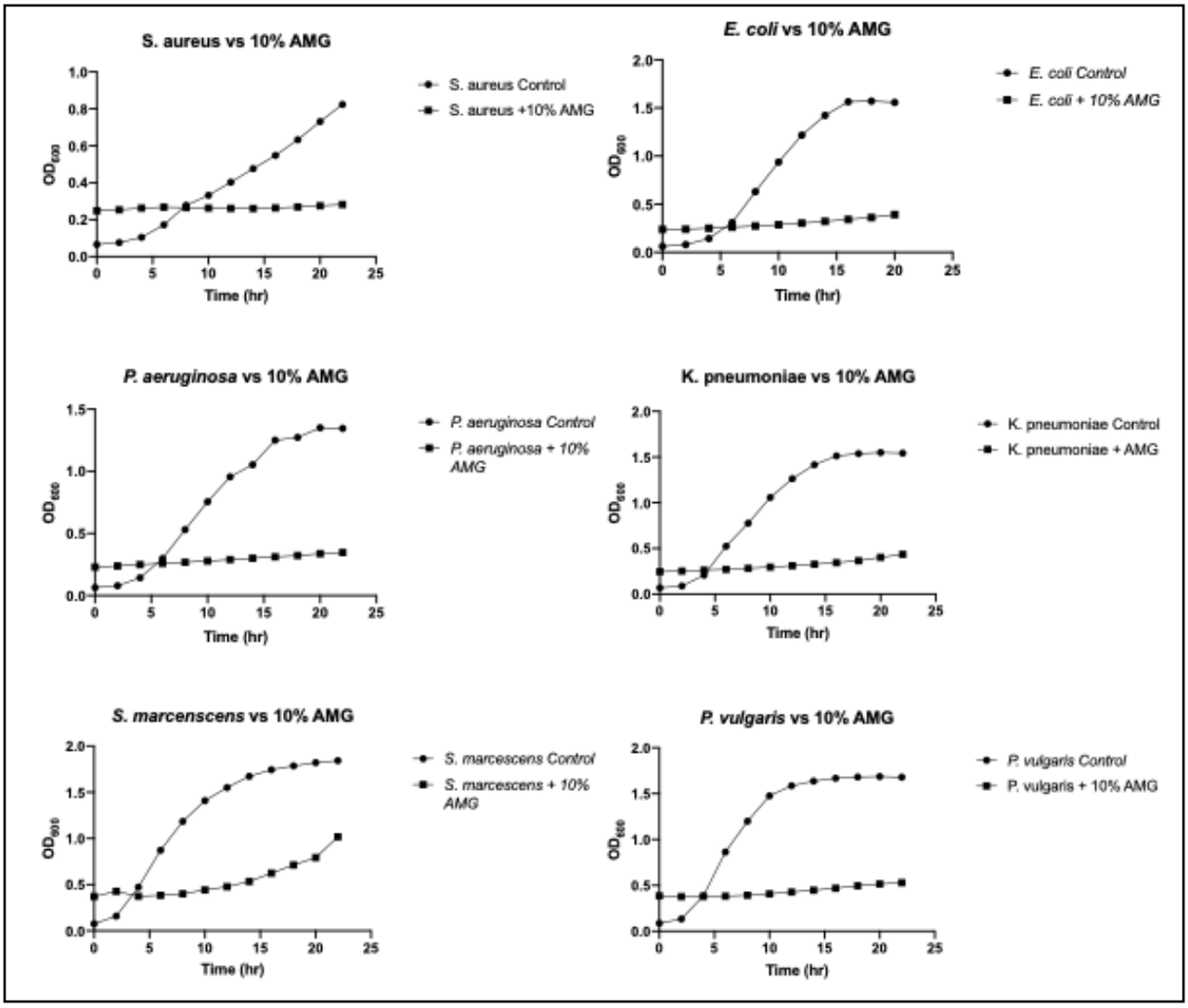


Figure 1: AMG inhibits planktonic cell growth of common bacteria associated with burn wound infections.

Bacteria were inoculated into 96-well plate containing wither LB or varying concentrations of AMG (1-10%) and OD_{600} readings taken every 2 hours for 24 hours at 20°C with shaking.

Additionally, *S. pseudintermedius*, the causative bacteria responsible for canine hot spots, was tested against AMG (1-10%). Planktonic cell growth was inhibited beginning at approximately 4 hours post-inoculation and continues to be inhibited through 24 hours when treated with 10% AMG (Fig 2).

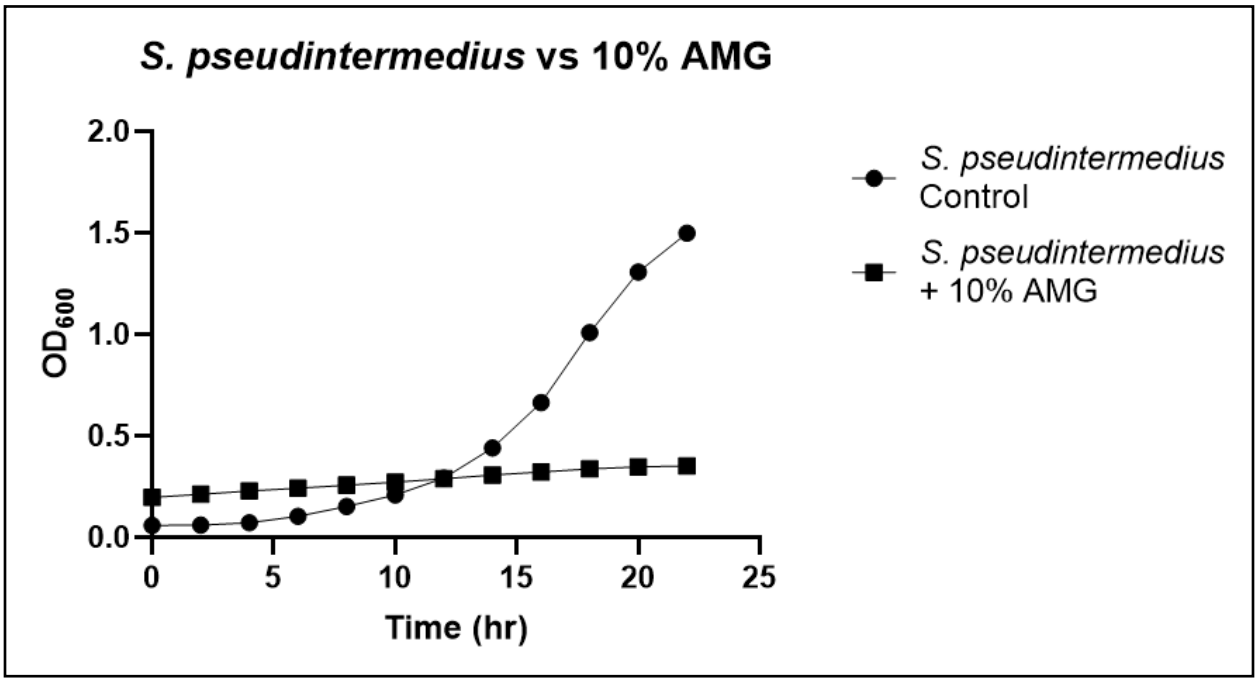


Figure 2: AMG inhibits planktonic growth of *S. pseudintermedius*.

Bacteria were inoculated into 96-well plate containing wither LB or varying concentrations of AMG (1-10%) and OD_{600} readings taken every 2 hours for 24 hours at 20°C with shaking.

To determine the effects of AMG on microorganisms other than bacteria, the fungus *Candida albicans* was also included in this study. This microorganism is found in burn wounds and is the most common culprit for fungal infections in humans due to its presence in the mouth, throat, and gut. This particular pathogen has the unique ability to transition from yeast to hyphal morphology. In yeast phase, *C. albicans* is part of the natural microbiome in humans. Once transitioned to hyphae, the pathogen begins to form biofilms. When exposed to AMG, *C. albicans* growth is inhibited after approximately 2 hours post inoculation. Due to its increased density alongside the density of AMG, OD readings appear increased in comparison to control samples (Fig 3).

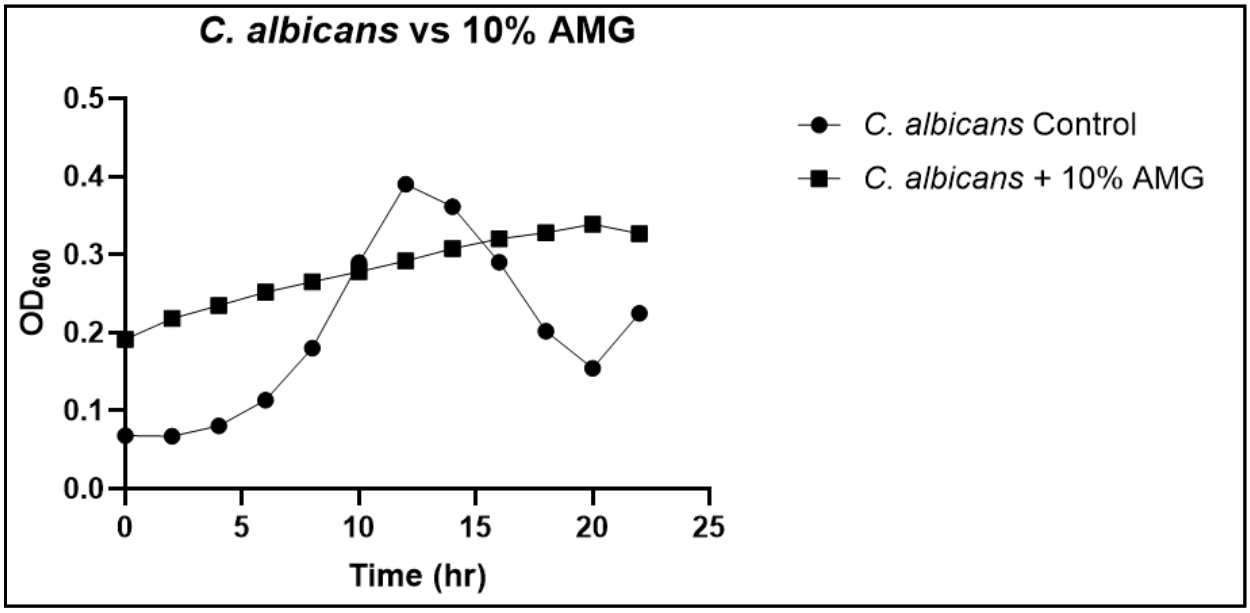


Figure 3: AMG inhibits planktonic cell growth of *C. albicans* associated with human skin and mucosal infections.

C. albicans was inoculated into 96-well plate containing wither LB or varying concentrations of AMG (1-10%) and OD_{600} readings taken every 2 hours for 24 hours at 20°C with shaking.

Given that *S. aureus* is one of the most prevalent microorganisms found on the human skin biome, its increasing resistance to antimicrobial drugs is of concern. After discovering *S. aureus* planktonic growth was inhibited by 81% when treated with AMG, we chose to examine this bacterium closer. Using a strain of *S. aureus* containing a constitutively expressed GFP plasmid (AH133) (14), we were able to monitor florescence of the pathogen over 24 hours. This provides an additional way to monitor growth and inhibition when treated with AMG, supporting our previous findings in Figure 1. Although considered stable, it is important to note that this plasmid can be expelled from the cell under environmental stress. This could result in incorrect florescent readings, indicating that the *S. aureus* strain is being inhibited by AMG when it may be expelling the plasmid.

AMG inhibits AH133 growth after 2 hours of exposure while control samples continue to express florescence through 18 hours. In fluorescent units, *S. aureus* growth is inhibited by 76% when treated with 10% AMG verses matched controls. Control samples appear to decline after 18 hours as nutrients begin to deplete in the media which follows the normal growth curve of all microorganisms (Fig 4).

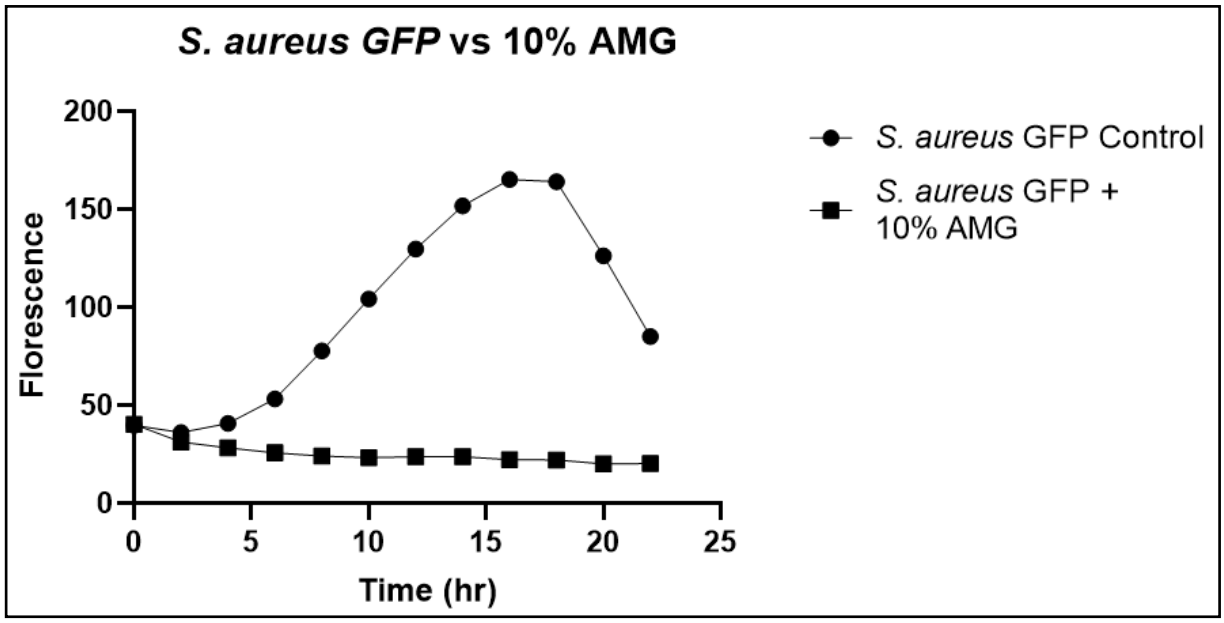


Figure 4: Growth Inhibition of the fluorescently labeled S. aureus strain AH133 in 10% AMG over 24 hours.

AH133 (*S. aureus* containing a constitutively expressed GFP plasmid) was inoculated into control wells with only LB or 10% AMG. Florescent readings were then taken at excitation 360/40 and emission 528/20 every 2 hours for 24 hours. Florescence of AH133 begins to decrease after inoculation with 10% AMG.

Although optical density readings are a reliable way to represent growth over time, they cannot represent the cell viability effects of AMG. To determine cell viability after treatment with AMG, aliquots of *S. aureus* grown in LB broth and grown in 10% AMG + LB were serially diluted, plated on LB agar, incubated, and observed for colony forming units (CFU) (Fig 5). AMG significantly inhibits *S. aureus* viable cells by 85% verses controls ($p=0.0003$). This

demonstrates that not only does AMG inhibit the optical density growth, but that it also inhibits the number of viable cells of *S. aureus*.

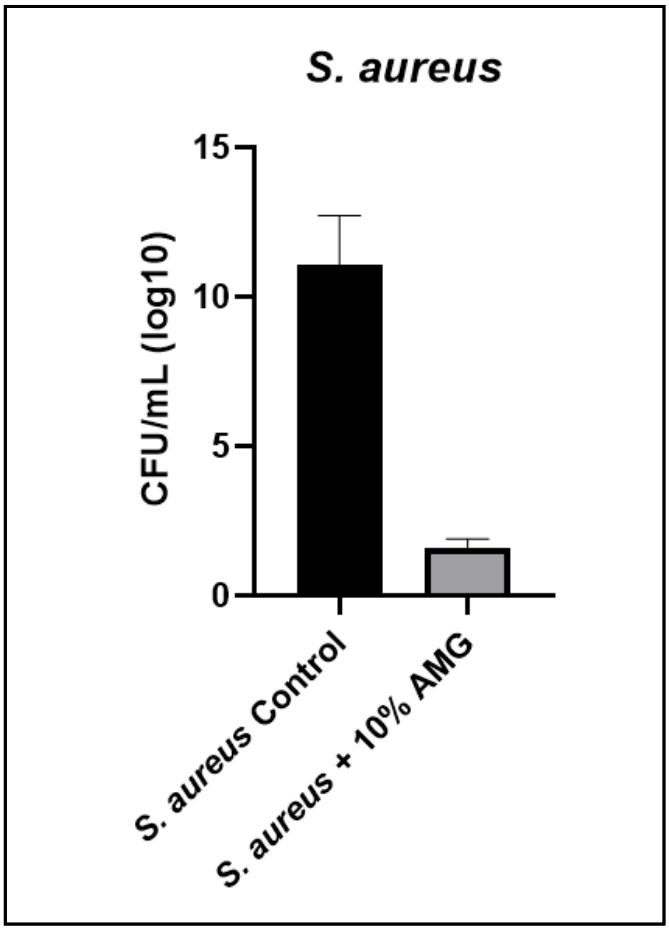


Figure 5: AMG inhibits *S. aureus* planktonic growth and viability.

Overnight cultures of *S. aureus* were normalized to $1x10^6$ and inoculated into 2ml of either LB broth or 10% AMG and incubated at 37C for 24 hours. Following incubation, cell suspensions were serially diluted 10-fold and spread onto LB agar plates. Plates were incubated and counted to determine CFU/ml (n=3)(p=0.0003).

AMG Inhibits Biofilm Growth of Microorganisms Associated With Burn Wounds

The ability for pathogens to form protective biofilms is crucial to their survival in many biotic and abiotic environments. As mentioned previously, this protects the pathogen not only from the immune system but also from antimicrobials. Biofilm development occurs in different phases. We chose to look at AMG's effects in two main phases, attachment and mature biofilms. In the attachment phase, we added 10% AMG at the beginning of the biofilm growth to determine if AMG inhibits the initial binding of planktonic bacteria. Inhibiting this crucial step is detrimental to biofilm development. Additionally, we wanted to look at AMG's effects on pre-established biofilms, in which bacteria were allowed to form biofilms and AMG was added after 24 hours. AMG is effective in inhibiting the development seven of the eight pathogens tested in our panel. With the exception of *S. pseudintermedius*, crystal violet biomass quantification demonstrates statistically significant differences between control and treatment groups (Fig 6). A significant change coming from *S. aureus* showing a 96.87% decrease in biomass (p-value = 0.000753) (Table S1). AMG reduced attachment for *E. coli* by 94%, *P. aeruginosa* by 76%, *P. vulgaris* by 100%, *K. pneumoniae* by 94%, *C. albicans* by 33%, and *S. marcescens* by 44%. For all biofilm experiments statistical analysis was performed on raw data. Control wells of LB only and 10% AMG only were included to be subtracted from the means of each sample trial. These values were included for comparison (Table S1). AMG is an effective means of treating abiotic surfaces and reducing bacterial loads in this model.

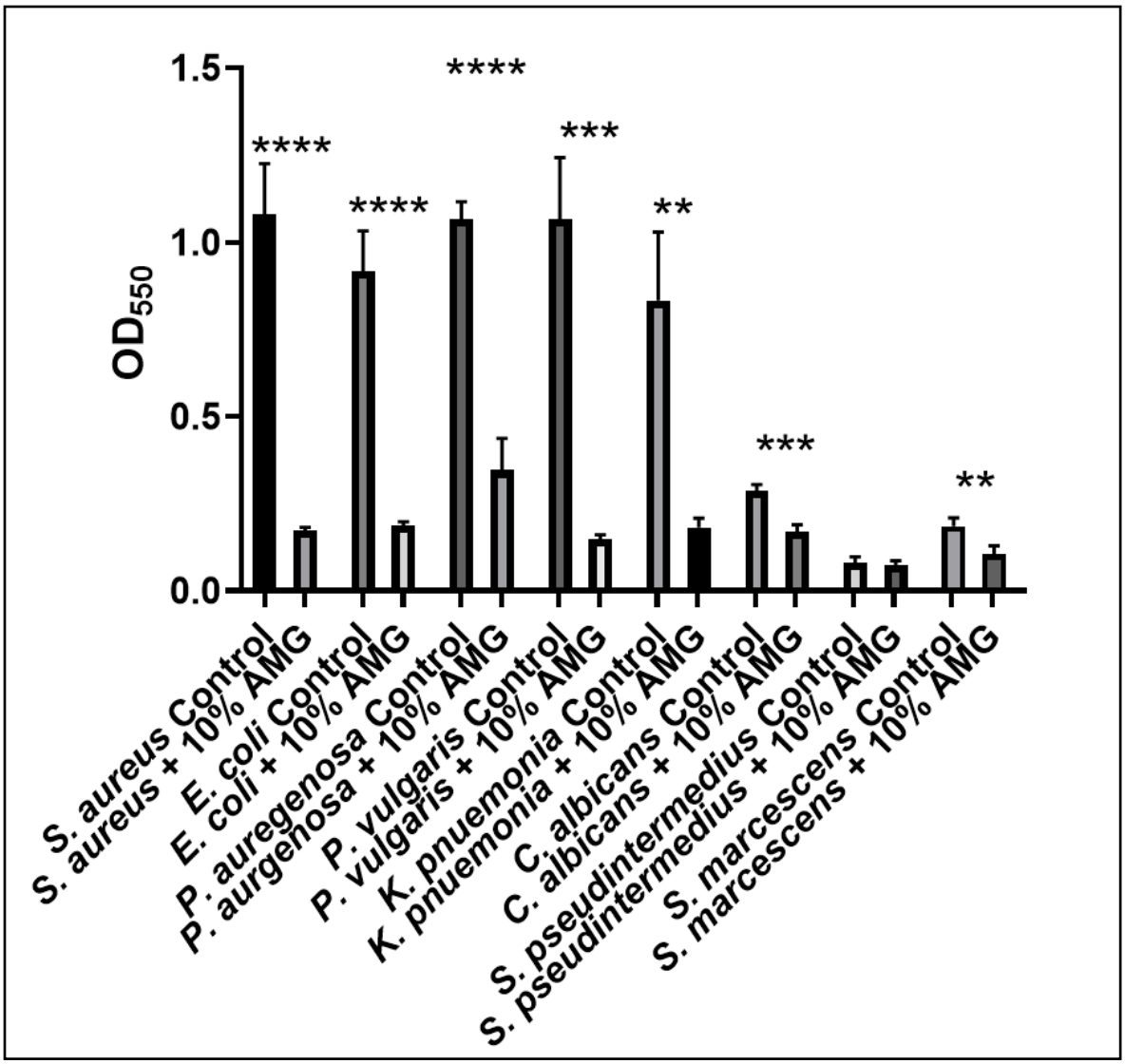


Figure 6: AMG Inhibits Biofilm Attachment of common Burn Wound Microorganisms.

Overnight cultures of microorganisms were normalized to $1x10^6$ cells/ml and inoculated into a sterile 96-well plate containing LB broth or 10% AMG. Plates were incubated overnight, washed, and stained with crystal violet. Stained wells were then lysed with 33% Acetic Acid and OD_{550} readings were taken. One-tailed t-tests completed using PRISM9 (* $P<0.05$; ** $P<0.01$; *** $P<0.001$, **** $P<0.0001$)

In many cases, wounds and thermal injuries are not immediately treated and pathogens have the opportunity to create mature biofilms. When this occurs, debridement and topical antimicrobial compounds are utilized to clear established infections. Therefore, we wanted to

turn our attention to biofilms that were already established before treatment occurs. Microbial biofilms were allowed 24 hours to grow and mature before being treated with 10% AMG. AMG was not as effective in penetrating and eradicating existing biofilms, only showing statistically significant changes in *E. coli* (p value = 0.00018) and *P. vulgaris* (p-value = 0.005). One- tailed student t-tests were performed using PRISM9 software. AMG decreased biofilm mass by 25% and 33% respectively (Fig 7) (Table S2). Interestingly, *P. aeruginosa* showed a significant increase in optical density when treated with 10% AMG. We believe this is due to the bacteria's ability to produce alginate and pyocyanin. Alginate, an exopolysaccharide, increases the bacteria's ability to attach and create new biofilms in response to environmental stress. Although biofilms were washed before staining, this polysaccharide is thick and difficult to remove without disrupting the biofilm. This leads to increased optical density readings when treated with AMG. It is well documented that biofilms resist numerous treatment methods, particularly antibiotic treatments, and that the three-dimensional structure and complex biofilm products protect the bacterial community more efficiently than in planktonic culture.

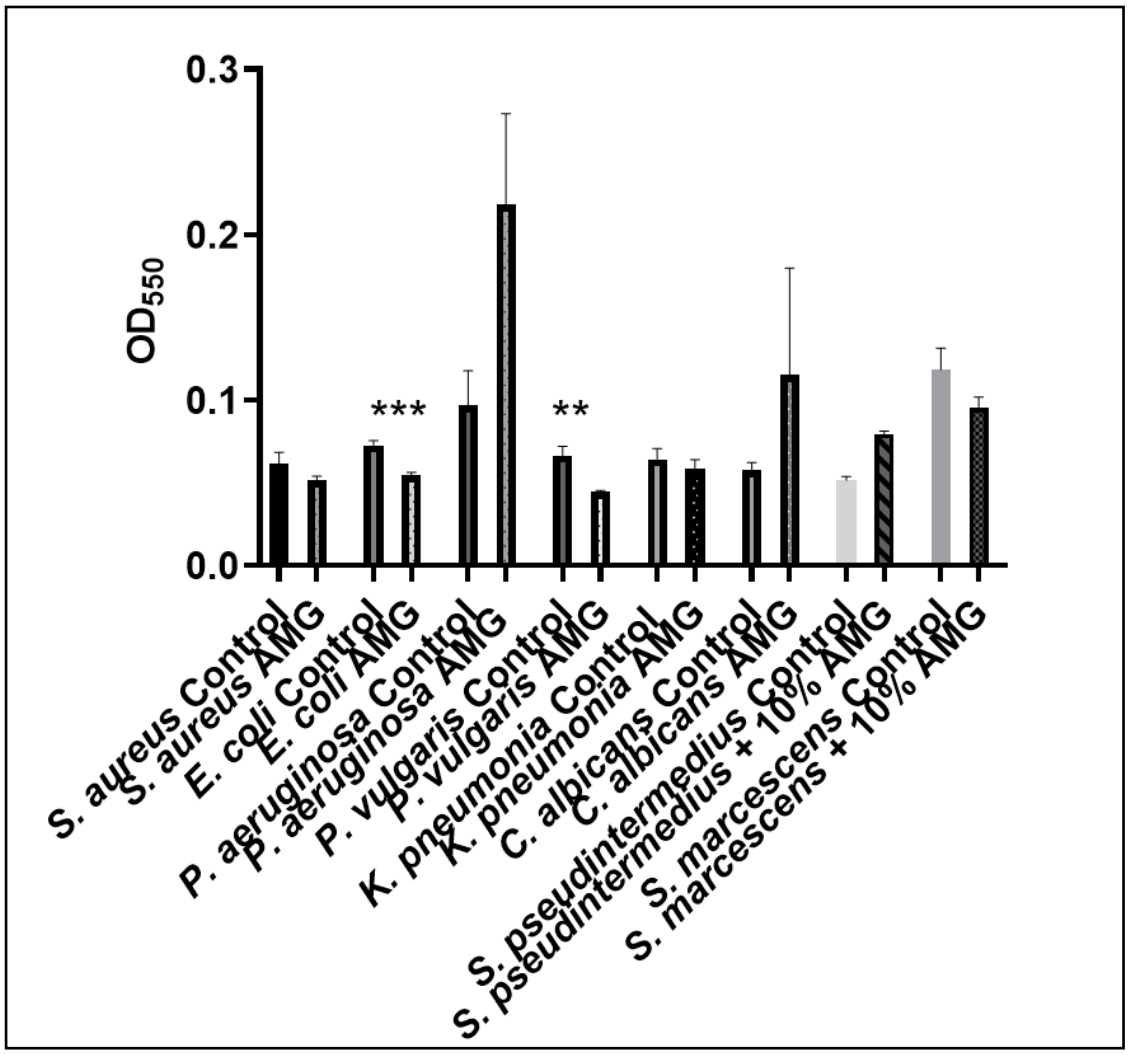


Figure 7:Mature biofilms are difficult to treat with AMG.

Overnight cultures of microorganisms were normalized to $1x10^6$ cells/ml and inoculated into sterile 96-well plates containing LB. Biofilm growth was developed over 24 hours then treated with 10% AMG and incubated for an additional 24 hours. Wells were washed, stained, and lysed using 33% Acetic Acid. Optical Density readings were taken at 550nm. Values represent the mean of 3 independent trails. One-tailed t-tests completed using PRISM9 (* $P<0.05$; ** $P<0.01$; *** $P<0.001$, **** $P<0.0001$)

Confocal Microscopy Allows for Visual Observations of AMG Inhibition of S. aureus

We wanted to visually examine the effects of AMG on both the attachment and mature phases of biofilm growth. To serve this, a florescent strain of *S. aureus* (AH133) and confocal microscopy were used to capture images of these stages when treated with AMG. Using the confocal microscopy protocol previously described, biofilm attachment, as well as mature biofilms, were imaged 24 hours post treatment with AMG. Consistent with our planktonic growth and biofilm growth inhibition assays, AMG's has an inhibitory effect on the attachment phase of *S. aureus* biofilm growth (Fig 8). Visually, there is a decrease in cell density and fluorescence in the AMG treated biofilms verses the LB control after 24 hours of treatment.

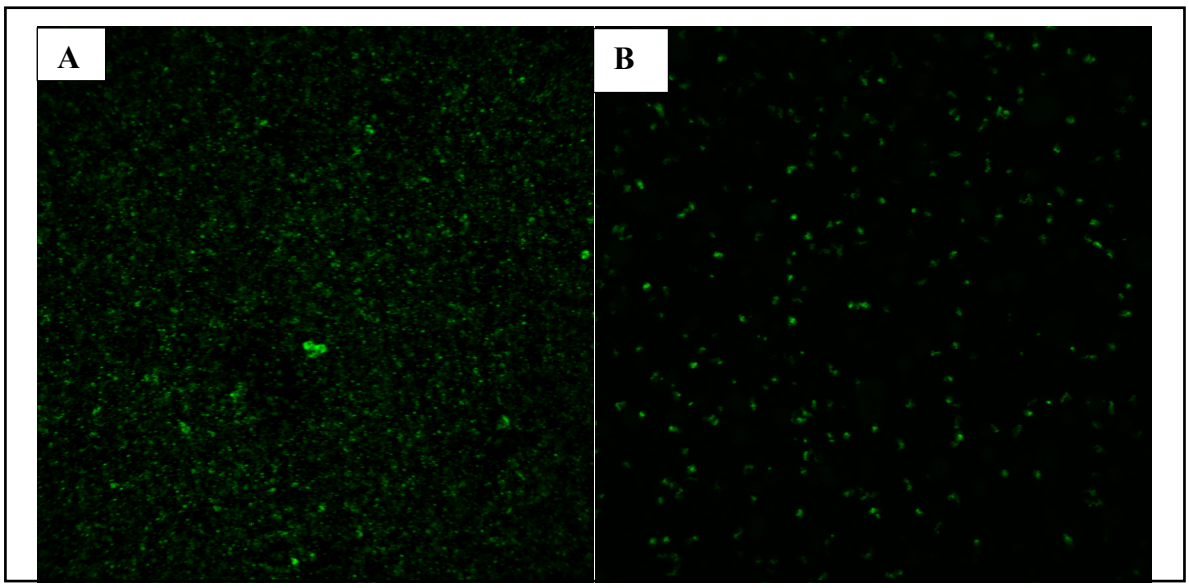


Figure 8: Confocal imaging of *S. aureus* AH133 on biofilm chambers.

(A) Biofilm attachment control of AH133 (B) Biofilm attachment inhibition of AH133 when treated with 10% AMG at the time of inoculation.

Mature *S. aureus* biofilms were treated 24 hours after inoculation with 10% and observed with confocal microscopy. Mature biofilms treated with 10% AMG (Fig 9B) are reduced in both cell density and fluorescence in comparison with LB controls (Fig 9A).

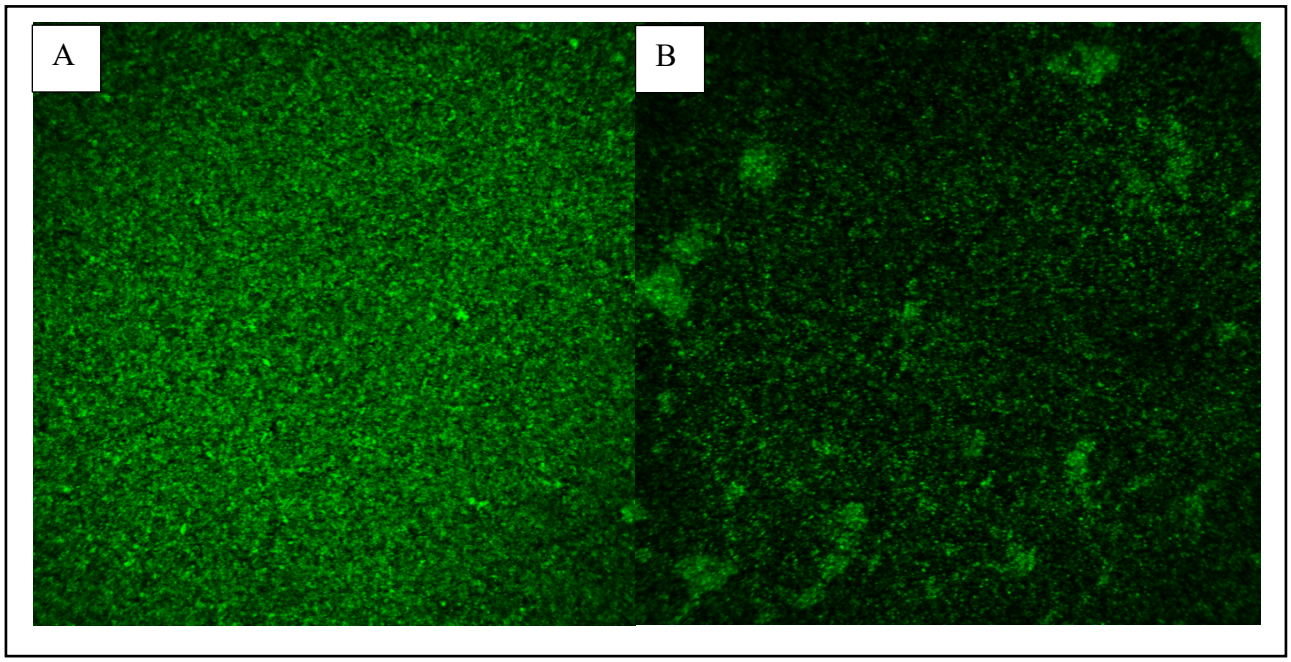


Figure 9: Confocal imaging of *S. aureus* AH133 on mature biofilm chambers.

(A) Biofilm maturation phase control of AH133 (B) Biofilm maturation phase inhibition of AH133 when treated with 10% AMG at the time of inoculation.

AMG Alteration of S. aureus Gene Expression

Currently, the exact mechanism of action of AMG on *S. aureus* is unknown. To attempt to delineate the pathway AMG inhibits in *S. aureus*, a panel of genes were selected for screening by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) of total RNA. Genes that show significance in RT-PCR could then be focused upon and supported using quantitative real-time polymerase chain reaction (qRT-PCR). Of the panel of genes, only two showed any changes in expression, but were not determined to be statistically significant (Fig 10).

SprX. A range of regulatory RNA molecules are expressed in *S. aureus* that, in general, have an unknown biological function. However, in 2014, researchers uncovered a *S. aureus* small RNA SprX that is highly conserved and shapes bacterial resistance to glycopeptides (16). Since the emergence of methicillin resistant staphylococcal species, glycopeptide antibiotics such as

vancomycin have been used to eradicate *S aureus* infections. This class of antibiotics inhibits the synthesis of peptidoglycan by binding to newly synthesized units, preventing repair and synthesis of cell wall structures. SprX specifically down regulates the expression of stage V sporulation protein (SpoVG). Previous studies have shown that SpoVG is the major factor in expression of the *yabJ-spoVG* operon, which is required in *S. aureus* for antibiotic resistance (17). SprX negatively regulates SpoVG expression through direct antisense pairing at the internal translational initiation signal. The regulation of SpoVG directly impacts oxacillin resistance by regulating cell wall metabolism in *S. aureus* strains (17).

SprX was chosen for this study due to its direct correlation to the inhibition of peptidoglycan synthesis.

PsrA. The methicillin-resistant phenotype of *S. aureus* results from the expression of penicillin-binding protein 2A (PBP2A). This binding protein can catalyze DD- transpeptidation of peptidoglycan due to its low affinity for ß- lactam antibiotics (5). PrsA is described as a ubiquitous protein lipid anchored to the outer surface of the membrane where it assists in the folding of extracellular proteins. Previous studies have shown that the alteration of PrsA altered oxacillin resistance and caused a decreased in PBP2A without changing the expression of mecA, the gene that transcribes PBP2A (5). This indicates that PrsA is responsible for some posttranscriptional maturation of PBP2A, possibly in the export and folding of newly synthesized PBP2A. In *B. subtilis,* PsrA is an essential component for peptidoglycan structure inside the cell membrane due to its post- transcriptional control of PBP2a. The mis-folding of PBP2a results in morphological defects and decreased cross-linking of peptidoglycan (18). For the purpose of this study, PrsA was chosen to indicate cell wall disruption

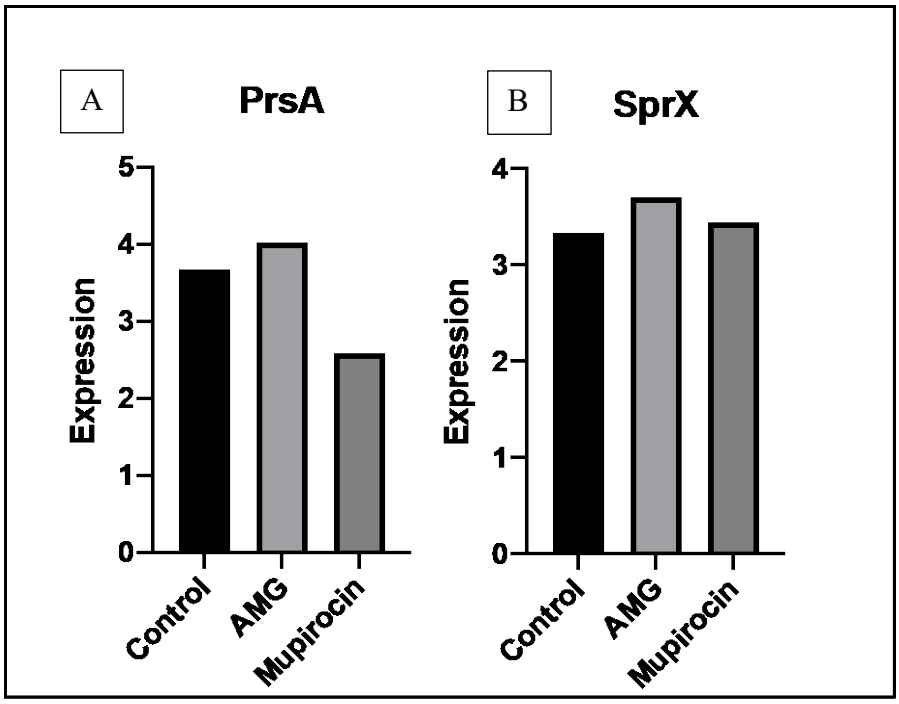


Figure 10. Gene expression levels of AMG and Mupirocin treated *S. aureus*.

AMG minimally, and not significantly, induced increased expression of PrsA (A) and SprX (B).

Gene expression analysis shows that *S. aureus* treated with 1% AMG minimally upregulates the expression of both PrsA, and SprX by 20.9% and 22.9% respectively (Fig 10). This indicates that AMG may be attacking the cellular membrane causing a disruption in repair mechanisms.

AMG Reduces the Number of Viable S. aureus Cells as Indicated by LIVE/DEAD Staining

Testing bacterial cell wall viability is an important aspect to determine the efficacy of an antimicrobial compound. The LIVE/DEAD staining technique is used to determine cell wall integrity as well as bactericidal effects. Control and AMG treated samples of *S. aureus* were stained as previously mentioned. Images of each sample were then quantitated for first green (SYTO9), and then red (PI) florescence. The images were then merged to determine the quantity of live and dead cells in the sample. While there are not necessarily more dead cells, the density of live cells is significantly inhibited by 10% AMG. This indicates that AMG inhibits bacterial growth as well as kills existing cells in the sample (Fig 11).

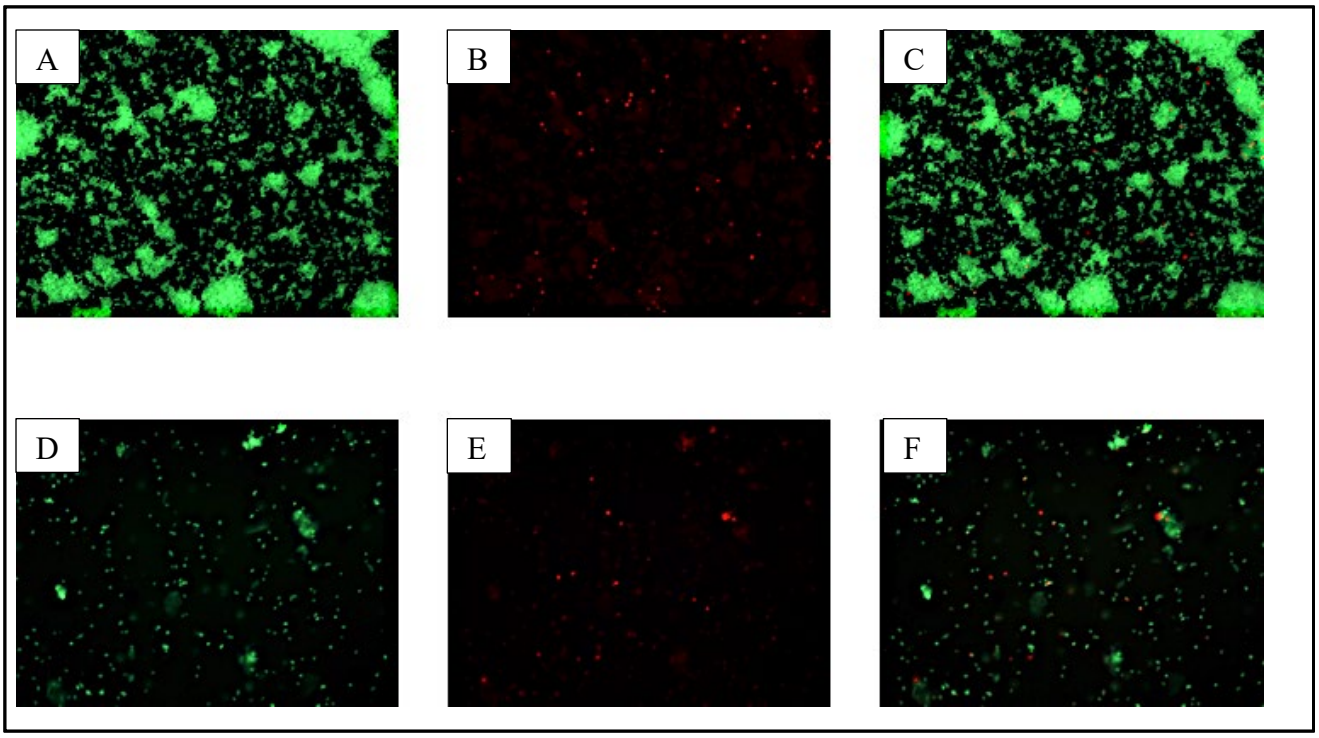


Figure 11. AMG inhibits growth of *S. aureus*.

LIVE/DEAD staining was utilized to demonstrate the bactericidal effects of AMG. Control samples were first imaged 480nm excitation/500nm emission to visualize STYO9 green florescence (A), and then 480nm excitation/635 emission to visualize propidium iodine red florescence (B). The Images were then merged to represent the ratio of live to dead cells in the control sample (C). The sample protocol was used to visualize *S. aureus* treated with 10% AMG. Treated samples were first visualized for green florescence (D), followed by red (E). The images were again merged to represent the ratio of live to dead cells in the treated sample (F).

To quantify the above LIVE/DEAD stain images, *S. aureus* was inoculated into LB control media or 10% AMG + LB media, incubated for 24 hours and stained with the LIVE/DEAD stain and quantitated by taking the SYTO9 (500nm) fluorescent reading and PI (635nm) fluorescent reading. Taking these readings and using the equation: % live cells = (100 x (SYTO9/PI)) / (1 + (SYTO9/PI)) we could determine the relevant fluorescent signals of each condition and the percentage of live and dead cells in the populations. Figure 12 illustrates that the fluorescent signal in the control *S. aureus* condition are very strong indicating large cell density for both SYTO9 and PI. In the 10% AMG treated *S. aureus* conditions, the SYTO9 and

PI signals are minimal indicating there are very few cells present. Using the above equation and setting their total cell count to 100%, AMG treated has an increased number of dead cells present (Fig 12) and reduced number of live cells (black bars) when matched with control cells.

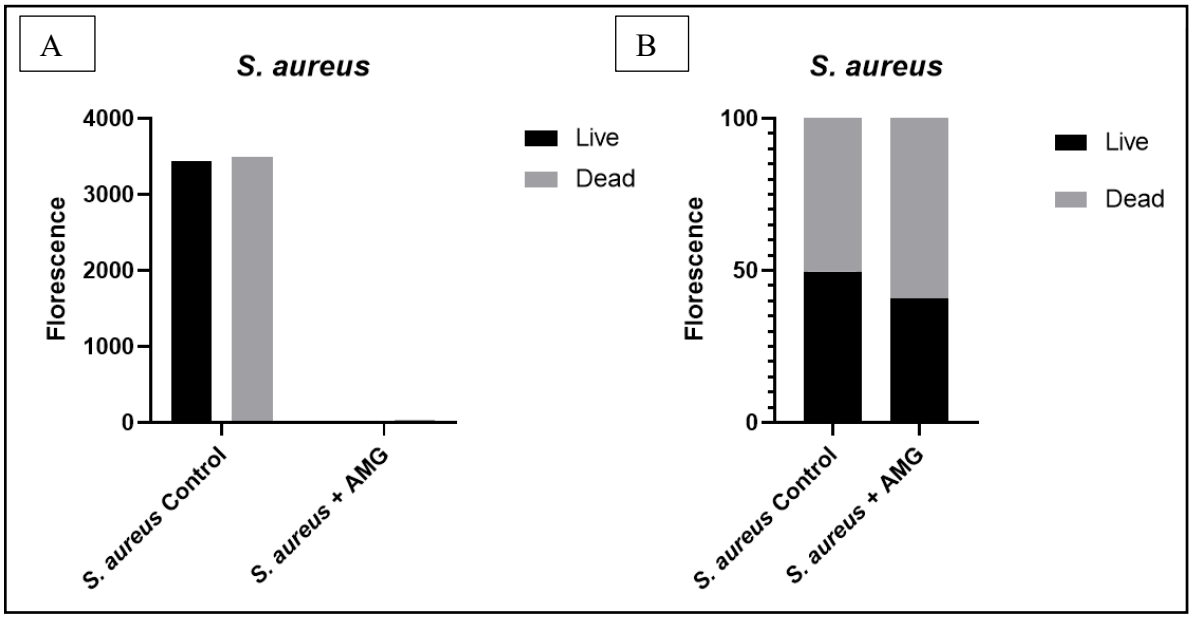


Figure 12. Quantification of LIVE/DEAD staining samples. LIVE/DEAD staining was utilized to quantitate the amount of live and dead cells in both control and 10% AMG treated samples. Florescence of control and 10% AMG treated samples were first read at 480nm excitation/500nm emission to measure STYO9 florescent intensity, and then 480nm excitation/635 emission to measure PI florescent intensity (A). These values were then set to 100% to create a ration of live to dead cells in each sample (B). AMG treated samples have increased amounts of dead cells compared to control samples.

In-Vitro Models of Wound Infection Demonstrate AMG can Inhibit S. aureus in Wound-Like Conditions

While AMG is effective at inhibiting numerous in-vitro models, we wanted to examine the effects of AMG in a more natural environment. Implementing a physiologically relevant tissue culture media and creating a wound-like impression, we tested the ability of AMG to inhibit *S. aureus* growth. Controls show thick healthy *S. aureus* lawns while the AMG gauze

treated *S. aureus* lawns show a decrease in bacterial growth and florescence (Fig 13). M199 wound media was used due to it similarity to the skin physiological environment. This media can also be used as a pH indicator and the application of AMG appeared to reduce the pH of the media after 24 hours.

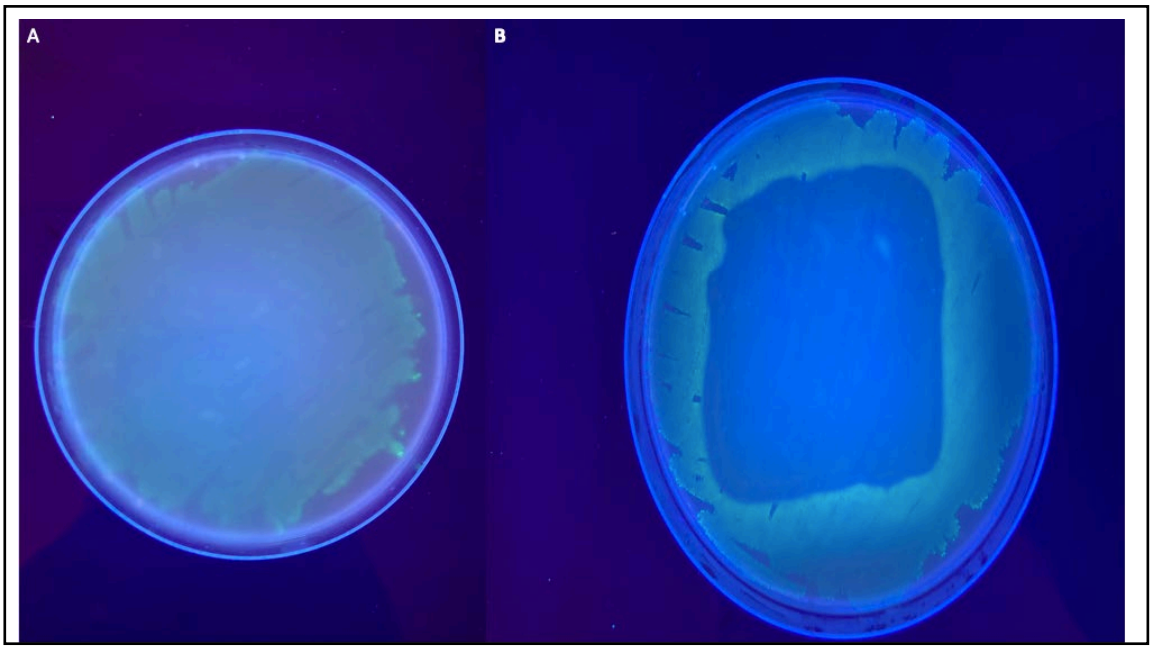


Figure 13. AMG infused gauze inhibits *S. aureus* growth on MI99 wound media.

Bacterial lawns of AH133 were treated with gauze containing sterile water (A), or 10% AMG (B). Plates were then incubated at 37°C for 24 hours. Plates were removed and imaged using UV light.

The gauze pad experiment was also performed on LB agar plates with non-florescent *S. aureus.* These results were consistent with the wound model showing a significantly decrease in bacterial growth in comparison to control plates (Fig 14).

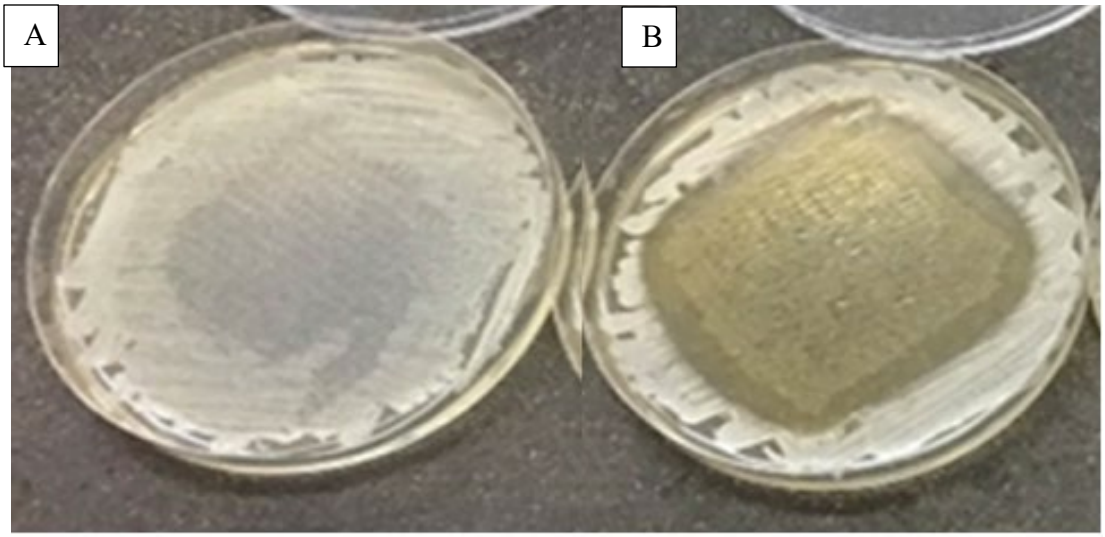


Figure 14. AMG inhibits *S. aureus* in gauze model.

Bacterial lawns of *S. aureus* were inoculated. Control plates were left untreated (A). Sterile gauze was seated with 10% AMG and applied to experimental plates (B). Each plate was incubated at 37°C for 24 hours. Gauze was then removed, and plates were imaged. Treated plates show reduced bacterial growth compared to control plates.

Our modified wound model was also used to show that AMG can be applied to disrupted skin areas such as wounds and burns as well as demonstrates that AMG can work synergistically with other common topical antimicrobials. Impressions were made in the M199 media to simulate the rough, uneven topography of a burn or puncture wound. The wound-like impression was then inoculated with *S. aureus* (AH133) and left untreated or treated with 0.125g 100% AMG, 0.125g 2% Mupirocin, or a combination of AMG and Mupirocin. Following an incubation period, these models were visualized by UV light (Fig 15). Figure 15A demonstrates a strong fluorescent signal and growth of *S. aureus* in the control untreated wound. In all three other conditions (AMG treated, Mupirocin treated, and AMG + Mupirocin treated) there was no fluorescent signal and no growth of the *S. aureus.*

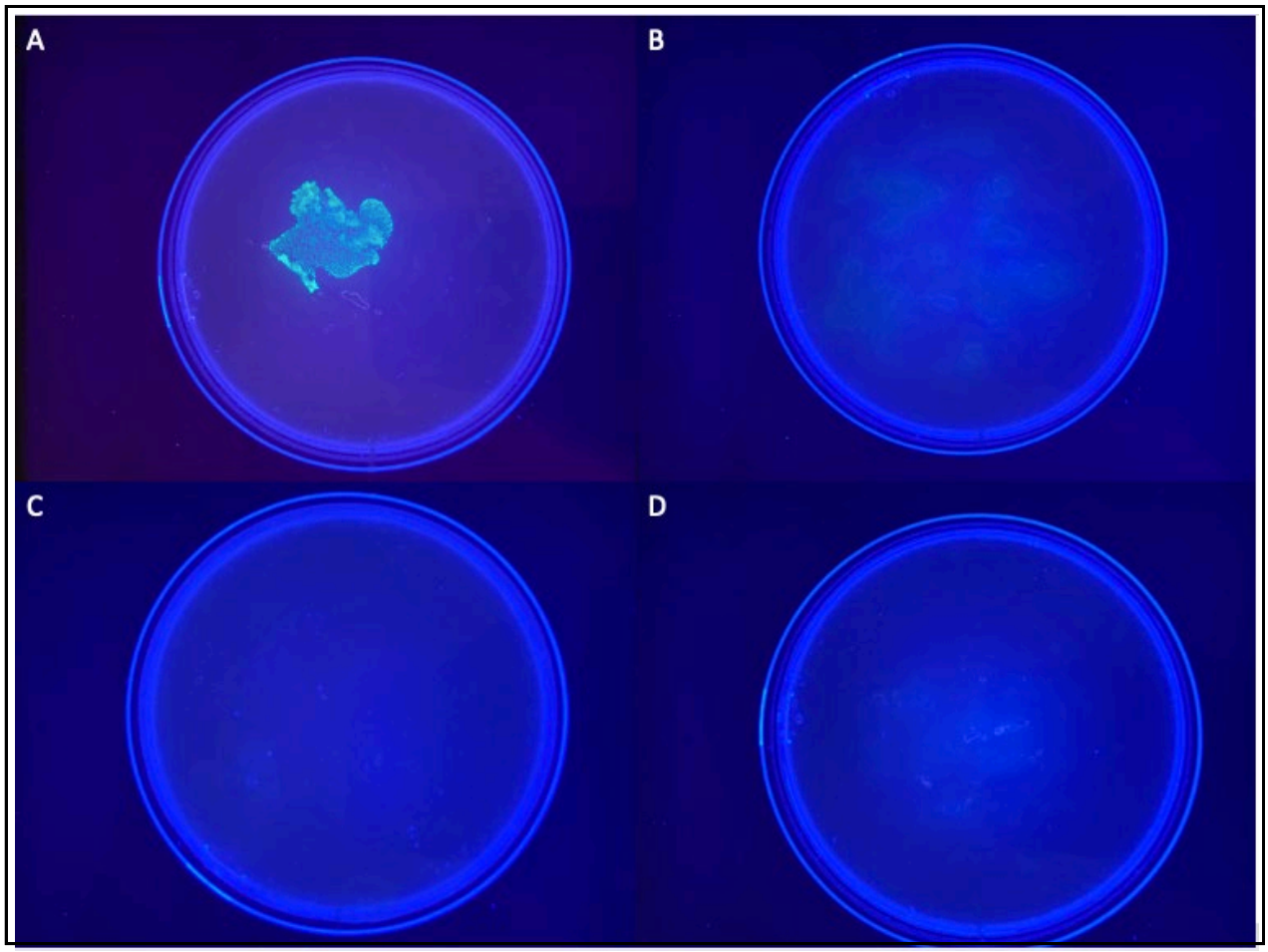


Figure 15. UV visualization of artificial wounds.

Wound beds were made as previous described and inoculated with AH133. Wounds were allowed to dry completely and then treated with either (B) 10% AMG, (C) 2% Mupirocin ointment, or (D) 10% AMG + 2% Mupirocin. Plates were then incubated at 37C for 24 hours. Plates were removed and imaged using UV light.

We also wanted to determine if there were any small pockets of *S. aureus* in the wound models. To do this, each wound model was washed, and serial diluted to obtain CFU/ml. For each model treated with either 10% AMG, 2% Mupirocin, or a mixture of both, there were no colony growth after 24 hours. AMG appears to work as effectively as Mupirocin in treating *S. aureus* infection reducing bacterial burden by 100%.

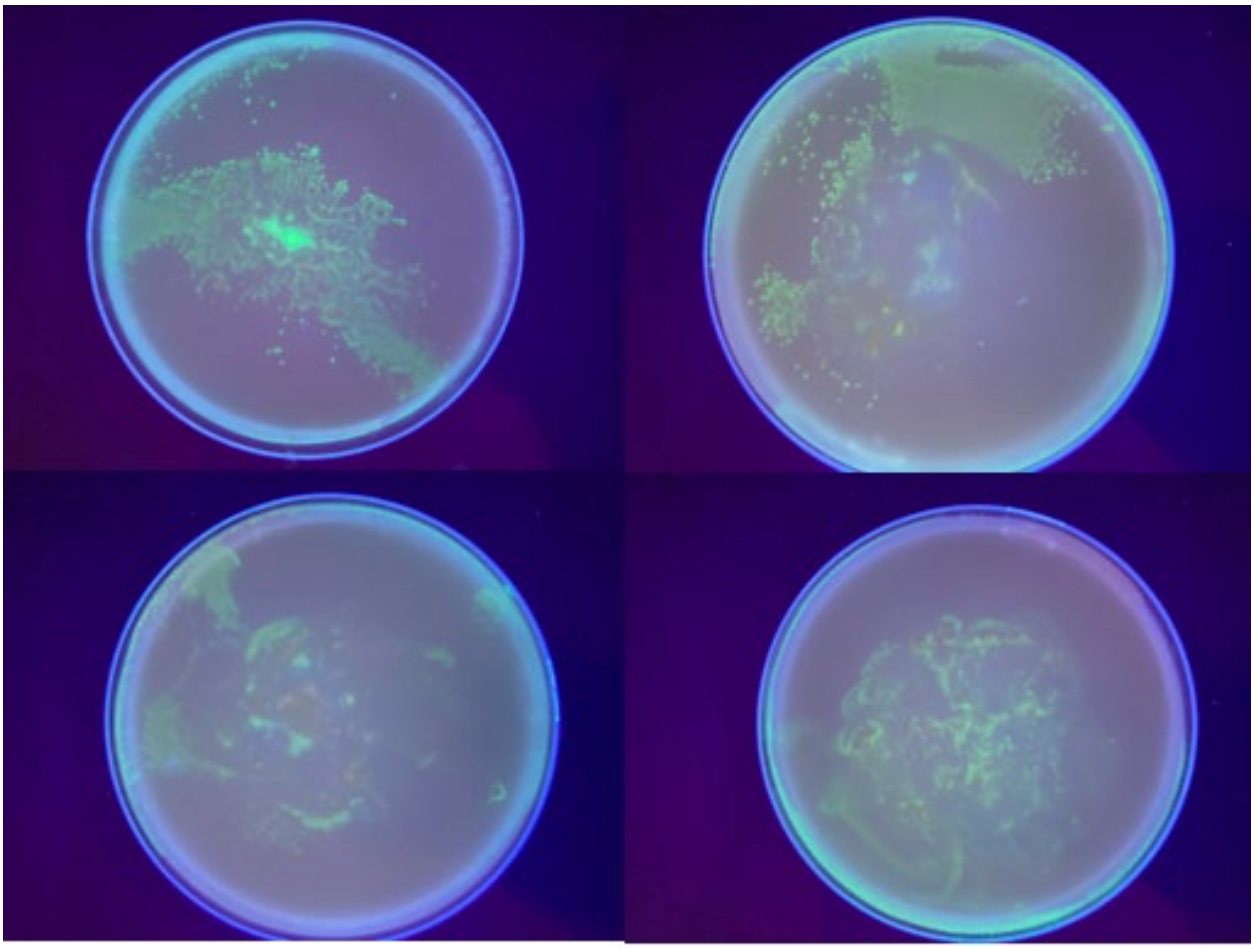


Figure 16. UV Visualization of Mature Artificial Wound Models**.** Wound beds were created as previously described and inoculated with AH133. Wounds were incubated for 24 hours and then treated with either (B) 100% AMG, (C) 2% Mupirocin, or (D) 100% AMG + 2% Mupirocin. Wounds were again incubated for 24 hours and imaged.

The same wound models were created as described and inoculated with AH133. These models were incubated at 37°C for 24 hours. Following incubation plates were treated with either (B) 100% AMG, (C) 2% Mupirocin, and AMG + Mupirocin. Models were again incubated at 37°C for 24 hours and then imaged using UV light. This model represents a wound that has been left untreated for 24 hours and bacteria to proliferate and begin biofilm formation. Each model was washed generously with 1X PBS and serially diluted to obtain CFU/ml (n=1). For wounds treated with 100% AMG there is a decrease in colony growth consistent with images (Fig 17).

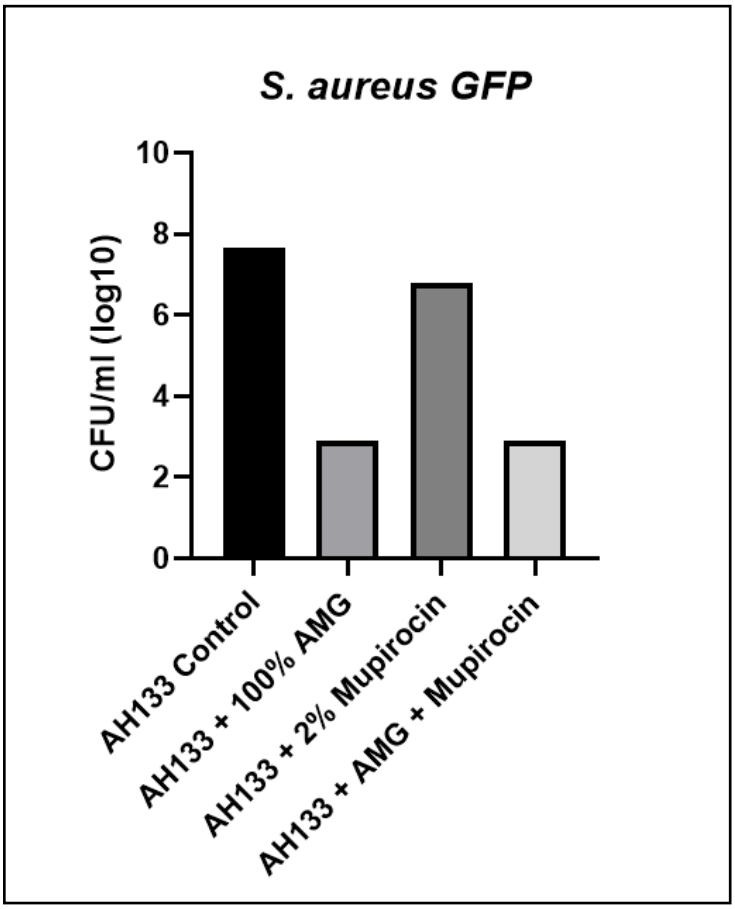


Figure 17. AMG treatment of mature artificial wound models. Artificial wounds inoculated with AH133 were incubated at 37C for 24 hours. Wounds were then treated with either 100% AMG, 2% Mupirocin, or a combination of both. Wounds were incubated an additional 24 hours, washed with PBS and serially diluted. CFU/ml were obtained. Colony growth was inhibited by both AMG treated groups in comparison with control plates (n=1).

CHAPTER 4. DISCUSSION

Infected wounds support a variety of pathogenic species and many carry resistance genes that help protect them from antibiotics and host immune responses. A component of this resistance is the ability to form biofilms. Biofilms can contain multiple microbial pathogens that can co-exist together as well as support one another's growth. Numerous topical antimicrobial products have been developed to combat these pathogenic species in wounds. These topical products vary in their ability to eradicate planktonic bacteria, prevent biofilm production, and penetrate existing biofilms. However, with each antimicrobial product created, microbes have stayed a step ahead and developed resistance. This is a concern of both human and veterinary medicine because without new antimicrobial treatment options, resistant pathogens will continue to cause substantial issues in healthcare.

In this study, we evaluated the efficacy of AMG (antimicrobial gel), a novel wound antimicrobial product designed to accelerate wound healing, reduce pain, and clear microbial infections. This all-natural compound was created to be a safe and effective alternative to traditional antibiotic products. AMG is effective in inhibiting microbial growth of Gram positive and Gram negative bacteria as well as the eukaryotic opportunistic fungal pathogen *Candida albicans.* The inhibitory effects of AMG appear to be fast acting, as each of the microorganisms in our panel were inhibited in planktonic growth immediately and continued over 24 hours. This included inhibition of bacteria commonly associated with both human, *S. aureus*, and canine, *S. pseudointermedius*, skin infections. This inhibition by AMG was also evident in other forms of microbial growth, namely microbial biofilms. AMG exerted a strong effect on inhibiting the attachment phase of biofilm growth. This is a necessary precursor for biofilms to become established and by being a potent inhibitor, AMG could be used in the early stages of skin injury

to prevent further development of microbial infections. AMG was able to inhibit pre-established biofilms, but not at a significant rate. Biofilms are naturally more resistant to treatments of any kind, so it stands to reason that it may require more than 10% AMG to see a significant decrease to established biofilms. As *S. aureus* was inhibited by AMG the greatest of the microbes in the panel and due to its ability to cause severe infections in humans and prevalence in healthcare settings, we decided to investigate AMG's effects on *S. aureus* further. Utilizing a GFP labeled strain of *S. aureus*, we were able to confirm the inhibition observed in planktonic cultures, but also the decrease in *S. aureus* attachment in biofilms thru observing the three-dimensional architecture of biofilms treated with AMG. Staining techniques also confirmed the inhibition of *S. aureus* by AMG. LIVE/DEAD staining showed decreased fluorescence of AMG treated *S. aureus* and a decrease in viable cells that was also supported by colony forming units. An artificial wound model demonstrated the practical and synergistic uses of AMG with the GFP labeled strain of *S. aureus* was employed to visually demonstrate AMG's ability to reduce bacterial burden within the wound-like media. Used both alone and in conjunction with the topical antibiotic Mupirocin, AMG is able to reduce the bacterial burden of *S. aureus* by 100%. Genetic analysis of *S. aureus* treated with AMG was not as conclusive but gives us a direction for further experiments as we found an upregulation in both Sprx and PrsA genes within *S. aureus* samples when treated with 1% AMG.

To end with, AMG is shown to be a novel formulation for inhibiting infections of skin injuries, particularly of those microorganisms associated with burn wound infections. We hypothesize that AMG works by creating a reducing environment and destabilizing the microbial cell. This destabilization affects the integrity of the cell and thusly the processes that are required to control what is allowed into and out of the cell. Due to the all-natural components of AMG,

we have not observed any resistance by *S. aureus*, nor do we anticipate there to be a development of resistance to AMG. As there is not a specific target that AMG uses and because it acts in a suppressive manner rather than a cidal manner, bacteria may not have the pressure to obtain resistance mechanisms. AMG show effective potential as a new tool in the treatment of and prevention of microbial topical infections.

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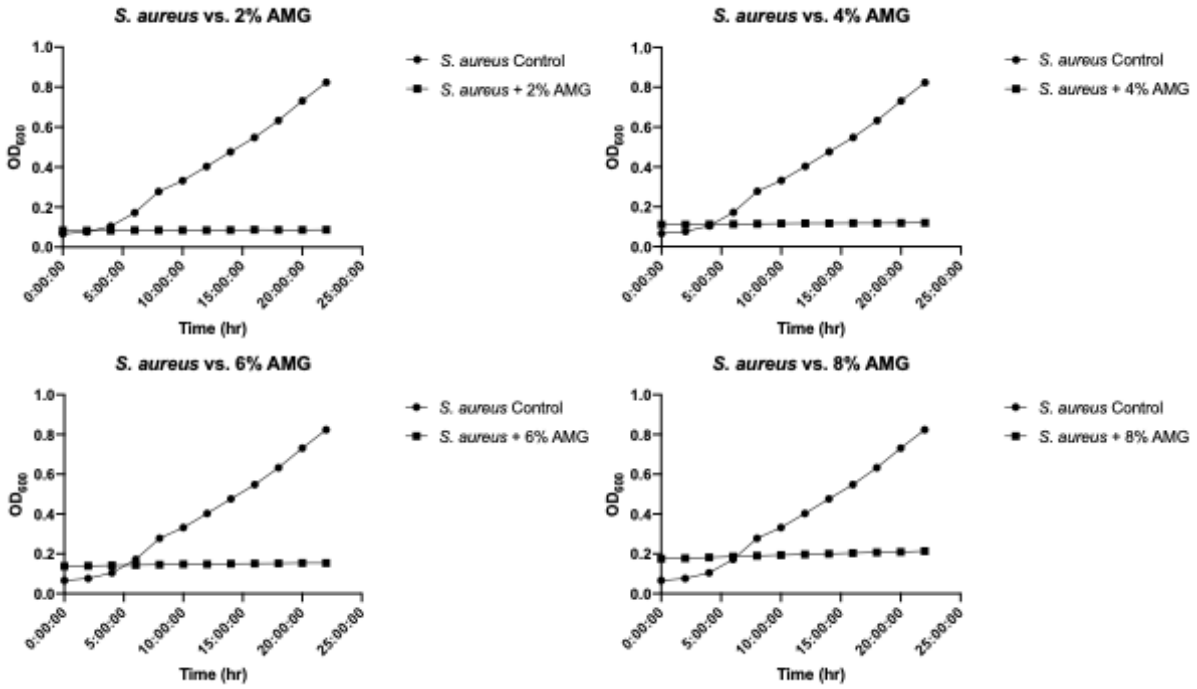
APPENDICES

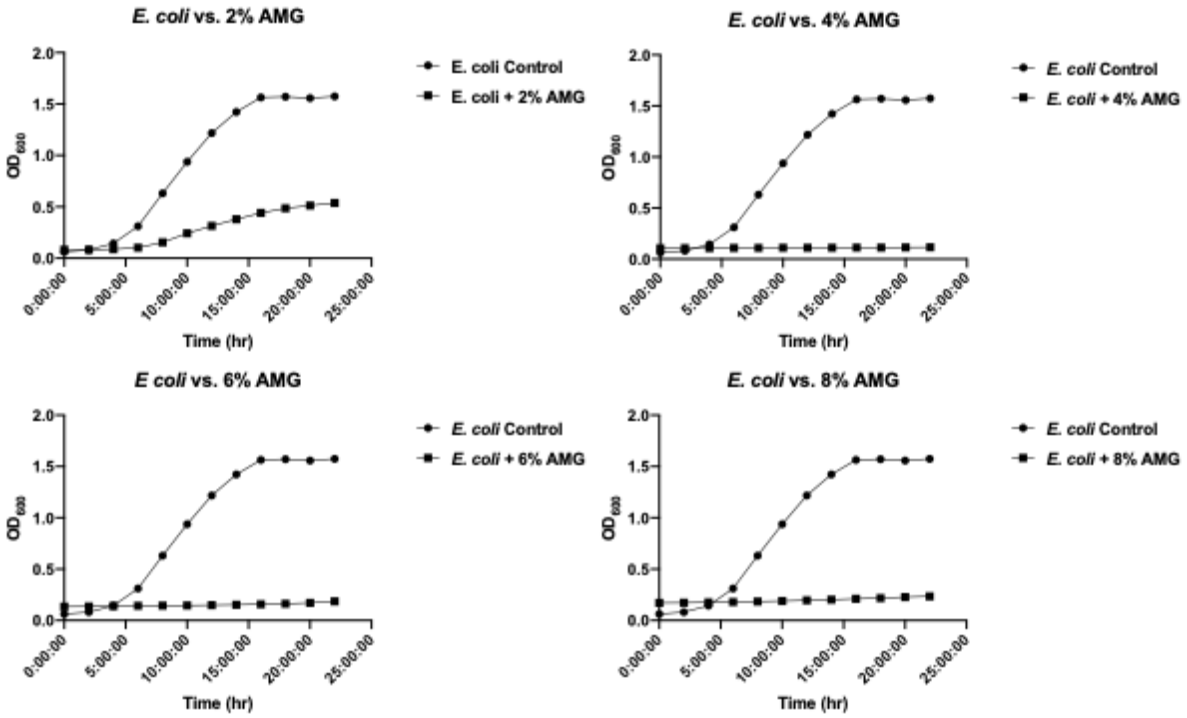
Appendix A: Supplemental Data

Supplemental Data:

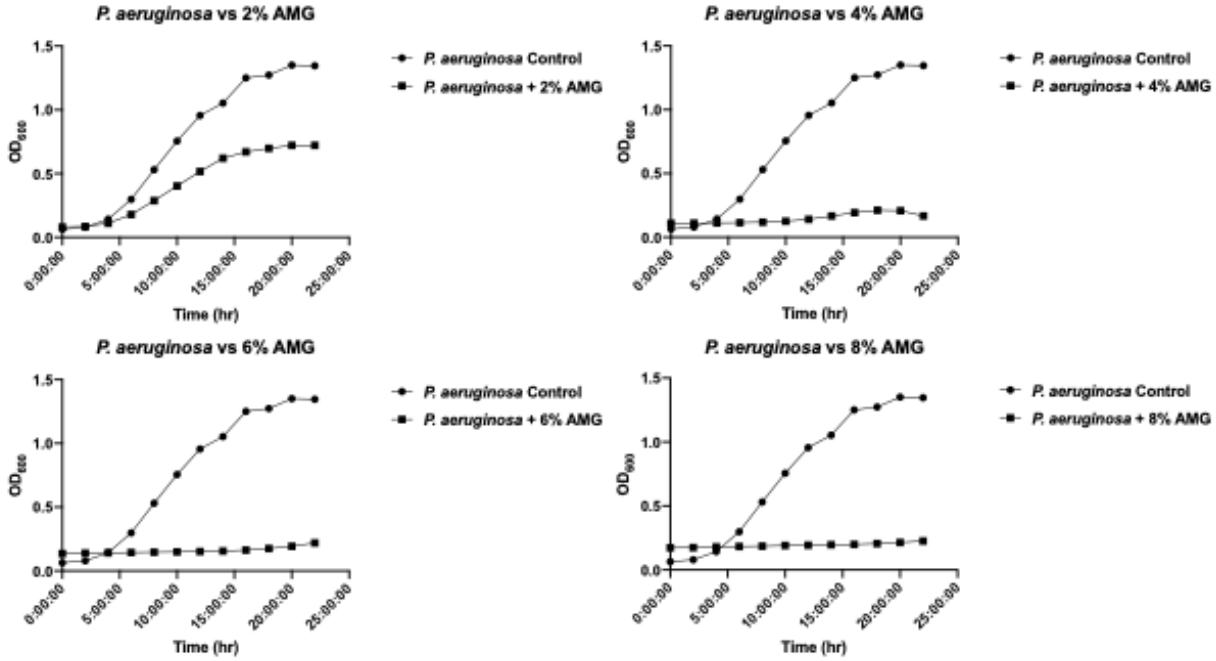
Kinetic Growth Curves



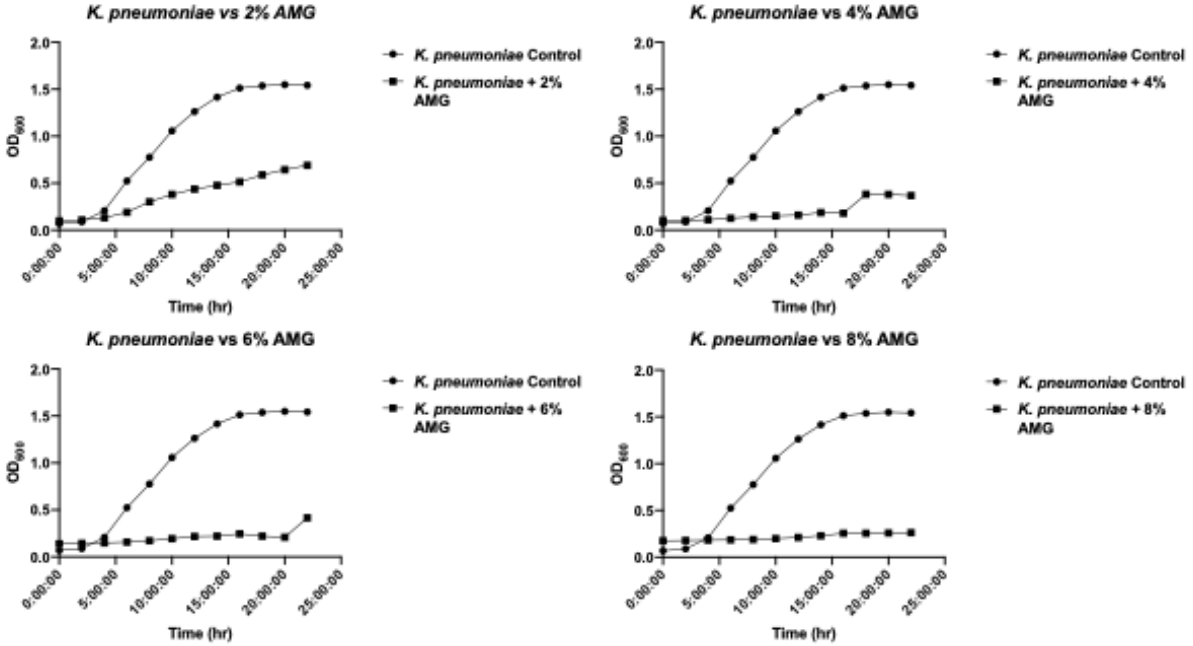
Supplemental fig A.



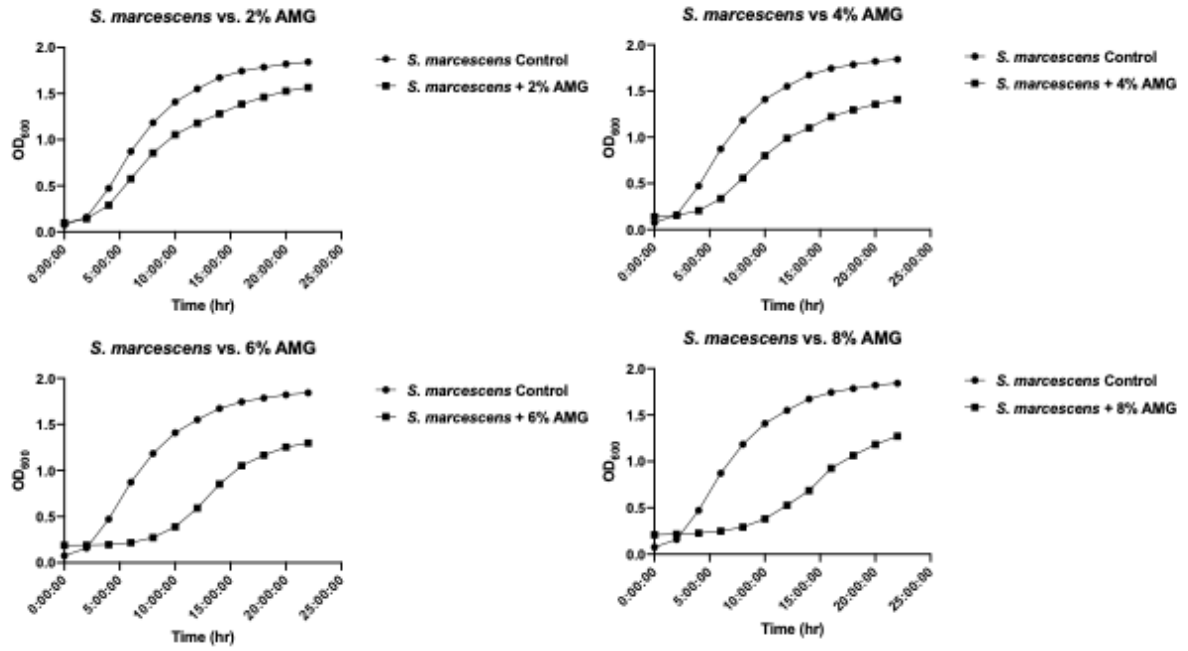
Supplemental fig B



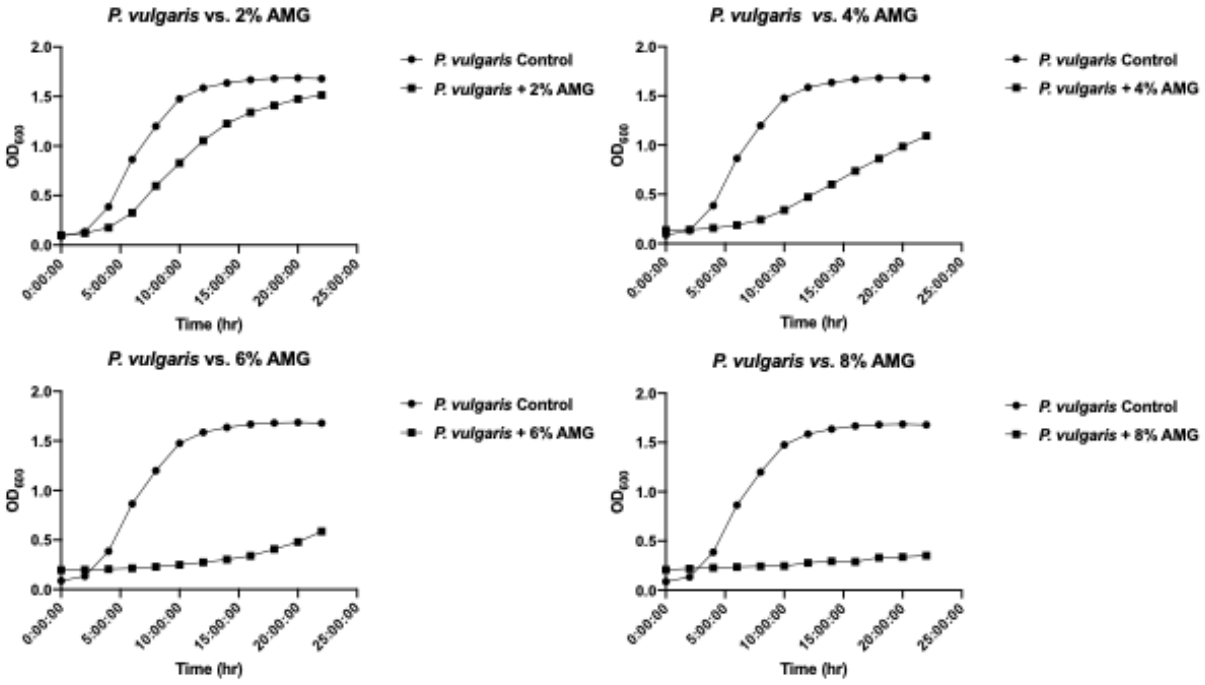
Supplemental fig C.



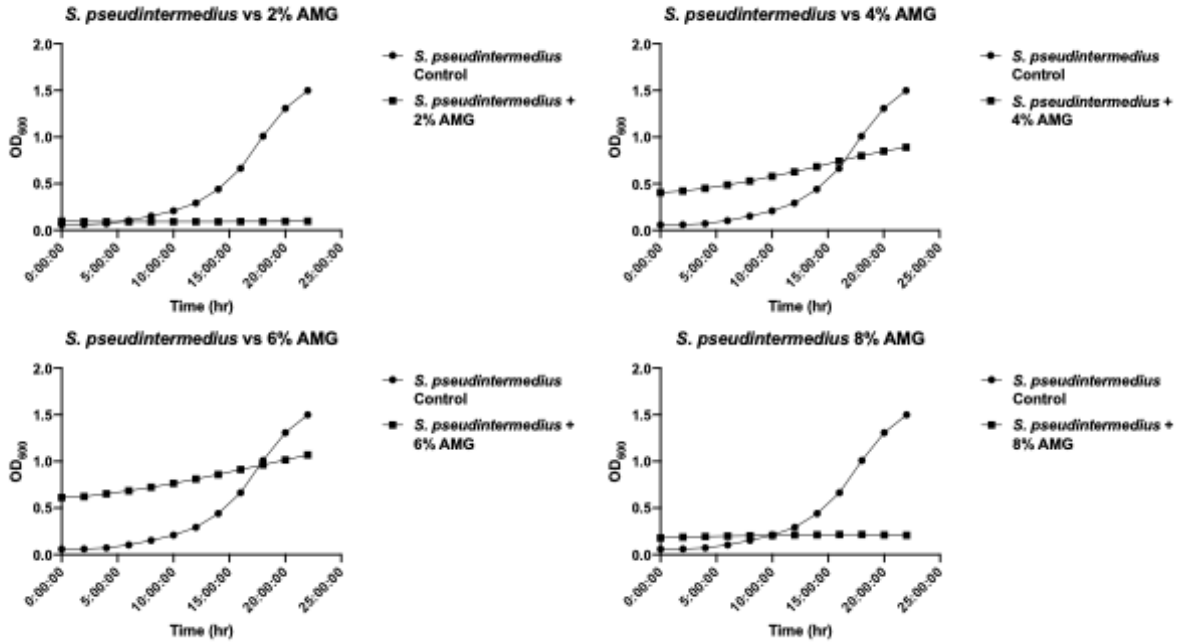
Supplemental fig D.



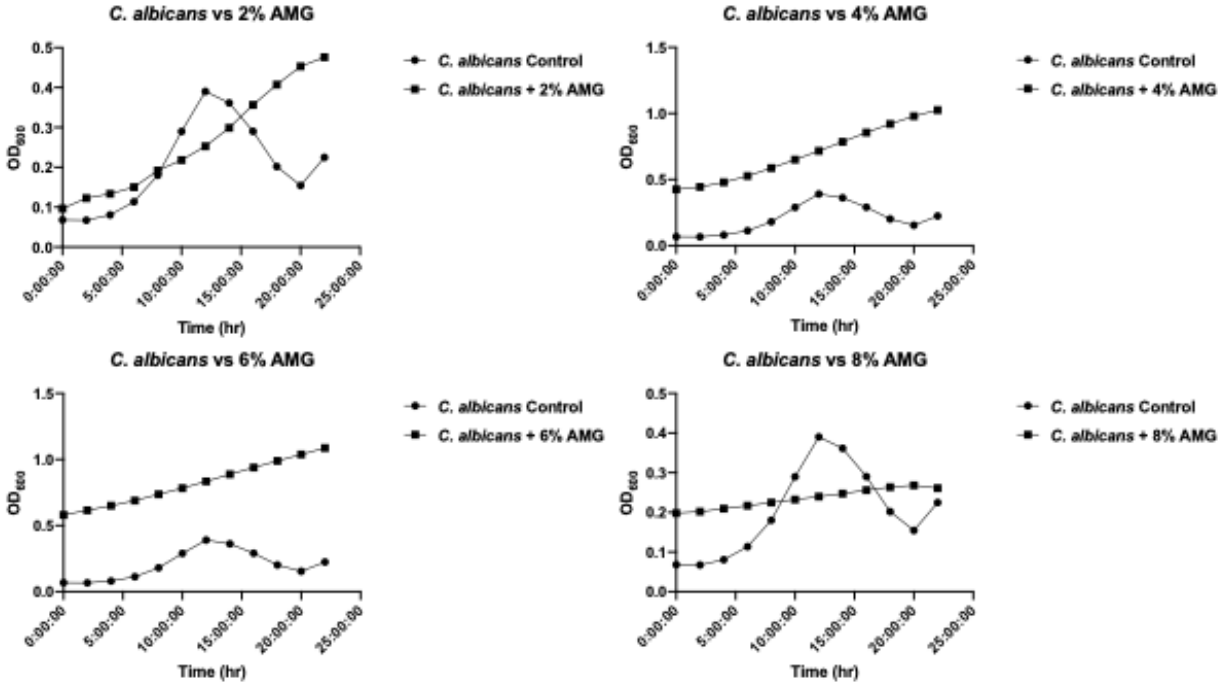
Supplemental figure E



Supplemental Figure F



Supplemental fig G



Supplemental fig H

Pathogen	Mean	Mean- Background Control	SD	P - Value	Percent Difference
S. aureus Control	1.08	0.832	0.191	0.0007	96.8
S. aureus + 10% AMG	0.173	0.026	0.007		
E. coli Control	0.917	0.666	0.256	0.0007	93.8
E. coli + 10% AMG	0.188	0.040	0.020		
P. aeruginosa Control	1.067	0.816	0.108	6.138E-05	75.6
P. aeruginosa + 10% AMG	0.346	0.198	0.185		
P. vulgaris Control	1.067	0.816	0.427	0.001	100
P. vulgaris + 10% AMG	0.147	0	0.030		
K. pneumonia Control	0.833	0.582	0.247	0.010	94.1
K. pneumonia + 10% AMG	0.182	0.034	0.053		
C. albicans Control	0.286	0.035	0.043	0.0009	37.2
C. albicans + 10% AMG	0.17	0.022	0.024		
S. pseudintermedius Control	0.081	0.029	0.028	0.349	11.0
S. marcescens Control	0.186	0.134	0.023	0.007	43.7
S. marcescens + 10% AMG	0.104	0.008	0.025		

Table S1. Statistical analysis of biofilm attachment groups. All analysis was completed using excel and PRISM9.

Pathogen	Mean	Mean-background control	SD	P-value	Percent Difference
S. aureus Control	0.062	0.021	0.016	0.094	16.9
S. aureus + 10% AMG	0.051	0.011	0.006		
E. coli Control	0.072	0.032	0.006	0.0001	24.9
E. coli + 10% AMG	0.054	0.014	0.004		
P. aeruginosa Control	0.096	0.056	0.051	0.042	-125.1
P. aeruginosa + 10% AMG	0.218	0.177	0.134		
P. vulgaris Control	0.066	0.026	0.013	0.005	33.2
P. vulgaris + 10% AMG	0.044	0.004	0.002		
K. pneumonia Control	0.064	0.024	0.015	0.258	9.06
K. pneumonia vs 10% AMG	0.058	0.018	0.014		
C. albicans Control	0.057	0.017	0.011	0.206	11.9
C. albicans vs 10% AMG	0.050	0.001	0.004		
S. pseudintermedius Control	0.051	-0.004	0.003	0.0002	-54.2

S. pseudintermedius + 10% AMG	0.079	-0.084	0.003		
S. marcescens Control	0.118	0.062	0.022	0.109	19.1
S. marcescens + 10% AMG	0.095	-0.068	0.011		

Table S2. Statistical analysis of Mature biofilms samples. All analysis was completed using excel and PRISM9.

Appendix B: AMG Patent

AMG Patent

US Patent Number 10,912,759 awarded on February 9, 2021. "Topical Gel Compositions for the Treatment of Staphylococcus Infections". Lavengel® is a Registered Trademark with the United States patent office

VITA

TASHA K. NELSON

Education:	B.S., Health Science, East Tennessee State University, Johnson City, Tennessee, 2019
	M.S., Biology, East Tennessee State University, Johnson City, Tennessee, 2021
Professional Experience:	Graduate Assistant, Department of Health Sciences, East Tennessee State University, 2019-2021
	Student Researcher, Department of Health Sciences, East Tennessee State University, 2019-2021
Award:	Graduate Student Research Grant, East Tennessee State University Graduate School, 2020