Modulating the Toxicity of Polyamines in *Staphylococcus aureus*

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USA300 is a recently discovered strain of community-acquired Methicillin-resistant *S. aureus* (CA-MRSA) that has been shown to be more virulent and transmissible than any other strain of MRSA. Like other strains of *S. aureus*, USA300 possesses resistance to *β*-lactam antibiotics; however, USA300's increased potency is owed to its resistance to the naturally occurring polyamines spermine and spermidine. While spermine and spermidine are toxic to most strains of *S. aureus,* USA300 encodes a gene known as *speG* that confers resistance to these compounds. Because the target of spermine and spermidine is cytosolic and *speG* is a cytosolic protein, polyamine toxicity is thus limited by cellular uptake, with polyamine resistance additionally being conferred from within *S. aureus* cells. This thesis thus serves to determine whether polyamine uptake can be increased to improve toxicity against USA300 clones.

We have shown that unsaturated fatty acids such as palmitoleic acid, oleic acid, and linoleic acid are highly toxic to USA300 clones, presumably through the fatty acids' incorporation into membrane phospholipids resulting in membrane disruption. In turn, we have shown that treatment of *speG*-deficient USA300 clones with combinations of palmitoleic acid, oleic acid, or linoleic acid with spermine results in higher levels of killing of these clones than the use of a fatty acid or spermine treatment alone. We have further shown that the treatment of wild-type USA300 clones with combinations of palmitoleic acid, oleic acid, or linoleic acid with the synthetic polyamines Bis(hexamethylene)triamine (HMTA) and Tris(3-aminopropyl)amine (TAPA) results in higher levels of killing of these clones that the use of a fatty acid or synthetic polyamine

treatment alone. Additionally, this thesis has identified the *S. aureus*-encoded gene *fakA* as a contributor to exogenous unsaturated fatty acid toxicity, presumably due to *fakA*-dependent incorporation of exogenous fatty acids into the bacterium's cellular membrane that could lead to increased membrane destabilization and the potential for increased polyamine uptake. As *S. aureus* is a skin and soft tissue infection, this thesis's findings provide evidence that a combination of unsaturated fatty acids and polyamines could serve as the key components of a future topical treatment for USA300-caused infections.

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Preface

As a high school senior, I completed an antibiotic resistance lab in an AP Biology course taught by Mr. Ryan Boylan. That lab would inspire me to pursue further research with antibiotic-resistant bacteria, and through this research, I had the privilege to work with several individuals whom I would like to acknowledge for their role in the completion of this thesis.

I would like to thank my thesis advisor, Dr. Anthony Richardson, for providing me with the mentorship necessary to complete this thesis. Dr. Richardson welcomed me into his lab as a collegiate freshman with minimal research experience, and through years of collaborative effort, he helped me gain a wealth of microbiological research experience that I will forever treasure. Within Dr. Richardson's lab, I would also like to thank Dr. Amelia Stephens, Ms. Kelly Hurley, and Dr. Srijon Banerjee for all their support over the past three years. Amie, Kelly, and Srijon, the time, patience, and understanding that you gave me allowed me to develop invaluable research skills, and simply put, this thesis would not have been possible with all your help.

I would like to additionally thank my academic advisor, Ms. Brenda McDevitt, for her assistance and compassion in helping me pursue an academic courseload that allowed me to actively work in my lab while taking classes that supplemented my work. I would also like to thank Dr. David Hornyak, Assistant Dean for the Honors College and coordinator for the BPhil program, for his willingness to work with me to allow me to complete the requirements of the BPhil program. Finally, to my parents, TR and Vimala, and my brother Arun, I would like to thank you for being an integral part of my support system as I completed this thesis. I am grateful for everything the three of you have done for me during my time at the University of Pittsburgh, and I appreciate all of you more than you can possibly believe.

1.0 Introduction

Staphylococcus aureus is a highly infectious bacterial pathogen that poses significant risks to humanity. Due to its nature as a bacterium capable of significant virulence factor production and antibiotic resistance, *S. aureus* has been a key contributor to the ongoing antibiotic resistance epidemic. As such, understanding the ways *S. aureus* infections can be effectively treated is paramount to addressing the increasing damage caused by *S. aureus*. This introduction will detail significant features of *S. aureus*, highlight potential non-antibiotic compounds that could prove toxic to *S. aureus*, and provide the basis for the experiments performed in this thesis.

1.1 Staphylococcus aureus

Staphylococcus aureus is a gram-positive bacterium capable of causing severe infection. *S. aureus* colonization is typically seen in the nose, throat, and skin, and it is estimated to be carried transiently by approximately 30% of the population and persistently by approximately 20% of the population [1]. Though this means that nearly half of the population is actively infected by *S. aureus*, the bacterium typically does not present with symptoms for individuals with healthy skin [2]. When *S. aureus* infection does causes symptoms, the bacterium present as a skin and soft tissue infection (SSTI) with the most severe symptoms including endocarditis and osteomyelitis [3].

S. aureus infections can be difficult to treat due to the bacterium's ability to evolve antibiotic-resistant determinants. The first penicillin-resistant strains of *S. aureus* were discovered shortly after the incorporation of penicillin into clinical practice in the 1940s, but the most

important development came in the 1960s when strains of *S. aureus* resistant to methicillin were discovered [4]. Since that time, the inability to readily treat methicillin-resistant *S. aureus* (MRSA) infections with penicillins or most β-lactam antibiotics has led to MRSA infections being responsible for longer hospital visits, increased healthcare costs, and increased morbidity rates [5].

Two major classifications of MRSA strains have been documented over the past half-century. Upon its initial discovery, MRSA was only typically associated with hospital or healthcare settings, but since their discovery in 1980s, the frequency of community-acquired MRSA (CA-MRSA) infections has significantly increased [5]. CA-MRSA strains possess significantly different phylogenetic traits than comparable strains of hospital-acquired MRSA (HA-MRSA), but more importantly, strains of CA-MRSA display increased virulence and transmissibility as compared to strains of HA-MRSA [5]. Ultimately, given the diminishing efficacy antibiotics have shown in treating MRSA infections, coupled with the increasing virulence and transmissibility associated with newly discovered strains of CA-MRSA, the use of new compounds that are effective in limiting MRSA's infectious activity is desirable.

1.2 *S. aureus* displays increased susceptibility to polyamines

Polyamines are non-antibiotic compounds that have shown bactericidal effects in most strains of *S. aureus*. There, insight is provided about the nature and function of polyamines, the mechanism of polyamine toxicity in sensitive strains of *S. aureus* is outlined, and the manner in which polyamine resistance in resistant strains of *S. aureus* is discussed.

1.2.1 Polyamines

Polyamines are aliphatic cations consisting of at least two amino groups. In all organisms, polyamines can be initially synthesized from L-arginine. In mammals, arginases are employed to convert L-arginine to L-ornithine, with L-ornithine then being decarboxylated to form putrescine and sequential addition of an aminopropyl group forming spermidine and spermine, respectively [6]. However, in bacteria, which typically lack arginases, L-arginine is decarboxylated to form agmatine, with agmatine then being converted to putrescine through either direct or indirect enzymatic conversion pathways [6].

Polyamines conventionally have positive pleiotropic effects in the majority of living organisms. Polyamines spread the positive charge from their attached amino groups along a flexible hydrocarbon backbone, thus allowing for the dissuasion of repulsive forces between nucleic acids and protein surfaces and the subsequent promotion of macromolecular synthesis [6]. Furthermore, inflammation, which sees high levels of nucleic acid synthesis, is accompanied by high amounts of polyamine production, suggesting that polyamines play a key role in modulating inflammatory processes [6]. Polyamines are also seen to regulate gene expression and outer membrane permeability in bacterial species [6]. Ultimately, the multifold function of polyamines seen in organisms across the three major kingdoms of life suggest that these compounds play a key role in facilitating important cellular activities essentially for organismal survival.

1.2.2 *S. aureus* interactions with spermine and spermidine

Though polyamine synthesis and use are seen to be an integral part of cellular function in organisms across the kingdoms of life, this phenomenon is not observed in *S. aureus*. *S. aureus*

has been shown to lack the biosynthetic genes necessary for the conversion of putrescine to spermidine and, subsequently, spermidine to spermine [6]. *S. aureus* has thus been shown to lack the capability for *de novo* polyamine synthesis, a phenomenon which is shared with other gram-positive bacteria such as *Streptococcus pyogenes* [6].

What is unique to *S. aureus* is its hypersensitivity to exogenous spermine and spermidine. Spermine and spermidine have been shown to be bactericidal in a variety of *S. aureus* strains, with spermine showing high levels of toxicity at a physiological concentration of 3 mM [6]. While *S. aureus* shares a lack of *de novo* polyamine synthesis with bacterial species such as *Francisella novicida* and *Haemophilus influenzae*, *S. aureus* is one of the only species shown to lack *de novo* polyamine synthesis and display hypersensitivity to spermine and spermidine [6].

There is evidence, though, that *S. aureus* can circumvent the toxic effects of spermine and spermidine. USA300 is a recently discovered strain of CA-MRSA that has shown resistance to exogenous spermine and spermidine. Unlike other strains of CA-MRSA, USA300 possesses a genetic island known as the arginine catabolic mobile element (ACME) that encodes for a gene known as *speG* [6]. *speG* encodes for an acetyltransferase, which is known as spermine/spermidine *N*-acetyltransferase, that acetylates spermine and spermidine to form a non-bactericidal derivative [6]. USA300 is thus able to grow effectively even when exposed to physiological concentrations of spermine and spermidine, with *speG* being shown to be responsible for conferring resistance to exogenous spermine and spermidine [6].

An important component of exogenous spermine and spermidine toxicity in *S. aureus* is the specific cellular target of the compounds. Spermine and spermidine are shown to have cytosolic targets within *S. aureus*, thus meaning that cellular uptake of these compounds is necessary for bactericidal effects to be observed [6]. The spermine and spermidine resistance-conferring *speG*

gene in USA300 clones has also been shown to be a cytosolic protein, thus showing that resistance to spermine and spermidine is conferred from within USA300 clones [6]. Ultimately, the interactions of polyamines with a variety of *S. aureus* strains demonstrates that polyamine toxicity in *S. aureus* is limited by both cellular uptake and the ability to encode resistance-conferring cellular components.

1.3 Exogenous fatty acids can destabilize *S. aureus*'s cellular membranes

As a gram-positive bacterium, the external surface of *S. aureus* consists of a layer of peptidoglycan surrounding a selectively permeable phospholipid bilayer [7]. Phospholipids consist of a hydrophilic "head" and two hydrophobic "tails," which themselves may consist of saturated fatty acid and unsaturated fatty acids [8]. Gram-positive bacteria lack the ability to synthesize unsaturated fatty acids, and thus rely on branched-chain fatty acids instead to modulate membrane fluidity. Importantly, unsaturated fatty acids contain at least one double bond that prevents the phospholipids they are a part of from stacking together, which in turn contributes to the fluid nature of the phospholipid bilayer [8].

S. aureus has been shown to have the ability to incorporate exogenous fatty acids, thus allowing the bacterium to use fatty acids to synthesize membrane phospholipids [9]. However, unsaturated fatty acids, such as oleic or linoleic acid, can have toxic effects on *S. aureus* due to membrane destabilization or peroxidation that can occur following incorporation of said fatty acids into the bacterium's cellular membrane [10]. Increased bacterial membrane fluidity leading to membrane destabilization has been linked to the observance of openings forming in bacterial membranes, which could be an avenue for increased cellular uptake of extracellular materials [11].

1.4 Hypotheses

S. aureus has shown hypersensitivity to polyamine treatments; however, the toxicity of treatments is limited by the cellular uptake of polyamines into *S. aureus* cells. Furthermore, in USA300 clones containing the resistance-conferring *speG* gene, cellular uptake of spermine and spermidine into *S. aureus* cells is limited by *speG* activity. Given this information, this thesis initially serves to examine if spermine and spermidine uptake and toxicity can be increased in sensitive strains of *S. aureus*. Furthermore, this thesis seeks to determine if the uptake of synthetic polyamine treatment that have shown to have bactericidal effects on USA300 clones can be increased in order to increase these polyamines' uptake and toxicity.

Given the ability of unsaturated fatty acids to cause membrane destabilization upon their incorporation into *S. aureus* cellular membranes, it is proposed that the use of select unsaturated fatty acids in tandem with spermine as a treatment in sensitive *S. aureus* strains will result in a synergetic effect, with the combined treatment showing higher efficacy in killing sensitive *S. aureus* strains than the use of a fatty acid or spermine treatment alone. Furthermore, given prior experimental findings showing the toxicity of the synthetic polyamines Bis(hexamethylene)triamine (HMTA) and Tris(3-aminopropyl)amine (TAPA) in USA300 clones, it is proposed that the use of unsaturated fatty acids with each of HMTA and TAPA as a treatment in USA300 clones will also result in a synergetic effect, with the combined treatment showing higher efficacy in killing the USA300 clones than the use of a fatty acid or synthetic polyamine treatment alone.

2.0 Methodology

2.1 Bacterial Assays

Bacterial plating efficiency assays were used to determine the rate of bacterial death seen in sensitive *S. aureus* strains and USA300 clones exposed to fatty acid and polyamine treatments.

2.1.1 Assays involving fatty acid-only treatments

Tested *S. aureus* samples were initially obtained from stock solutions stored at -80 °C, plated on plates containing brain-heart infusion (BHI) agar, and grown overnight at 37 °C. Subsequently, one to three colonies of each *S. aureus* sample to be tested were suspended in 5 mL of BHI broth.

Fatty acid treatments were initially prepared as 5 mL 10X solutions. Palmitic, palmitoleic, oleic, conjugated linoleic, and nitro-conjugated linoleic acid solutions were each prepared in 100% ethanol, and linoleic acid solutions were prepared in dimethyl sulfoxide (DMSO).

Tested *S. aureus* samples were inoculated in a sterilized 96-well plate. For any given inoculated sample, approximately 200 µL of each sample would be added to a well, and the appropriate fatty acid treatment would be added to the sample in a 1:10 volumetric ratio. For each type of fatty acid treatment, a non-inoculated *S. aureus* sample would be prepared for use as a control by adding approximately 200 µL of the sample to a designated well. Once each sample was inoculated, the 96-well plate was shaken for 18 hours at 250 rpm.

Following the 18-hour incubation period, approximately 100 µL of each of the incubated samples were removed and serially diluted with phosphate-buffered saline (PBS) solution to yield dilutions between 10^0 and 10^{-6} for each sample. Approximately 10 µL of each sample's dilutions were plated on plates containing BHI agar and subsequently grown overnight.

To determine the rate of bacterial death caused by the fatty acid treatments, the number of colony forming units (CFUs) per mL on the most diluted sample of the inoculated sample was determined. This value was divided by the value obtained for the number of colony forming units (CFUs) per mL on the most diluted sample of the non-inoculated control sample to yield the approximate rate of bacterial death caused by the fatty acid treatment. It should be noted that the amount of ethanol and DMSO used as fatty acid solvents has no effect on *S. aureus* viability.

2.1.2 Assays involving fatty acid and polyamine combination treatments

Assays involving the use of fatty acids and polyamines as treatment options for tested *S. aureus* samples were performed in a similar manner to assays involving only the use of fatty acids as a treatment for said samples. These assays, however, also included the preparation of polyamine treatments, which were initially prepared as 5 mL 10X solutions. Spermine, HMTA, and TAPA were each prepared in deionized water.

These assays involved the use of a non-inoculated *S. aureus* control sample, a tested sample inoculated with only a fatty acid treatment, a tested sample inoculated with only a polyamine treatment, and a tested sample inoculated with both a fatty acid and a polyamine treatment. Each treatment option was added into approximately 200 µL of each tested sample in a 1:10 volumetric dilution before being incubated for 18 hours at 250 rpm.

As with the assays involving only fatty acid treatments, after the 18-hour incubation period, approximately 100 μL of each of the incubated samples were removed and serially diluted with phosphate-buffered saline (PBS) solution to yield dilutions between 10^0 and 10^{-6} for each sample. Approximately 10 μL of each sample's dilutions were plated on plates containing BHI agar and subsequently grown overnight.

To determine the rate of bacterial death caused by each treatment, the number of colony forming units (CFUs) per mL on the most diluted sample of the inoculated sample was determined. This value was divided by the value obtained for the number of colony forming units (CFUs) per mL on the most diluted sample of the non-inoculated control sample to yield the approximate rate of bacterial death caused by each treatment option.

2.2 Phage transduction of *fakA::Erm^R* into LAC

fakA is a *S. aureus*-encoded kinase responsible for the phosphorylation of exogenous fatty acids, thus allowing for their subsequent incorporation into the *S. aureus* bacterial membrane [9]. To determine the impact of *fakA* in the synergetic treatment of USA300 with unsaturated fatty acids and polyamines, the *S. aureus* mutant NE229 (*S. aureus* JE2 Δ*fakA*::erm^R) was transduced into wild-type USA300 using Φ11 phage transduction.

To produce the Φ11-linked NE229 phage, an overnight culture of NE229 was grown at 37 °C, diluted at a 1:100 ratio in CY broth, and grown again at 37 °C to a OD_{660} reading of approximately 1.0. The dilute NE229 sample was then combined with Φ-11 phage and phage buffer and incubated at 30 °C.

Subsequently, wild-type USA300, classified as LAC, was grown up overnight at 37 °C before being treated with the Φ11-linked NE229 phage. The Φ11 phage transduced-LAC sample was plated on a plate containing tryptic soy agar (TSA), 0.5% sodium citrate, and 5 μg/mL erythromycin and grown at 37 °C to verify successful transduction, with polymerase chain reaction (PCR) and gel electrophoresis of the subsequently obtained transduced colonies performed to verify the genetic identity of the transduced samples.

2.3 qRT-PCR of macrophage samples

In addition to their uptake in *S. aureus*, spermine and spermidine uptake is observed to occur in efferocytotic macrophages, thus leading to immunomodulation of said macrophages [12]. Macrophage activity in the immune response to infection plays a critical role in eliminating *S. aureus*, so to understand the role of immunomodulation in macrophages responding to *S. aureus* infection, real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed on macrophages responding to *S. aureus* infection.

Purified RNA transcripts for GLUT-1 and HIF-1-α were obtained and quantified from efferocytotic macrophages responding to *S. aureus*. Using a Applied Biosystems Power SYBR green RNA-to Ct 1-step kit, qRT-PCR reactions involving the GLUT-1 and HIF-1-α were prepared and run in a BioRad iQ5 machine. C_t values were obtained through the BioRad iQ5 machine and iQ5 software, and through comparison to the housekeeping gene *18S*, $\Delta\Delta C_t$ values were obtained.

2.4 Statistical Analyses

Statistical analyses were performed to determine if the combination of fatty acids and polyamines as a treatment option results if significant higher rates of *S. aureus* death than the use of a fatty acid or polyamine treatment alone. Rates of bacterial death for the treatment options combining fatty acids and polyamines were compared to the treatments options using either a fatty acid treatment or a polyamine treatment using Student's t-tests. These tests were performed in the statistical analysis software Prism, with tests being run with one-tailed, nonparametric, and unpaired parameters.

3.0 Results

3.1 Select unsaturated fatty acid treatments cause bactericidal effects in USA300 clones

As a gram-positive bacterium, *Staphylococcus aureus* lacks the ability to synthesize unsaturated fatty acids, with these compounds causing membrane destabilization when incorporated into the *S. aureus* cellular membrane [9]. To confirm the types of fatty acids that would most effectively cause membrane destabilization, wild-type USA300 clones (classified as LAC) and USA300 clones lacking the *speG* gene (classified as *ΔspeG*) were treated with a variety of fatty acids. This section highlights how specific unsaturated fatty acids exert toxicity towards USA300 clones, potentially through the destabilization of USA300 clones' cellular membranes.

3.1.1 Use of unsaturated fatty acids is necessary to cause bactericidal effects in USA300 clones

To determine if fatty acid saturation can affect toxicity towards USA300 clones, *ΔspeG* and LAC were treated with a variety of concentrations of palmitic and palmitoleic acid. Both palmitic and palmitoleic acid are fatty acids with 16-carbon tails; however, palmitic acid is a completely saturated fatty acid, and palmitoleic acid is a monounsaturated fatty acid with a double bond at position C-9 [13].

Bacterial assays of *ΔspeG* and LAC with palmitic acid demonstrate that saturated fatty acids are unable to cause toxicity in USA300 clones (**Figure 1A**). Palmitic acid treatments of *ΔspeG* and LAC at concentrations less than 100 μM showed a lack of significant bacterial death,

with bacterial death virtually unobserved when *ΔspeG* and LAC were treated with 100 µM palmitic acid. This concentration vastly exceeded the highest concentration of palmitoleic acid administered to *ΔspeG* and LAC that still resulted in bacterial growth, hence showing that saturated fatty acids like palmitic acid are unable to cause cellular membrane destabilization in USA300 clones.

On the other hand, bacterial assay of *ΔspeG* and LAC with palmitoleic acid demonstrate that unsaturated fatty acids can cause toxicity in USA300 clones (**Figure 1B**). Concentrations of palmitoleic acid of 10 µM and lower do not appear to result in bacterial death of *ΔspeG* and LAC; however, the use of 25, 30, and 40 µM palmitoleic acid results in an average of 3-4 logs of bacterial deaths of *ΔspeG* and LAC, with the use of concentrations of palmitoleic acid of 50 µM and higher causing the virtual sterilization of tested *ΔspeG* and LAC cultures. Palmitoleic acid's demonstrated toxicity thus suggests that unsaturated fatty acids may cause cellular membrane destabilization in USA300 clones, potentially affecting polyamine uptake.

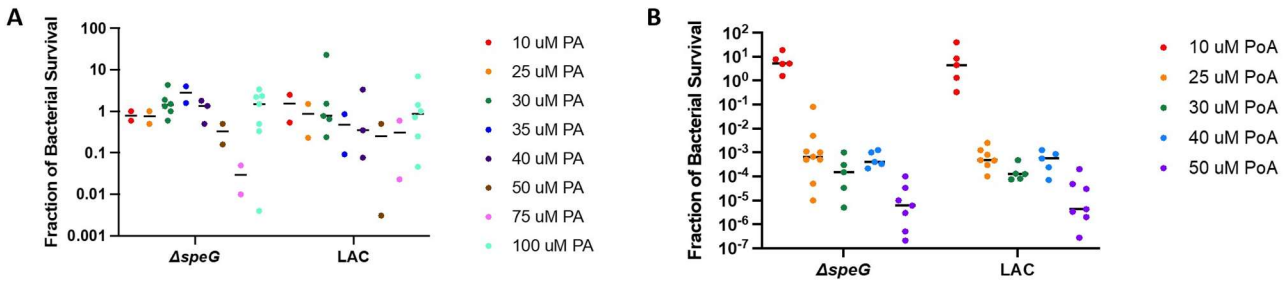

Figure 1. Bactericidal effects are observed in USA300 clones treated with unsaturated fatty acids. The use of the saturated fatty acid palmitic acid (PA) did not produce bacterial death in either *ΔspeG* and LAC (A), but treatment of the same strains with the unsaturated fatty acid palmitoleic acid (PoA) resulted in significant amounts of USA300 death.

3.1.2 Use of long unsaturated fatty acids causes bactericidal effects in USA300 clones

Like palmitoleic acid, oleic acid is a monounsaturated fatty acid; however, oleic acid has an 18-carbon tail and a double bond at position C-9 [13]. Bacterial assays of *ΔspeG* and LAC with oleic acid demonstrate that despite the increase hydrocarbon tail length, oleic acid is still able to cause toxicity via in USA300 clones (**Figure 2**). Notably, concentrations of oleic acid that were 100 times larger than concentrations of palmitoleic acid shown to be bactericidal in *ΔspeG* and LAC were needed to cause similar bactericidal effects in the same strains of USA300. Specifically, the use of oleic acid treatments at concentrations between 1 and 5 mM resulted in an average of 1-3 logs of bacterial deaths of *ΔspeG* and LAC. Oleic acid's demonstrated toxicity thus provides evidence that increased hydrocarbon tail length in unsaturated fatty acids does not preclude the ability of said compounds to display toxic effects in USA300 clones.

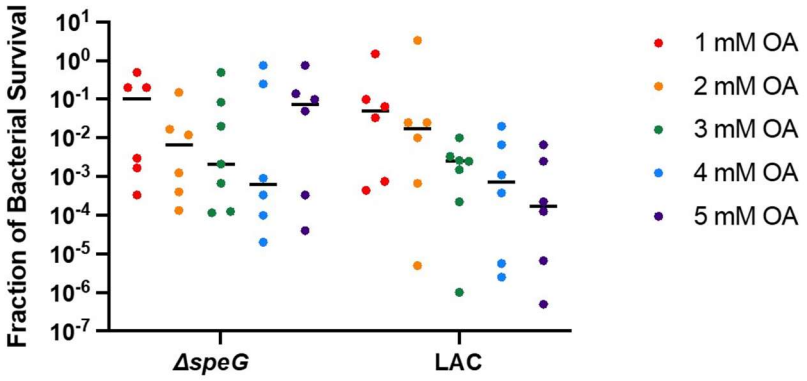

Figure 2. Treatment of USA300 strains with oleic acid (OA) produces bactericidal effects. The ability of oleic acid to cause bacterial death in USA300 strains means that hydrocarbon tail length in unsaturated fatty acids is not necessarily an obstacle to said compounds having bactericidal effects in USA300 strains.

3.1.3 Use of poly-unsaturated fatty acids causes bactericidal effects in USA300 clones

Like palmitoleic acid, linoleic acid is a 16-carbon tail; however, while palmitoleic acid contains only one double bond, linoleic acid contains double bonds at both positions C-9 and C-12 [14]. Bacterial assays of *ΔspeG* and LAC with linoleic acid demonstrate that despite the presence of multiple double bonds in the hydrocarbon tail, linoleic acid is still able to cause toxicity in USA300 clones (**Figure 3**). The concentrations of linoleic acid that were able to result in bactericidal effects in *ΔspeG* and LAC compare similarly to the concentrations of palmitoleic acid that were bactericidal in the same strains. Specifically, concentrations of linoleic acid of 10 µM and lower do not appear to result in bacterial death of *ΔspeG* and LAC; however, 25 µM linoleic acid caused an average of one log of bacterial death of *ΔspeG* and LAC, with 30, 40, 50, and 60 µM linoleic acid causing an average of 4-5 logs of bacterial death of *ΔspeG* and LAC. Linoleic acid's demonstrated toxicity thus provides evidence that increasing the number of double bonds in the hydrocarbon tail of unsaturated fatty acids does not necessarily preclude the ability of said compounds to display toxic effects in USA300 clones.

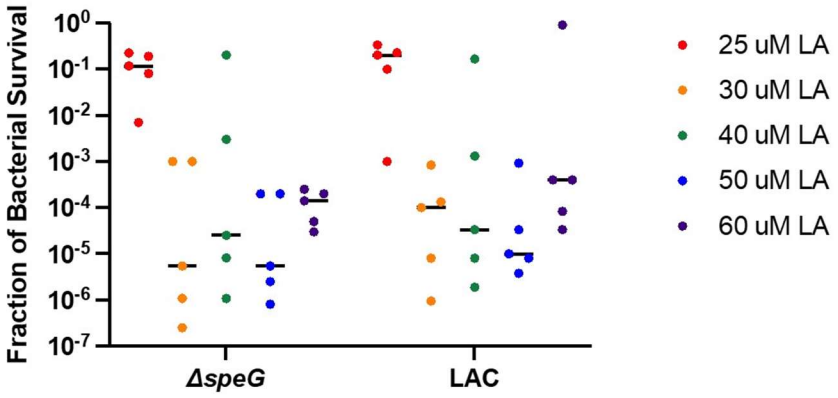

Figure 3. Treatment of USA300 strains with linoleic acid (LA) produces bactericidal effects. The ability of linoleic acid to cause bacterial death in USA300 strains means that the number of double bonds in the hydrocarbon

tail of unsaturated fatty acids is not necessarily an obstacle to said compounds having bactericidal effects in USA300 strains.

3.1.4 Conjugated poly-unsaturated fatty acids do not cause bactericidal effects in USA300 clones

Though linoleic acid treatments in *ΔspeG* and LAC suggest that the addition of double bonds to the hydrocarbon tail of unsaturated fatty acids does not necessarily preclude the ability of said compounds to display toxic effects in USA300 clones, the location of the double bonds within the unsaturated fatty acids' hydrocarbon tail could affect the ability of said compounds to display bactericidal effects in USA300 clones. Like linoleic acid, conjugated linoleic acid contains a 16-carbon tail with two double bonds; however, these double bonds are located at positions C-9 and C-11 [15].

Bacterial assays of *ΔspeG* and LAC with conjugated linoleic acid demonstrate that this compound does not have bactericidal effects on USA300 clones (**Figure 4A**). Treating *ΔspeG* and LAC with concentrations of conjugated linoleic acid between 1 and 10 μM showed a lack of significant bacterial death. Conjugated linoleic acid's lack of toxicity in USA300 clones hence suggests that the location of multiple double bonds in a hydrocarbon tail in an unsaturated fatty acid plays a role in dictating said compound's ability to cause bacterial cell death

Additionally, as previously stipulated, unsaturated fatty acids can have bactericidal effects in *S. aureus* because of either cellular membrane destabilization or peroxidation that occurs following unsaturated fatty acid incorporation into the bacterium's cellular membrane [10]. To test the potential for membrane peroxidation following incorporation of an unsaturated fatty acid, *ΔspeG* and LAC were treated with nitro-conjugated linoleic acid. Like conjugated linoleic acid,

nitro-conjugated linoleic acid contains a 16-carbon tail and two doubles bonds at positions C-9 and C-11 [16]. Significantly, though, nitro-conjugated linoleic acid contains a nitro group at position C-9 [16]. Nitro groups can be broken down into nitric oxide and reactive nitrogen species, both of which are bactericidal compounds [17]. As such, if nitro-conjugated linoleic acid toxicity was to be observed in USA300 clones, it could be attributed to either membrane destabilization caused by nitro-conjugated linoleic acid incorporation into the membranes of USA300 clones or peroxidation of the nitro-conjugated linoleic acid incorporated into the aforementioned cellular membranes.

Despite these potential modes of toxicity, bacterial assays of *ΔspeG* and LAC with nitro-conjugated linoleic acid revealed that this compound does not have bactericidal effects on USA300 clones (**Figure 4B**). Treating *ΔspeG* and LAC with concentrations of nitro-conjugated linoleic acid between 1 and 10 μM showed a lack of significant bacterial death. Nitro-conjugated linoleic acid's lack of toxicity in USA300 clones suggests that despite the compound's potential for causing bacterial death through either membrane destabilization or peroxidation effects, the compound does not possess the mechanisms necessary for causing bactericidal effects in USA300 clones.

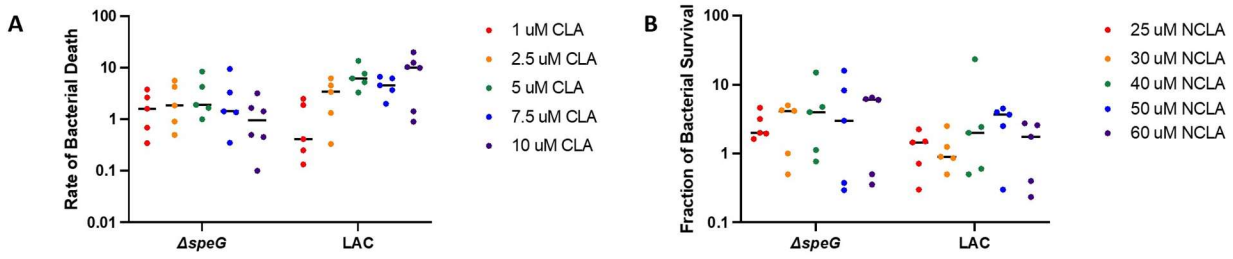

Figure 4. No bactericidal effects are seen in USA300 clones treated with conjugated poly-unsaturated fatty acids. The use of both conjugated linoleic acid (CLA) did not produce bacterial death in either *ΔspeG* and LAC (A). The same phenomenon was observed when nitro-conjugated linoleic acid (NCLA) was used to treat *ΔspeG* and LAC (B).

3.2 Combinations of unsaturated fatty acids with polyamines result in synergetic bactericidal effects in USA300 clones

Palmitoleic acid, oleic acid, and linoleic acid each were determined to have the capability to cause bactericidal death in USA300 clones. To test if these compounds could modulate the uptake of polyamines by USA300, *ΔspeG* and LAC were inoculated with treatments combining each of the aforementioned unsaturated fatty acids with either spermine, HMTA, or TAPA.

Based on the bacterial assays of *ΔspeG* and LAC with each of palmitoleic acid, oleic acid, and linoleic acid, the working concentrations of each unsaturated fatty acids for these experiments were 25 μM palmitoleic acid, 2 mM oleic acid, and 30 μM linoleic acid. The working concentrations of the tested polyamines were 3 mM spermine, 5 mM HMTA, and 10 mM TAPA. Rates of bacterial death in *ΔspeG* and LAC treated with both an unsaturated fatty acid and polyamine were compared to the rate of bacterial death experienced by each strain when exposed to a treatment consisting of only a corresponding unsaturated fatty acid and polyamine.

3.2.1 Combined unsaturated fatty acid and spermine treatments produce increased bactericidal effects in *speG*-deficient USA300 clones

Treatment of *ΔspeG* with each of palmitoleic acid, oleic acid, and linoleic acid in tandem with spermine demonstrated that the combined treatment option was highly effective in causing bactericidal effects. Multiple *ΔspeG* samples inoculated with a combination of an unsaturated fatty acid with spermine were seen to be completely sterilized, thus showing high efficacy of the combined treatment option. Furthermore, each treatment option combining an unsaturated fatty acid with spermine resulted in significantly higher levels of bacterial death of *ΔspeG* that the

treatment of this strain with either the corresponding unsaturated fatty acid or spermine alone (**Figure 5**).

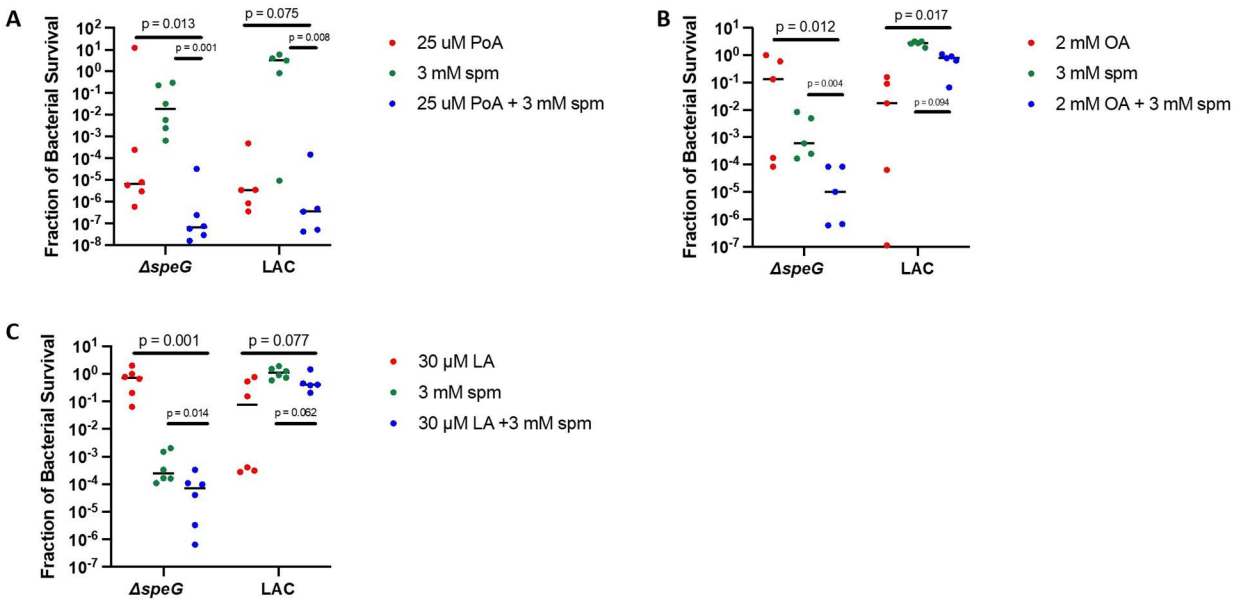

Figure 5. Treatment of *ΔspeG* with combinations of individual unsaturated fatty acids and spermine produces increased bactericidal effects than the use of one treatment option alone. Each of palmitoleic acid (PoA), oleic acid (OA), and linoleic acid (LA), when combined with spermine (spm), yielded significantly higher death rates of *ΔspeG* than the treatment of this strain with an individual unsaturated fatty acid or with spermine. Apart from the treatment of LAC with PoA and spm, no treatment combining an individual saturated fatty acid with spermine yielded significantly higher death rates of LAC than the treatment of this strain with an individual unsaturated fatty acid or with spermine.

As expected, LAC's possession of the spermine resistance-conferring *speG* gene meant that the treatment of this strain with a combination of each of palmitoleic acid, oleic acid, and linoleic acid in tandem with spermine resulted in similar levels of bacterial death of LAC to the levels seen when treated with the corresponding fatty acid alone (**Figure 5**). Ultimately, while the treatment options combining an unsaturated fatty acid with spermine may not have proved

effective in causing high levels of bacterial death in LAC, the results of the treatment of *ΔspeG* with the combination of an unsaturated fatty acid with spermine provides evidence that a mechanism exists for increasing polyamine toxicity in USA300 clones.

3.2.2 Combined unsaturated fatty acid and synthetic polyamine treatments produce increased bactericidal effects in wild-type USA300 clones

Previous lab members screened a library of synthetic non-canonical polyamines to identify the compounds that displayed bactericidal effects in both *ΔspeG* and LAC. Of these polyamines, HMTA and TAPA were identified as being able to cause toxicity in *ΔspeG* and LAC, with both compounds able to circumvent the resistance-conferring activity of *speG*. Knowing the susceptibility of *ΔspeG* to polyamines in general and the need to use canonical polyamines to cause bactericidal effects in LAC, samples of LAC were thus inoculated with treatments combining each of the synthetic polyamines HMTA and TAPA with each of palmitoleic acid, oleic acid, and linoleic acid. Treatment of LAC with these combined treatment options demonstrated that the combined treatment option was highly effective in causing bactericidal effects. In fact, as observed with the treatment of *ΔspeG* with combinations of each of palmitoleic acid, oleic acid, and linoleic acid with spermine, treatment of LAC with combinations of each of palmitoleic acid, oleic acid, and linoleic acid with either HMTA and TAPA resulted in complete sterilization of the LAC samples. Furthermore, nearly every treatment option combining an unsaturated fatty acid with either HMTA or TAPA resulted in significantly higher levels of bacterial death of LAC that the treatment of this strain with either the corresponding unsaturated fatty acid or spermine alone (**Figure 6**). These findings exempt the treatment of LAC with a combination of oleic acid and HMTA, as this treatment option was shown to cause similar rates of bactericidal death of LAC as

treatment of LAC with either oleic acid or HMTA alone (**Figure 6C**). The results of the treatment of LAC with each of palmitoleic acid, oleic acid, and linoleic acid with either HMTA and TAPA demonstrates the capability for increased toxicity of polyamine compounds in spermine and spermidine-resistance LAC when modulated by unsaturated fatty acids.

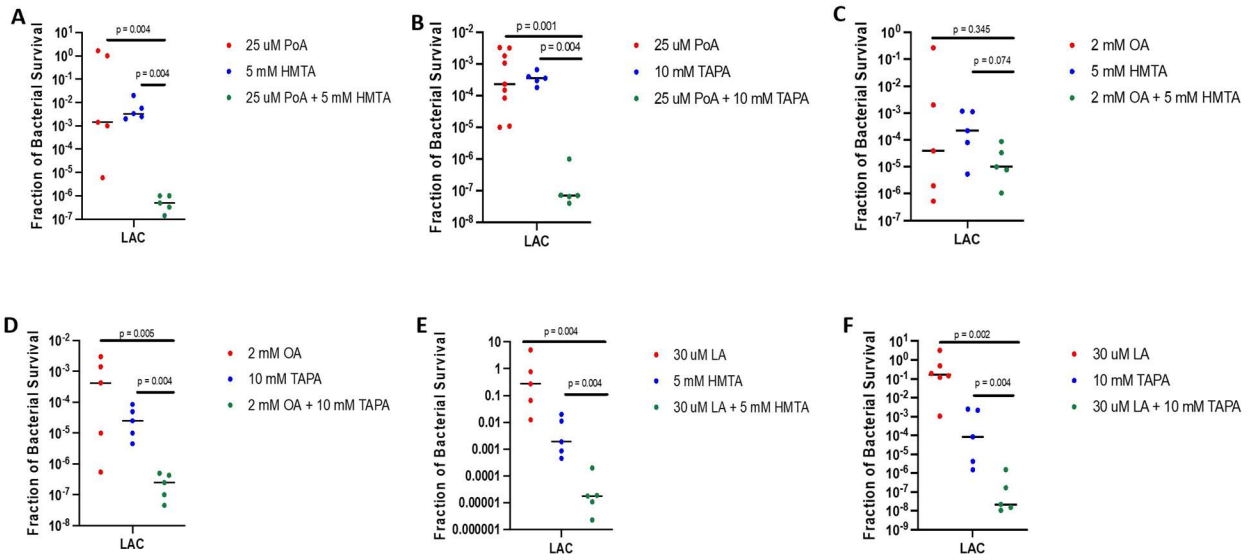

Figure 6. Treatment of LAC with combinations of individual unsaturated fatty acids and synthetic polyamines produces increased bactericidal effects than the use of one treatment option alone. Each of palmitoleic acid (PoA) and linoleic acid (LA), when individually combined with each of the synthetic polyamines Bis(hexamethylene)triamine (HMTA) and Tris(3-aminopropyl)amine (TAPA) yielded significantly higher death rates of LAC than the treatment of this strain with palmitoleic acid alone, linoleic acid alone, HMTA alone, or TAPA alone (A, B, E, F). While the combination of oleic acid (OA) with TAPA yielded a higher rate of death of LAC than the treatment of the strain with either oleic acid or TAPA (D), treatment of LAC with a combination of oleic acid with HMTA did not yield a higher rate of death of LAC than the treatment of the strain with either oleic acid or HMTA (C).

3.3 *fakA* is a key contributor to exogenous fatty acid incorporation into USA300 cellular membranes

The ability of *S. aureus* to incorporate exogenous fatty acids into the bacterial cellular membrane is for increased polyamine uptake and subsequently increased toxicity [9]. *S. aureus* has been shown to encode a fatty acid kinase, known as *fakA*, that has been shown to be responsible for the incorporation of exogenous fatty acids into the *S. aureus* cellular membrane [9].

To examine the effect of *fakA* on the ability of unsaturated fatty acids to be incorporated into the cellular membranes of USA300 clones and the subsequent ability for polyamine uptake to be increased, LAC and a mutant of LAC, classified as LAC Δ*fakA*::erm^R, were treated with working concentrations of palmitoleic, oleic, and linoleic acid. Rates of bacterial death in LAC Δ*fakA*::erm^R treated with each of the aforementioned unsaturated fatty acids were compared to the rates of bacterial death in LAC treated with the same unsaturated fatty acids.

3.3.1 Inactivation of *fakA* allows for partial rescue of wild-type USA300 clones treated with unsaturated fatty acids

Treatment of LAC Δ*fakA*::erm^R with palmitoleic acid, oleic acid, and linoleic acid demonstrated that inactivation of the *fakA* gene from LAC results in lower rates of bacterial death following fatty acid treatment (**Figure 7**). As compared to the treatment of LAC with palmitoleic acid, oleic acid, and linoleic acid, LAC Δ*fakA*::erm^R demonstrated lower levels of bacterial death; however, LAC Δ*fakA*::erm^R treated with oleic and linoleic acid still experienced an average of 1 log of bacterial death, with the same strain experiencing approximately 4 logs of bacterial death when treated with palmitoleic acid. These findings hence confirm the role of *fakA* in fatty acid

incorporation into the membrane of *S. aureus*; however, they also indicate that even after the removal of *fakA*, only partial rescue of USA300 can be seen.

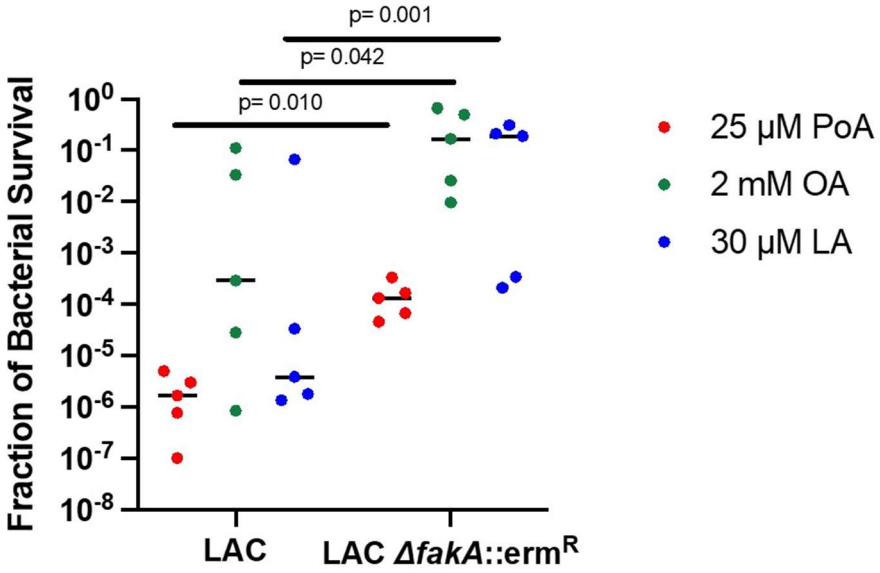

Figure 7. Inactivation of *fakA* in LAC allows for the partial rescue of LAC treated with unsaturated fatty acids. The LAC mutant LAC Δ*fakA*::erm^R, which is unable to incorporate exogenous fatty acids due to the deletion of the fatty acid kinase *fakA*, experienced significantly less bacterial death when treated with palmitoleic acid (PoA), oleic acid (OA), and linoleic acid (LA) as compared to samples of LAC treated with the same unsaturated fatty acids. Bacterial death was still observed in LAC Δ*fakA*::erm^R samples treated with palmitoleic acid, oleic acid, and linoleic acid, suggesting that even after the deletion of *fakA*, complete rescue of LAC following exogenous unsaturated fatty acid treatment cannot occur.

4.0 Discussion

The treatment of *S. aureus* with non-antibiotic options has been necessitated ever since the discovery of the first strains of MRSA in the 1960s. Though *S. aureus*'s susceptibility to polyamines indicated their promise in use in treatments against infections caused by the bacterium, the discovery of USA300 and its ability to confer resistance to spermine and spermidine thus means that a polyamine-only treatment would not necessarily be highly effective in treating USA300-caused infection.

This thesis demonstrates that high rates of bacterial death in USA300 clones can be achieved through the treatment of the strain with select unsaturated fatty acids and synthetic polyamines. Palmitoleic acid, oleic acid, and linoleic acid have all been demonstrated to allow for increased membrane destabilization with USA300 clones, which in turn allows for the increased uptake of polyamines like HMTA and TAPA into said clones. Given that the mechanism of toxicity for polyamines requires cellular uptake of said compounds, the ability for palmitoleic acid, oleic acid, and linoleic acid to provide an avenue for increased polyamine uptake indicates that an effective treatment option for USA300-caused infections would be one that combines unsaturated fatty acids with synthetic polyamine compounds.

Results obtained for bacterial assays involving each of palmitoleic acid, oleic acid, and linoleic acid suggest that the individual efficacy of these compounds can be quite variable. Each of the palmitoleic acid, oleic acid, and linoleic acid treatments repeatedly yielded significantly higher or lower rates of bacterial death of USA300 clones than the average obtained for each of these treatments. As such, a key factor to consider in future experiments is determining way in

which to decrease the variability in bacterial death rates observed with treatments including the use of unsaturated fatty acids such as palmitoleic acid, oleic acid, and linoleic acid.

While palmitoleic acid, oleic acid, and linoleic acid were identified in this thesis as the main contributors to causing bactericidal effects in USA300 clones, that is not to say that these acids alone are the only types of fatty acids that can contribute to causing the bacterial death of the aforementioned strain of *S. aureus*. This thesis has demonstrated that direct administration of exogenous nitro-conjugated linoleic acid to USA300 does not cause bacterial death via membrane destabilization; however, we have shown that nitro-conjugated linoleic acid enhances the activity of efferocytotic macrophages responding to USA300-caused infection. As such, unsaturated fatty acids can serve in the response to USA300-caused infection by both interacting against invading bacteria and with the immune response, thus indicating a high level of versatility to unsaturated fatty acids' use in responding to USA300-caused infections.

While this thesis provides evidence that USA300 infections could potentially be treated using treatments combining unsaturated fatty acids like palmitoleic acid, oleic acid, and linoleic acid with synthetic polyamines like HMTA and TAPA, further research would allow for determinations of the extent of combinations of unsaturated fatty acids and synthetic polyamines that can serve to effectively treat said infections. Compounds such as arachidonic acid and AEPD, which are an unsaturated fatty acid and synthetic polyamine, respectively, have been shown to have bactericidal effects in USA300, so testing with these compounds may provide even more options for potential synergetic treatment options for USA300 infections [10]. Increasing the scope of potential treatment options thus provides more options for responding to infection and prevents future mutations in USA300 that confer resistance to the synergetic treatments from readily

developing. USA300 infections have shown to cause debilitating effects in infected individuals, but as this thesis shows, there are means that exist to help more readily treat these infections.

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