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**The Uptake and Incorporation of Exogenous Fatty Acids Confer Phenotypic Advantages to *Acinetobacter baumannii***

**by**

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**Chairperson, University Departmental Honors Committee**

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## **II. ABSTRACT**

**AIM:** *Acinetobacter baumannii* is a ubiquitous gram-negative bacterium found on a variety of surfaces that include skin, hair, and soil. Some gram-negative bacteria like *A. baumannii* have the ability to acquire and incorporate fatty acids into their phospholipid membranes. Known as ‘Iraqibacter,’ *A. baumannii* has emerged as a significant cause of nosocomial infections in the United States and abroad. This study was designed to determine whether structural alterations occur in *A. baumannii* upon exposure to a wide range of polyunsaturated fatty acids (PUFAs) as well as the significance of this phenomenon in terms of survival and resistance to stress.

**METHODS:** Thin layer chromatography of isolated phospholipids indicated phospholipid profile variation depending on the exogenous PUFA supplied. To assess the incorporation of exogenous fatty acids with *A. baumannii* phospholipids, isolated lipids were analyzed by ultra-performance liquid chromatography/mass spectrometry (UPLC/MS). Membrane permeability was assessed with a crystal violet hydrophobic compound uptake assay. An assay for biofilm formation was performed to indicate the production of biofilms among bacterial growth in the presence of each fatty acid. To observe additional phenotypic responses, environmental stresses such as hydrogen peroxide, antimicrobial peptide, and antibiotic pressures were assayed.

**RESULTS & CONCLUSION:** The fatty acids affected membrane permeability, as determined by a hydrophobic compound uptake test. At least a 20% range of uptake was observed between the PUFAs examined. Bacterial growth in the presence of each PUFA caused an increase in biofilm production. As expected, numerous unique phospholipid species were identified and were bioinformatically predicted to contain the exogenously supplied PUFA as one of their acyl chains. While no differences in

minimum inhibitory concentrations were observed with peroxide and beta-lactam antibiotic stresses, sensitivity of *A. baumannii* to two different antimicrobial peptides, colistin and polymyxin B, increased following growth in several of the PUFAs, with arachidonic acid displaying the highest inhibition. Since cationic antimicrobial peptides are believed to interact with bacterial lipid membranes for eventual pore formation, these results implicate exogenous fatty acids as modification moieties that may impact resistance to environmental stresses.

### **III. INTRODUCTION**

One of the most prevalent challenges in the realm of healthcare has arisen in the form of multidrug-resistant bacteria. Examples of flourishing bacteria include *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii*. These are four of six ‘ESKAPE bugs’ that were classified by the Infectious Diseases Society of America in 2008. ESKAPE bugs “are of immediate US health concern that collectively cause the majority of nosocomial infections and can escape therapeutic activity of currently available antibiotics.”<sup>1</sup> *A. baumannii* does not desiccate easily and is notably responsible for a number of hospital-acquired diseases that affect various systems in the body. These diseases are typically difficult to treat and mainly affect individuals who are on ventilation or have previous wounds.<sup>2,3</sup> It was also given the nickname “Iraqibacter” after causing a spike in *A. baumannii* infections on wounded soldiers during the war in Iraq, which in turn caused a global spread of the bug as soldiers returned home. One publicized example of this occurred when ABC news correspondent Bob Woodruff became infected by *A. baumannii* in Iraqi soil from a roadside bomb in January of 2006, causing sepsis and pneumonia. He ultimately lived after losing part of his skull, but this instance is only one of numerous cases of *A. baumannii* occurring all over the world.<sup>4</sup>

*A. baumannii* is a gram-negative bacterium. Unlike gram-positive bacteria that have a single cellular membrane, gram-negative bacteria such as *A. baumannii* are defined by an inner and outer cell membrane with a periplasmic space and thin peptidoglycan layer in between. Therefore, import and export transport systems in these bacteria must exhibit cooperation between inner and outer membranes.<sup>5,6</sup> *A. baumannii* is regarded as a universal organism and can inhabit a variety of surfaces including human skin and hair as well as soil, and it is able to persist and survive in numerous types of

environments.<sup>7</sup> Infections of *A. baumannii* can lead to problems in the lungs, spinal cord, blood, urinary tract, or skin. While some *A. baumannii* infections are treatable, many of them can be fatal, especially if they are not noticed or treated quickly.

Some gram-negative bacteria have evolved pathways to utilize fatty acids as carbon sources, with their degradation ultimately leading to production of ATP. More recently, it has been discovered that exogenous fatty acids can be taken up by *A. baumannii* and incorporated into their membranes (Giles lab, unpublished data). The mechanism of the uptake of fatty acids by gram-negative bacteria begins with FadL, a transporter that resides in the outer membrane of the cell. When FadL binds to and transfers a fatty acid through the outer membrane of the cell, the fatty acid navigates through the periplasm and travels to the cytosolic face of the inner membrane, where it is joined with coenzyme A (CoA) via the enzyme FadD, yielding acyl-CoA. Now joined to a carrier molecule, acyl-CoA can be used as a carbon energy source in the  $\beta$ -oxidation pathway or can go back to the membrane; in the membrane, acyltransferases integrate the new fatty acid into membrane phospholipids.<sup>8</sup> Although several bacteria possess machinery to recycle exogenous fatty acids into their own membrane, there is no data to show how these scavenged fatty acids benefit the bacteria. The aim of this study is to explore fatty acid assimilation in *A. baumannii* and to examine potential survival and resistive advantages gained by lipid membrane remodeling.

The scale of infections and diseases that *A. baumannii* has the ability to cause is extremely vast and broad. The Centers for Disease Control and Prevention estimate that approximately 80% of all reported infections in healthcare facilities arise from *A. baumannii*.<sup>9</sup> Healthy people do not have as great a probability of contracting disease from *A. baumannii*, as it is typically seen within all types of healthcare settings, but



specifically in veterans' hospitals and intensive care units. Those who do contract the bacteria usually have compromised immune systems, diabetes, lung problems, open wounds, long hospital stays, or have invasive devices in them such as catheters or ventilators. *A. baumannii* can be transmitted either by contact with an infected individual or a contaminated surface.<sup>10</sup> Soldiers infected by "Iraqibacter" in Iraq and Afghanistan have reported cases of osteomyelitis, bacteremia, and various respiratory and wound infections. Researchers believe that rather than being infected via the soil or debris directly, military medical establishments are the source of most *A. baumannii* infections among soldiers. It is likely that a few individuals contracted it from soil or debris, which was then subsequently spread in medical facilities. Mortality rates related to *A. baumannii* infections are fairly high, whether their deaths are directly linked to the bacteria or other underlying problems.<sup>2</sup> This is most likely a result of the pre-existing medical conditions associated with the general population of people affected by *A. baumannii*, as mentioned before.

Due to its persistent nature, *A. baumannii* will always be around as long as there are vulnerable patients and lack of compliance towards infection control by healthcare providers.<sup>2</sup> While there are several preventative measures that can be taken to avoid exposure to the bacteria, the most common method of treating it is through antibiotics. Common treatment agents against *A. baumannii* include carbapenems, lactamase inhibitors, tigecycline, aminoglycosides, and polymyxin therapy.<sup>2</sup> Most forms of antibiotics, however, have become an issue as the number of multidrug-resistant strains of *A. baumannii* has risen.

To address our hypothesis that exogenous fatty acids can be utilized by *A. baumannii*, we first determined how the membrane of *A. baumannii* is modified

structurally and how these alterations may benefit the bacteria's survival and resistance to stress. We analyzed phospholipid species to see if the bacteria were in fact incorporating fatty acids in their membranes. Ultra-performance liquid chromatography/mass spectrometry confirmed that new phospholipid species were produced depending on the fatty acid supplied. After this confirmation, we tested the effects of exogenous fatty acids on hydrophobic compound uptake. Finally, the effects of environmental stresses were investigated through a series of tests and assays that established how various phenotypic stresses affect *A. baumannii* based on its ability to uptake exogenous fatty acids.

Understanding the capabilities of *A. baumannii* is of great importance in microbiology and public health fields. Studying *A. baumannii* can also have a potential effect on the global scale, as "Iraqibacter" is highly multi-drug resistant in nature and has rapidly spread in a short period of time. Ideally, the tests conducted in this experiment will help better understand the survival and virulence of *A. baumannii* so that we may be better prepared to contend with the bacteria holistically. These studies may also give insight to methods that may improve treatments of diseases caused by *A. baumannii* worldwide in the most effective and efficient manner.

## **IV. METHODOLOGY**

### ***MATERIALS***

The following fatty acids were collectively used throughout the entirety of this project: linoleic acid (18:2), -linolenic acid (18:3 ), -linolenic acid (18:3 ), dihom- -linolenic acid (20:3), arachidonic acid (20:4), eicosapentaenoic acid (20:5), docosapentaenoic acid

(22:5), and docosahexaenoic acid (22:6). The strain of *Acinetobacter baumannii* used was ATCC 17978. Fatty acids and bacteria were obtained through Cayman Chemical and the American Type Culture Collection, respectively. Bacteria were grown in either Luria broth (LB) or G-56 minimal media. G-56 was buffered with Hepes (pH 7.4) and supplemented with  $\text{KH}_2\text{PO}_4$  (0.3 mM), KCl (10 mM),  $(\text{NH}_4)_2\text{SO}_4$  (10 mM), glucose (0.2%),  $\text{FeSO}_4$  (0.03 mM), and thiamine (0.075 mM) as well as additional additives of  $\text{CaCl}_2$  (1 mM),  $\text{MgSO}_4$  (1 mM), and casamino acids (0.4%). G-56 was prepared the same day it was used after being filter sterilized. Crystal violet (CV) was purchased from Fisher Scientific, imipenem from LKT Laboratories, Inc., colistin (polymyxin E) from Adipogen International, and polymyxin B (PMB) from Cayman Chemical.

### ***PREPARATION OF BACTERIAL CULTURES***

Bacterial cultures were prepared uniformly each time an assay was performed. To begin, *A. baumannii* (ATCC 17978) was streaked onto an LB agar plate and left in the 37°C incubator overnight. The plate was removed the next day and placed into the refrigerator, where it remained fresh for at least two weeks. Every two weeks a new plate was streaked to maintain consistently fresh bacterial colonies. Overnight cultures were set each evening prior to running an experiment, which were grown in LB broth tubes in a 37°C shaking incubator.

### ***GROWTH CURVE***

Overnight cultures were pelleted, washed in G-56 (pH 7.4), and used to inoculate fresh cultures in G-56 at a starting optical density of 0.05. 300  $\mu\text{M}$  of each fatty acid (18:2, 18:3, 18:3, 20:3, 20:4, 22:5, and 22:6) was supplemented to the appropriate cultures. The prepared bacteria was placed in the 37°C shaking incubator (200 rpm). Optical densities were read every 30 minutes for a total of eight hours, followed by an additional

reading five hours later as well as twelve hours after that, yielding a total observation of twenty five hours of growth.

### ***LIPID EXTRACTION AND THIN LAYER CHROMATOGRAPHY***

Bacteria were grown at 37°C. Once reaching an optical density between 0.8 and 1.0, cultures were pelleted for 10 minutes. The supernatant was poured off, followed by the washing of the residual pellet in 5mL of phosphate buffered saline (PBS) that was then centrifuged for an additional 10 minutes. Again, the supernatant was removed, and the remaining pellet was resuspended in 5 mL of single phase Bligh/Dyer mixture. This mixture is made up of chloroform/methanol/water in a 1:2:0.8 ratio. Once resuspended, the culture is vortexed and incubated for a total of 20 minutes at room temperature, followed by a 10-minute centrifugation. The supernatant was drawn off and put into a new glass tube while the pellet was disposed of, as it was no longer necessary. In order to make the single phase Bligh/Dyer mixture into a two-phase mixture, 1.3 mL of chloroform and 1.3 mL of water were supplemented to the single phase mixture that was then vortexed and centrifuged for 10 minutes. Centrifugation creates a lower and upper phase of the mixture; the lower phase was drawn into a fresh glass vial, while 2.6 mL of chloroform was added to the upper phase. The vortex, centrifugation, and extraction step was performed once more, and the collective extractions were further washed by adding 5.2 mL of methanol and 4.7 mL of water. The final extraction was collected and dried down under a stream of nitrogen. The remaining lipids were kept in -20°C until the thin layer chromatography took place.<sup>10, 11</sup>

Thin layer chromatography was used to analyze lipids via a solvent made up of chloroform/methanol/acetic acid in a 65:25:10 ratio.<sup>12</sup> The chromatography creates lipid

profiles that were visualized using a spray of 10% sulfuric acid in 100% ethanol and heating the plate to 200°C.

## ***ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY & MASS***

### ***SPECTROMETRY***

Ultra performance liquid chromatography (UPLC) and mass spectrometry were used to determine the mass to charge ratio of phospholipid species in *A. baumannii* after they were grown in the presence and absence of various fatty acids. These are two separate processes, with UPLC used to separate lipids and mass spectrometry used to detect and assign specific mass to charge ratios to the species as they are ionized. Structural assignments were generated using the Lipid Metabolites And Pathways Strategy database (Lipid MAPS).<sup>12</sup> Mobile phase A consisted of 30:70 (25 mM ammonium acetate [pH 6.7]: methanol), and mobile phase B was 100% methanol. Isolated phospholipids were weighed, and 300 parts per million (ppm) were paired in 1 mL diluent (50:50 mobile phase A: mobile phase B) prior to injection into a Waters UPLC equipped with a reversed-phased C8 column. The injection volume was 5 µL, the run time was 15 minutes, and the flow rate was 0.4 µL/minute. The gradient was 50:50 up until the 2-minute mark, where it shifted to 20:80 until the 12-minute mark. Between 12 and 12.2 minutes, the gradient shifted back to 50:50 until the end of the run. Detection was by quadrupole mass spectrometry using electrospray ionization in the negative mode.

### ***CRYSTAL VIOLET UPTAKE***

Overnight cultures of *A. baumannii* were pelleted, washed, and used to inoculate in G-56 media at a starting optical density of 0.1. These cultures were supplemented with each of the following fatty acids: 18:2, 18:3 , 18:3 , 20:3, 20:4, 20:5, 22:5, and 22:6. Once the cells had grown to an OD<sub>600nm</sub> of approximately 1.0, they were transferred into sterile

plastic tubes and centrifuged for 10 minutes at 4000 rpm. Media was removed and the cells were washed with PBS. Each culture was suspended in 1 mL PBS and the optical density was determined. Cultures of equal starting OD were prepared in 5 mL of PBS, and 5  $\mu$ L of 5 mg/mL crystal violet solution was added (final concentration of 5  $\mu$ g/mL). The tubes were agitated at room temperature, and the optical density was read every five minutes for a total of 25 minutes. To read the optical density, 800  $\mu$ L of each culture was micro-centrifuged for one minute at 1200 g. The supernatant was analyzed spectrophotometrically for absorbance at OD<sub>590nm</sub>.

### ***BIOFILM FORMATION***

The protocol to test for biofilm formation was borrowed for a method by the O'Toole assay.<sup>13</sup> Overnight cultures were pelleted, washed in PBS, and prepared at an OD of 0.1. In the appropriate media, 300  $\mu$ M of each fatty acid (18:3 , 20:3, 20:4, and 22:6) was added to each culture. Microtiter plates were used for this assay, with each inoculum placed into its respective wells and grown overnight statically or shaking in the 30°C or 37°C incubator. After 24 hours, the biofilm was to be stained following O'Toole's protocol again. Excess cells were first removed by shaking the liquid out of each microtiter plate. The plate was submerged in a small tub of distilled water and then shaken to remove additional liquid. This step was repeated two or three times until planktonic cells were confidently discarded. At this point, 125  $\mu$ L of 0.1% crystal violet solution was added to each of the wells in the microtiter plate and incubated at room temperature for 10-15 minutes. The crystal violet was rinsed out of the plate by gently submerging it in distilled water three to four times followed by vigorous blotting on a paper towel. Once the crystal violet was rinsed out, the plate was dried upside down overnight. After 24 hours, 125  $\mu$ L of a prepared solution of 30% acetic acid was added to

each well in the microtiter plate to solubilize the crystal violet. This incubated at room temperature for 10-15 minutes as well. 125  $\mu$ L of the solubilized crystal violet was transferred into a new microtiter dish and read on a Biotek Microplate reader with Gensys software. The biofilm assay was conducted several times, with manipulating variables including temperature, pH, and incubation (shaking vs. static). A two-tailed T-test was done to determine which data points reflect significant differences.

### ***MOTILITY TEST***

To begin the setup for the motility test, five plastic tubes were prepared, each with 1 mL of LB and the appropriate amount of fatty acids required (18:3 , 20:3, 20:4, and 22:6). These were grown in a 37°C shaking incubator until they reached an OD of 0.1 and the calculated amounts of culture were added to each tube. The motility plates were prepared with 0.3% and 0.4% LB agar in sterile petri dishes. Each of the five tubes was calculated to an OD of 0.5 and 0.1. Once the agar plates were solidified and ready to use, four spots of *A. baumannii* were injected into them, with two at an OD of 0.5 and two at an OD of 0.1 for each plate. A total of ten plates were inoculated, five in 0.3% agar and five in 0.4% agar, with and without each specific fatty acid. These plates were incubated overnight at 37°C and analyzed for growth and motility the next day.

### ***BACTERIAL RESISTANCE TO PEROXIDE STRESS, ANTIMICROBIAL PEPTIDE RESISTANCE, AND ANTIBIOTIC RESISTANCE***

The peroxide stress, antimicrobial peptide, and antibiotic resistance tests were prepared and conducted identically, differing only in the concentrations and types of stresses used against the bacteria. Overnight cultures were pelleted, washed with G-56, and used to inoculate fresh culture in G-56 (with and without fatty acids) at a starting OD of 0.1. The fatty acids used in this assay included 18:3 , 20:3, 20:4, 20:5, and 22:6. Triplicate and

quadruplicate microtiter plates were prepared with a range of twofold dilutions of hydrogen peroxide (10 mM - 640 mM), colistin (0.4 µg/mL - 25.6 µg/mL), imipenem (0.1 µg/mL - 0.64 µg/mL), and polymyxin B (1.6 µg/mL - 102.4 µg/mL). Each well was inoculated with bacteria at a starting OD of 0.1, followed by incubation at 37°C in a shaking incubator for 20 hours. The plates were read the next day via the Biotek Microplate reader with Gensys software at an absorbance of 600 nm.

## V. RESULTS

### *GROWTH CURVE*

The growth curve assay provided an overview pattern of growth for *A. baumannii* in the presence

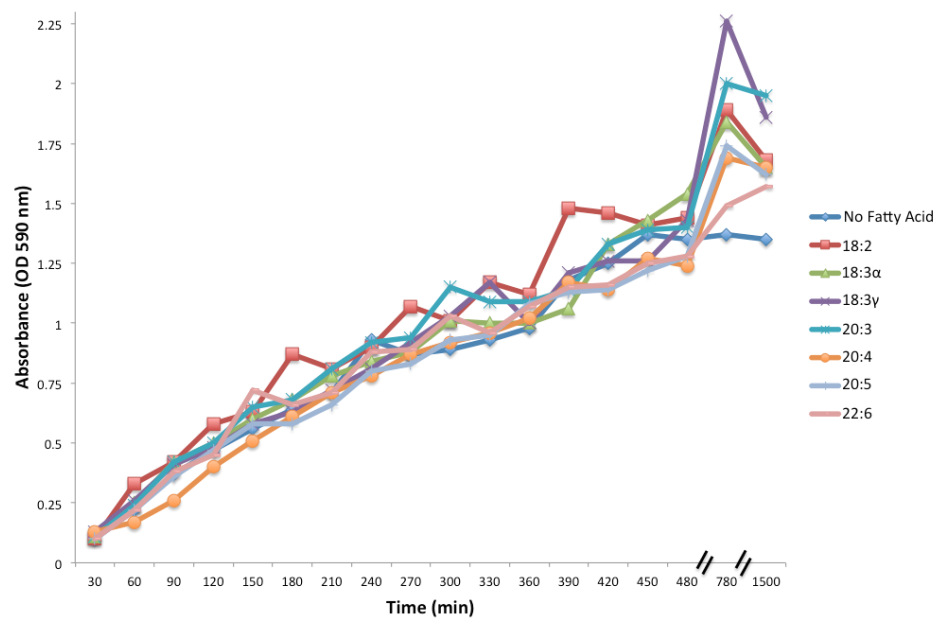
and

absence

various

fatty

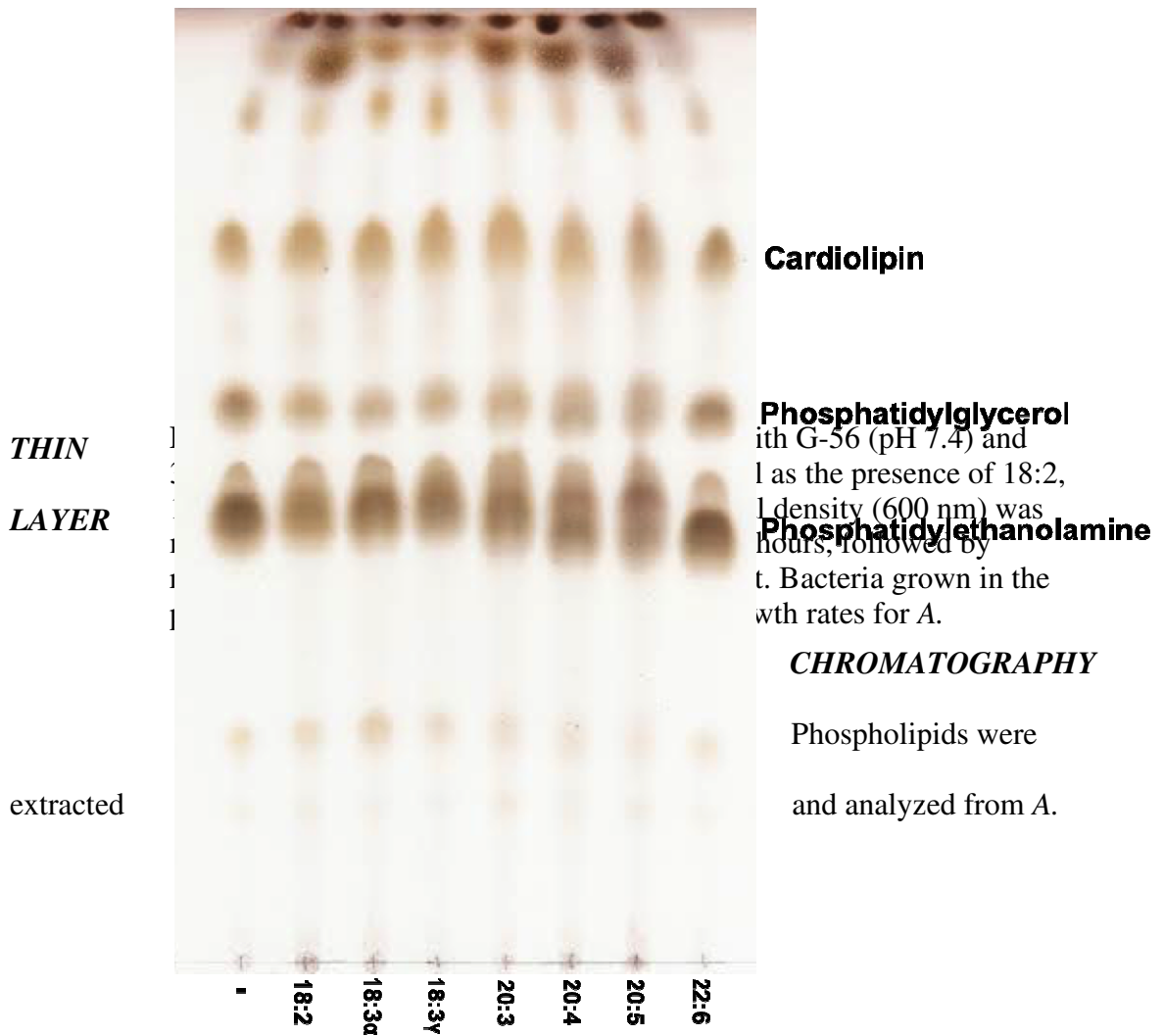
acids.



of



Flocculation, or cell aggregation, began to appear as early as the 60-minute mark for *A. baumannii* enriched with 18:2 and 18:3. All other cultures displayed flocculation within 120 minutes of growth. All of the media for this assay was prepared at 37°C in G-56 at pH 7.4. As shown in the graph, growth patterns begin to diverge at around 480 minutes between OD's of 1.25 and 1.5 (Fig 1). 18:3 reached the greatest optical density around 2.25, and the bacteria without fatty acids almost consistently had the slowest growth levels, specifically when measured after the eight-hour mark. This indicates that supplementation of *A. baumannii* with fatty acids may affect growth, particularly long-term growth.



*baumannii* in order to verify uptake of exogenous fatty acids in their own membranes, as many other gram-negative bacteria have exhibited. The bacteria were grown in the presence of various fatty acids, and their lipids were extracted and subjected to thin layer chromatography. The chromatography showed different patterns and shifts of migration with each of the different fatty acids. Specifically, *A. baumannii* with 18:3 , 20:3 and 20:5 displayed higher migration compared to the other fatty acids (Fig 2).

**ULTRA**  
**PERFORMA**  
**NCE LIQUID**

**Figure 2:** Phospholipid profiles of *A. baumannii* grown in minimal media (pH 7.4) and 37°C. The Bligh and Dyer method was used to extract lipids, followed by thin layer chromatography.

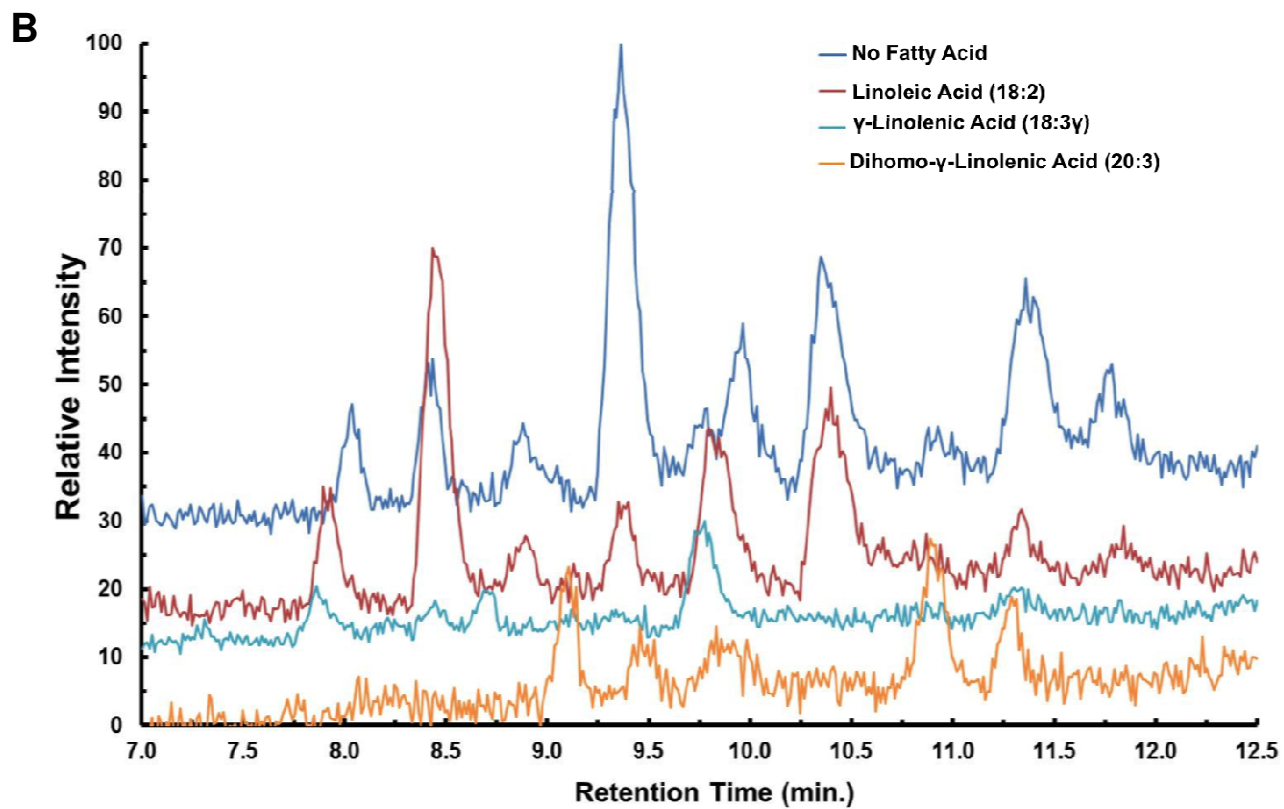
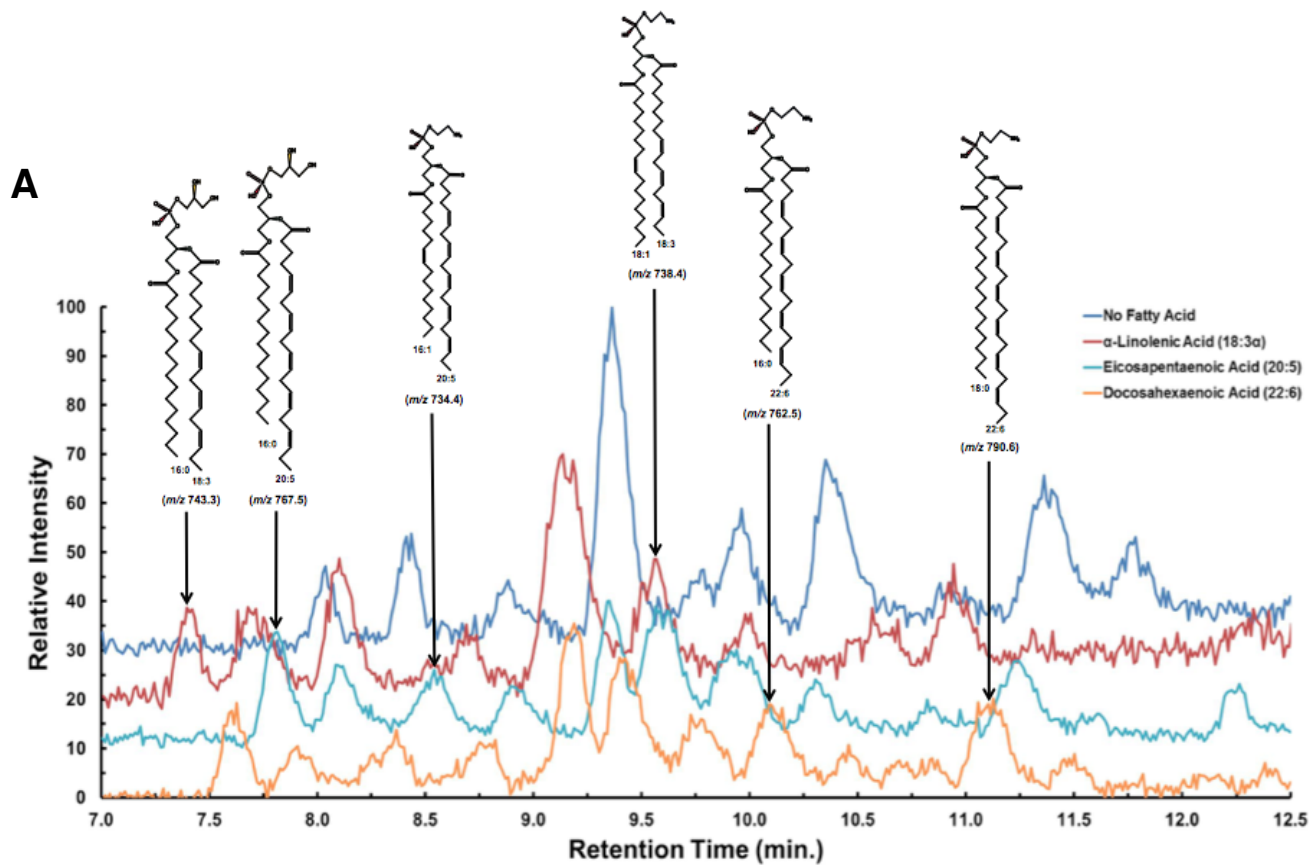
## **CHROMATOGRAPHY**

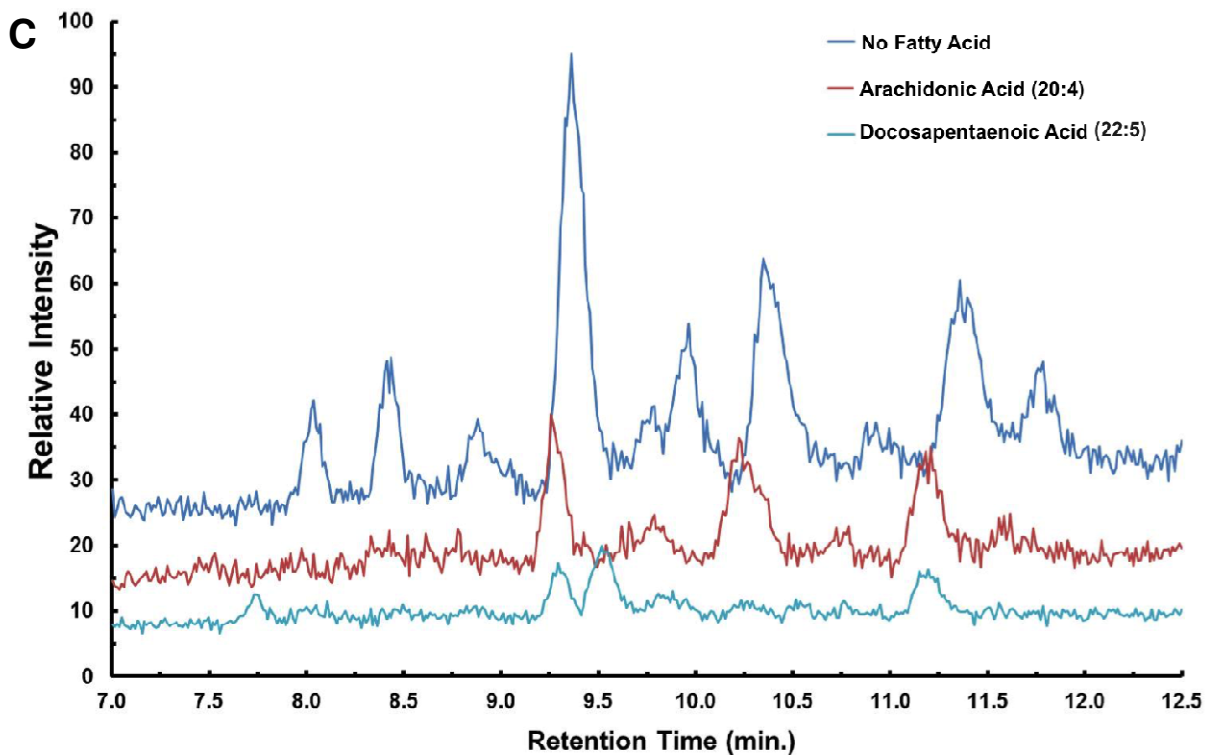
The Bligh and Dyer extraction method for phospholipids was used to prepare *A. baumannii* for phospholipid extraction. An extra wash step was included to increase purity of the isolated lipids. Table 1 represents all significantly detected lipid species that eluted from the column during chromatography and were detected by mass spectrometry. The three chromatograms shown below (Fig 6) indicate quantitative and structural differences among phospholipid species from each culture. The changes in retention time as compared to the control indicate production of new phospholipid species when grown with the indicated fatty acids. Many of the corresponding mass spectra are consistent with the incorporation of the exogenous fatty acids into the phospholipids of *A. baumannii* during growth at 37°C. Representative structures of predicted phosphatidylglycerol and phosphatidylethanolamine species are shown for the selected exogenous fatty acids in the first chromatogram. Corresponding phospholipid species were also identified for linoleic acid (18:2),  $\alpha$ -linolenic acid (18:3), dihomogamma-linolenic acid (20:3), arachidonic acid (20:4), and docosapentaenoic acid (22:5), as shown in the second and third chromatograms.

No FA	18:02	18:3 $\alpha$	18:3 $\gamma$	20:3	20:4	20:5	22:5	22:6
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				654.6				
						680.6		
						682.3	682.6	682.4
		684.5	684.5	684.2		684.2		
686.4	686.2	686.2	686.4			686.4		686.4
688.4	688.5	688.5		688.5	688.5	688.5	688.4	688.5
690.3	690.4		690.4		690.6	690.4	690.6	690.6
700.2								
702.2						702.1		702.6
			706.1					
						708.4		708.5
	710.3	710.2	710.4			710.4	710.4	710.4
	712.5	712.5	712.4	712.5	712.3			
714.4	714.5	714.3	714.4		714.3	714.4	714.5	714.5
716.4	716.5	716.5		716.4	716.5	716.4		716.5
	717.4							
719.6	719.4	719.2	719.6	719.4		719.3	719.2	719.4
		721.3				721.4	721.7	721.3
			728.4					
		734.4	734.5			734.4	734.4	734.5
					736.5	736.5	736.5	736.6
	738.3	738.4	738.4		738.6	738.4		
	740.3	740.1		740.5				
	741.5							
742.2	742.2	742.4			742.5	742.6		742.6
	743.4	743.3	743.3	743.6				
744.3								744.5
745.5	745.5	745.4			745.3	745.5	745.2	745.4
747.4	747.4	747.4	747.4	747.4	747.4	747.4	747.6	747.6
								752.5
						756.4		
								760.5
						762.4	762.3	762.5
					764.5	764.4	764.5	764.5
						765.4		
				766.4				
						767.4	767.4	767.4
	769.4	769.2	769.2		769.6	769.7		
	771.6			771.5				
773.3	773.5	773.3			773.3			773.3
775.4								775.5
						782.5		782.4
								788.5
								790.6
						793.5		793.4
							795.3	

**Table 1:** Spreadsheet representing all significantly detected lipid species ( $m/z$  of [M-H]<sup>-</sup>) that were eluted from the C8 column during chromatography and detected by mass spectrometry. Lipid species are categorized by the various fatty acids used throughout UPLC.



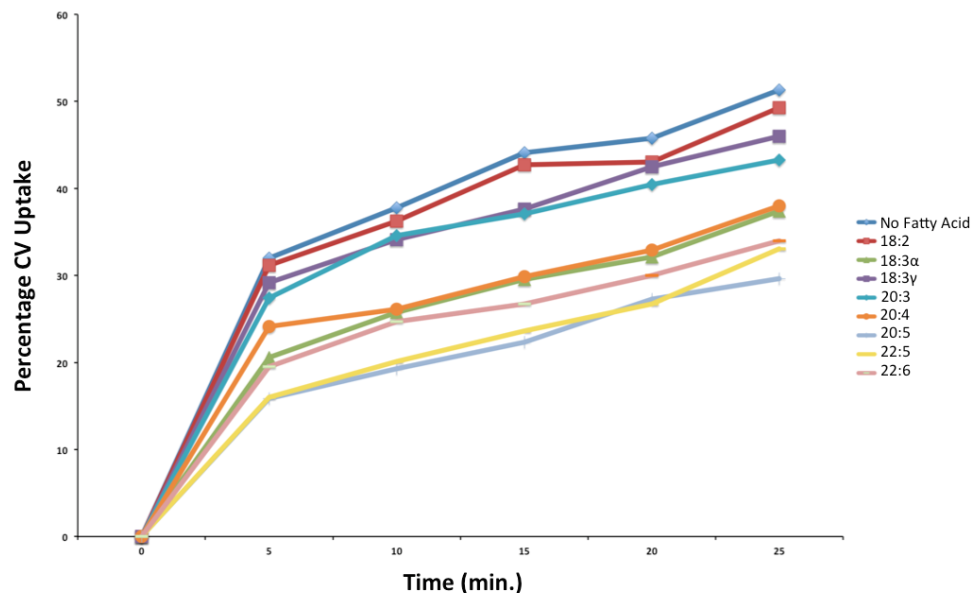


**Figure 6:** Ultra performance liquid chromatography (UPLC) and mass spectrometry of *A. baumannii* phospholipid species. Bacteria were grown in G-56 (pH 7.4) at 37°C with or without various fatty acids. Phospholipids were dissolved in 50:50 mobile phase A:B and injected into a Waters UPLC with reversed-phase C8 column. Detection was quadropole mass spectrometry. The changes in retention time (and mass spectra) are consistent with incorporation of the exogenous fatty acids into the phospholipids of *A. baumannii* during growth in 37°C. In Figure A, chromatograms are shown for bacteria grown with 18:3 , 20:5, and 22:6; figure B represents chromatograms for bacteria grown in the presence of fatty acids 18:2, 18:3 , and 20:3; figure C shows chromatograms for *A. baumannii* grown with 20:4 and 22:5. Predicted structures are provided in A, reflecting the eluted phospholipid species.

### ***CRYSTAL VIOLET UPTAKE***

The purpose of the crystal violet uptake assay was to identify changes in membrane permeability in the presence of various fatty acids. While most of the experiments used five core fatty acids, others were additionally incorporated for the crystal violet assay to gain a better understanding of the role that fatty acids play. This hydrophobic compound uptake test was conducted following bacterial growth in G-56 (pH 7.4) at 37°C.

Membrane permeability of *A. baumannii* was affected as evidenced by differential uptake of hydrophobic compounds (Fig 3). Growth in the presence of fatty acids resulted in reduced uptake of CV. Five fatty acids (arachidonic, -linolenic, docosahexaenoic, docosapentaenoic, and eicosapentaenoic) exhibited 15-20% difference in permeability as reflected by CV uptake. The decrease in membrane permeability was evident for the duration of the test.

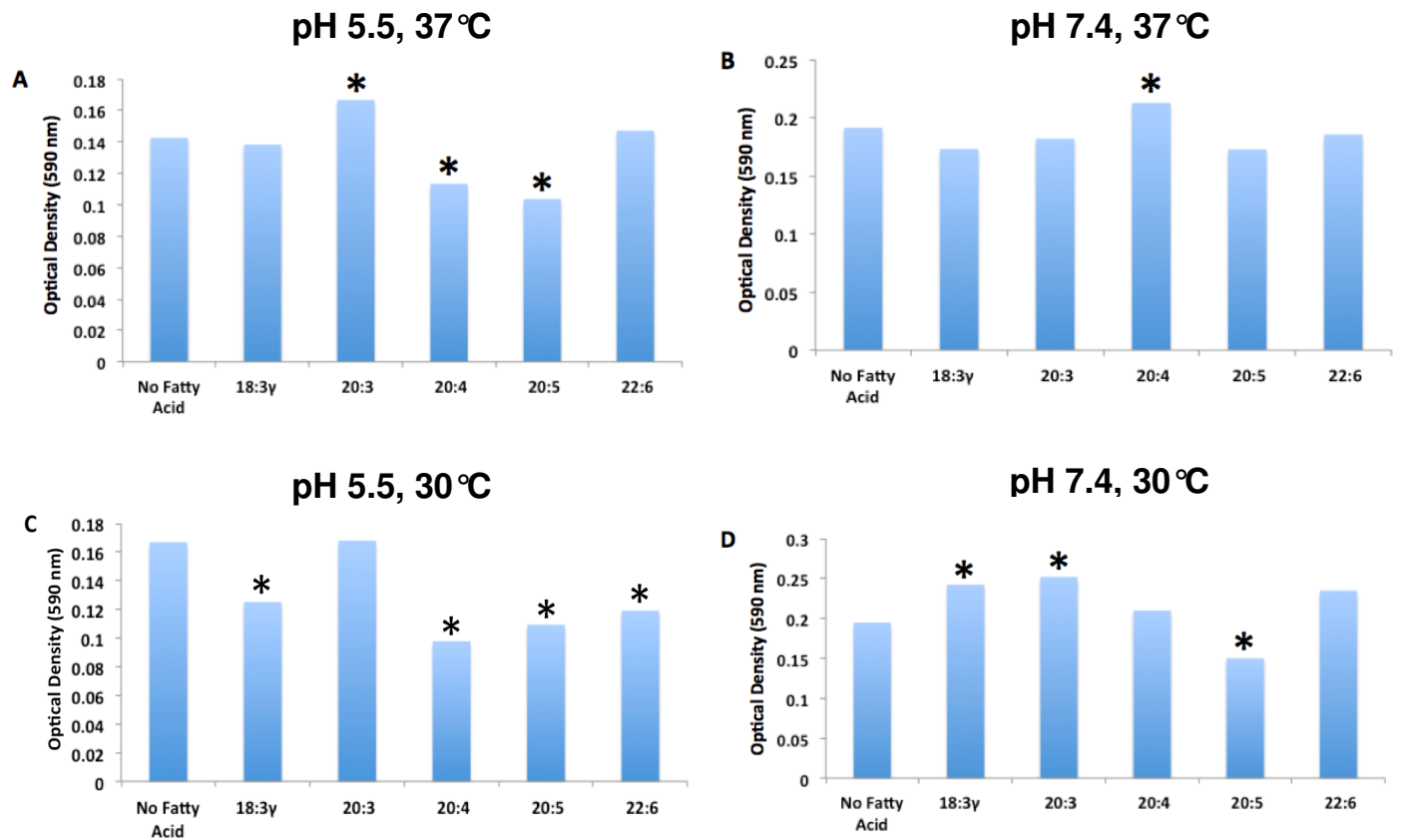


**Figure 3:** Crystal violet hydrophobic compound uptake assay shown with *A. baumannii* prepared in G-56 (pH 7.4) at 37°C. The fatty acids used with the cultures were 18:2, 18:3 , 18:3 , 20:3, 20:4, 20:5, 22:5, and 22:6. Crystal violet uptake was measured via optical densities (590 nm) of the supernatant every five minutes for a total of 25 minutes. All fatty acids inhibited crystal violet uptake to some degree.

**BIOFILM FORMATION**

Biofilms are persistent, thick clusters of adhesive cells that increase their survival and virulence on certain environmental surfaces, and assessing their formation is useful to understanding their resistive nature and role in bacterial survival and persistence.<sup>14</sup> Bacteria that can form biofilms inherit a great deal of advantages, including their resistivity, stability, and ability to cling to a variety of surfaces.<sup>15</sup> The Centers for Disease Control and Prevention and the National Institutes of Health claim that 65-80% of microbe-related infections are related to the development of biofilms.<sup>16</sup> The biofilm assay was unique from the other experiments in that a number of different variables were tested in the presence and absence of fatty acids, including temperature, pH, and type of incubation. While the data looks similar and lacks significant growth of biofilms for *A. baumannii*, as evidenced by OD, there are still some conclusions that can be drawn. Based on experiments performed in octuplet, there were several observations of both stimulatory and inhibitory effects of fatty acids on biofilm formation. At pH 5.5 and 37°C, 20:3 enhanced biofilm production, whereas 20:4 and 20:5 inhibited biofilm production. At pH 7.4 and 37°C, 20:4 slightly increased the production of biofilms, while 18:3, 20:3, and 20:5 had very little effect. Under conditions with pH 5.5 and 30°C, no fatty acid enhanced biofilm production, but 18:3, 20:4, 20:5, and 22:6 decreased it by a relatively significant amount. On the other hand, at pH 7.4 and 30°C, all fatty acids boosted biofilm production to some degree except for 20:5, which inhibited it (Fig 4). This is interesting to note because 30°C is our approximate skin temperature, and biofilms have been known to cause numerous dermatologic diseases.<sup>17</sup> When incubated under shaking conditions, very little biofilm was detected (data not shown).





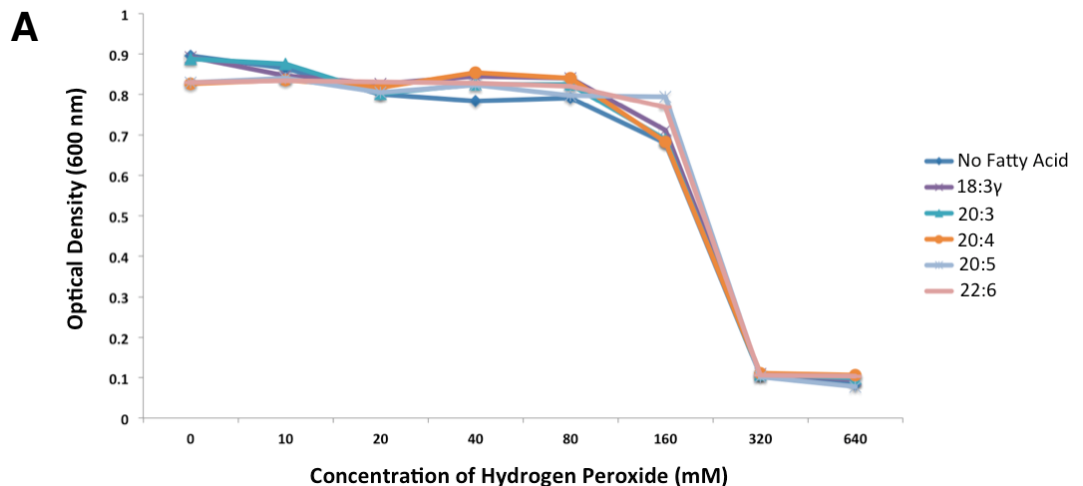
### ***MOTILITY TEST***

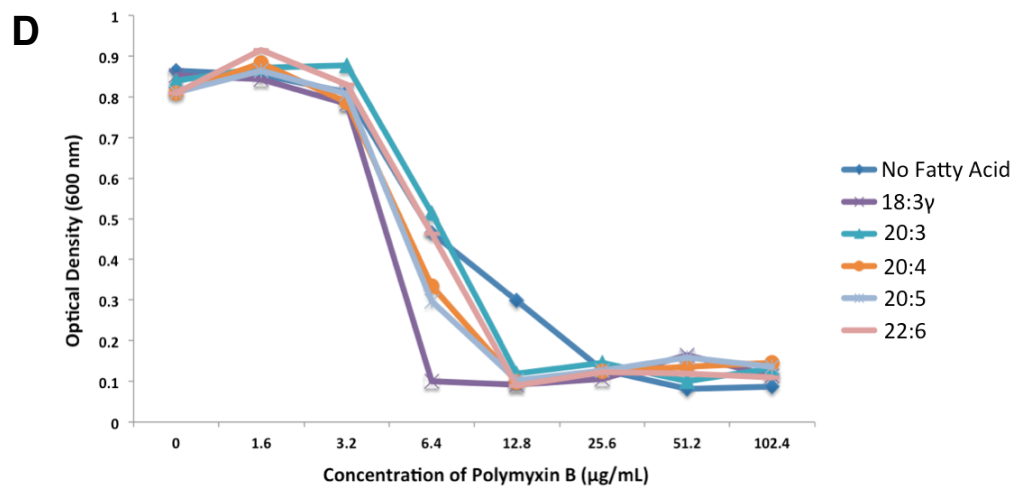
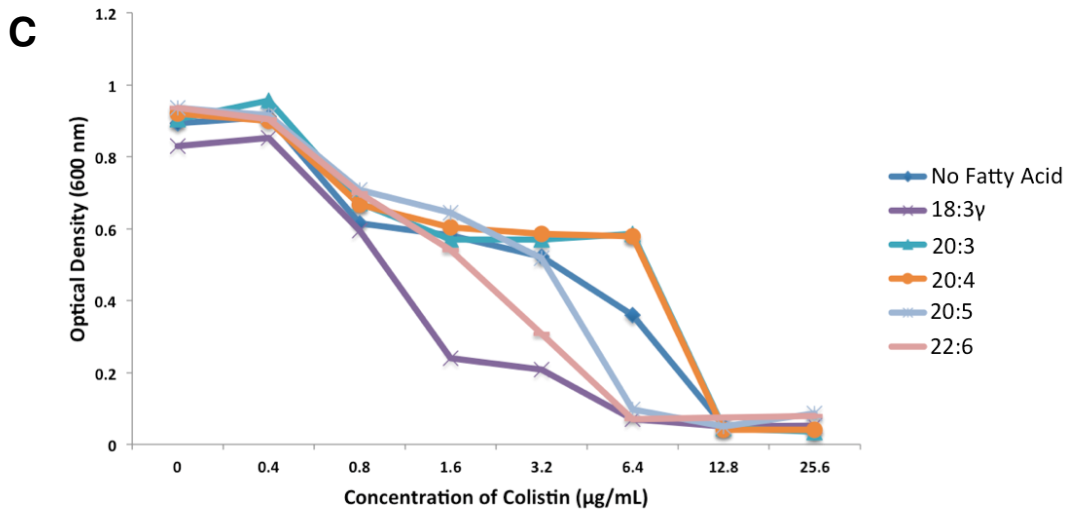
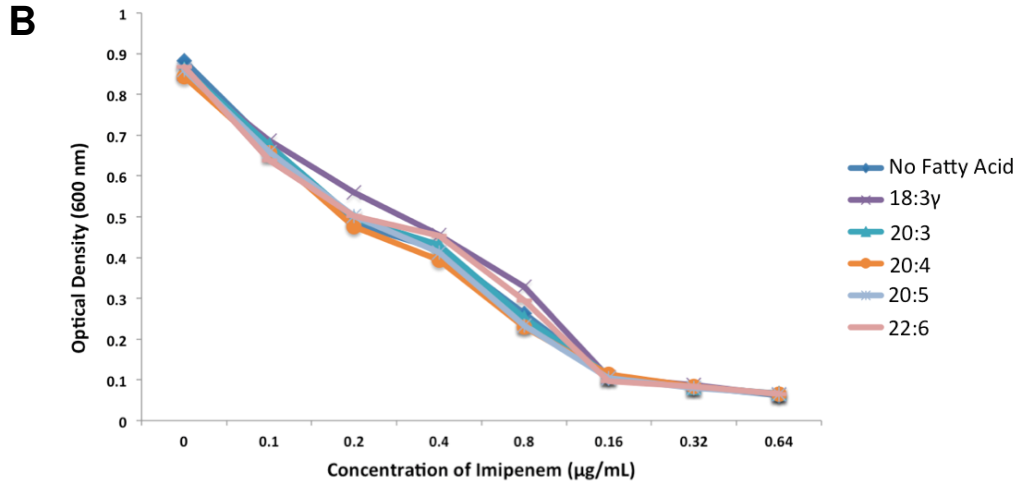
The literature cites motility as a difficult characteristic to examine with *A. baumannii*, calling for a specific media called Eiken agar as an appropriate media for testing surface motility.<sup>4</sup> Because we did not have the means to access this agar from Japan, we attempted our own assay based on previous research regarding *A. baumannii* motility.<sup>18,19</sup> In lieu of Eiken agar, we conducted our motility test on 0.3% and 0.4% LB agar plates. Unfortunately, no motility was exhibited on any of the plates after 24 hour incubation at 37°C. The strain of *A. baumannii* that is used in our lab (ATCC 17978) is non-motile due to its absence of a flagellum, although it is known to exhibit a twitching motility.<sup>20</sup>

### ***BACTERIAL RESISTANCE TO PEROXIDE STRESS, ANTIMICROBIAL PEPTIDE***

## ***RESISTANCE, AND ANTIBIOTIC RESISTANCE***

Because *A. baumannii* has become undoubtedly resistant to a number of stresses and environments, we thought it was important to test whether their stress survival was affected by potential modifications to phospholipids or the cell membrane. The results for these assays are shown in Figure 5. Prepared cultures were exposed to twofold concentrations of hydrogen peroxide, imipenem, colistin, and polymyxin B for these experiments. Hydrogen peroxide and imipenem minimum inhibitory concentrations (MICs) were not affected by the presence of fatty acids. In the peroxide stress and antibiotic experiments, bacteria grown in the presence and absence of all fatty acids followed a similar pattern, effectively being killed off at around 320 mM hydrogen peroxide and 0.16  $\mu\text{g/mL}$  imipenem. Exposure to polymyxin B resulted in increased susceptibility for all fatty acids, with the MIC decreasing from 25.6 to 12.8 for every fatty acid except 18:3, which decreased to 6.4  $\mu\text{g/mL}$ . Growth in the presence of 18:3, 20:5, and 22:6 led to increased susceptibility to colistin. As with PMB, 18:3 had the greatest effect on survival.





**Figure 5:** MIC assays for *A. baumannii* following growth with fatty acids and environmental stresses. Bacteria was grown in minimal media (G-56, pH 7.4) at 37°C, followed by introduction of various concentrations of A) hydrogen peroxide, B) imipenem, C) colistin, and D) polymyxin B. The graphs above show the various degrees of resistance of *A. baumannii* to these environmental stresses.

## VI. DISCUSSION

The literature states that strains of *A. baumannii* mainly synthesize 18:1, 16:0, and 16:1 de novo.<sup>19</sup> This means that all of the fatty acids used in our experiments had to be incorporated into the bacteria through the mechanisms described previously. It was certainly clear that the uptake of these fatty acids modified the cellular membrane, yet the implications from experiment to experiment varied from one another. For example, in the growth curve assay, it was observed that implementation of all fatty acids increased

absorbance of the bacteria to some degree, with 18:3 and 20:3 peaking highest of all at the 780 minute mark. By adding fatty acids to *A. baumannii*, we were adding an external carbon source that had the potential of increasing the growth rate of the bacteria, which is what we found. While we do not know whether or not the fatty acids are being utilized as extra carbon sources to make ATP by  $\beta$ -oxidation, we do know that the bacteria is capable of incorporating the fatty acids into their membrane. It is likely that the carbon source is used for longer survival rather than anything related to modifying the membrane.

In the crystal violet assay, it was shown that incorporation of all fatty acids used inhibit crystal violet uptake, which verified that the membrane had indeed been affected by the introduction of various fatty acids. Nevertheless, *A. baumannii* with 18:3, 20:4, 20:5, 22:5, and 22:6 exhibited a greater membrane alteration compared to the bacteria with 18:2, 18:3, and 20:3. It is interesting to note that the two groups of fatty acids created that provided less or more crystal violet inhibition are also similarly classified as  $\omega$ -3 and  $\omega$ -6 fatty acids, respectively. There is a clear distinction between  $\omega$ -3 and  $\omega$ -6 fatty acids, and they both play an integral role in human health. From the PUFAs that were used throughout my experiments,  $\omega$ -3 fatty acids include 18:3, 20:5, and 22:6, while the  $\omega$ -6 fatty acids are 18:2, 18:3, 20:3, and 20:4.<sup>21</sup> These fatty acids are naturally occurring in a lot of marine bacteria, which is of significance because some of these marine bacteria have been found to be viable natural sources that hold antibiotic and antitumor characteristics.<sup>22</sup> Many of the  $\omega$ -3 fatty acids in marine organisms have a different pathway of interconverting PUFAs within their biological systems, and they are beginning to become important in research related to human diet, health, and disease.<sup>23</sup> Perhaps the differences observed in the crystal violet assay reflect differential

incorporation between -3 and -6 fatty acids. If true, then our results indicate that -3 fatty acids may be preferentially assimilated into the membrane; or alternatively, -3 fatty acids are intrinsically more refractory to passage of hydrophobic compounds.

One of the reasons why *A. baumannii* is such a highly resistant bacteria can be attributed to its ability to form biofilms.<sup>24</sup> The biofilms that we observed on our microtiter plates grew on a hydrophobic material. Although the data initially did not seem to show much, statistical analyses proved that there was an underlying implication to the experiments. Two of the four graphs above exhibited a very significant p-value ( $p < 0.002$ ), and interestingly enough, they were the two replicates performed under conditions of pH 5.5. pH may play a bigger role than temperature for biofilm formation, and this information could potentially be related to the pH values of the soil and environments that *A. baumannii* thrive in. Biofilms are known to have the capability of forming on plastic and glass materials, which are found in various pieces of medical equipment throughout hospitals. This is noteworthy, as fatty acids are amphiphilic, composed of hydrophobic and hydrophilic components, which should help biofilms form more efficiently.

Although we tried manipulating various aspects while preparing *A. baumannii* for these assays, the observed biofilm growth was modest without any overall consistency. One reason why this occurred is because *A. baumannii* creates thicker biofilms at the liquid-air border because of its aerobic nature, and the lack of oxygen at the bottom and walls of microtiter wells are not conducive environments for them.<sup>23</sup> The mechanism by which many bacteria create biofilms is directly related to their locomotory moieties (pili, flagella, glycopeptidolipids), but since none of these were detected in our motility experiments, biofilm formation was most likely due to the ability of *A. baumannii*

colonies to grow on these hydrophobic materials such as our polystyrene microtiter plates.<sup>25</sup>

Strains of *A. baumannii* that are discovered in intensive care units and hospitals carry antibiotic resistance that has proven to be troublesome over time. The antibiotics and antimicrobial peptides used in this project had varying effects on the bacteria. Out of the four environmental stresses that were tested in this portion of the project, colistin and PMB showed the greatest variance of resistance when grown with different bacteria; hydrogen peroxide and imipenem had little to no effect. The reason why a difference is seen between the effects of imipenem versus colistin or polymyxin B is related to their specific modes of inhibiting the bacteria. Imipenem is a  $\beta$ -lactam antibiotic that works by binding to penicillin binding proteins (PBPs) and obstructing formation of the bacterial cell wall.<sup>26</sup> Since *A. baumannii* is a gram-negative bacterium, there is less peptidoglycan for the antibiotic to get through to inhibit the cell altogether, and the pores formed provide an easy route to inhibit the bacteria. On the other hand, colistin and polymyxin B are cationic microbial peptides that have almost identical structures, differing in only one amino acid. They work solely against gram-negative bacteria by interacting with lipopolysaccharide (LPS).<sup>27</sup> Lipopolysaccharide molecules are comprised of lipid A, a core oligosaccharide, and a distal polysaccharide. Cationic microbial peptides disrupt the calcium and magnesium bridges that hold LPS together and ultimately disturb the outer membrane.<sup>28</sup> This yields a cell membrane with amplified permeability and penetrability, and the cell ultimately lyses.<sup>29,30</sup> The addition of fatty acids contributes to this process by altering the MICs of the antibiotics, as described in our results. Since the exposure to PUFAs has no negative chemical or physiological effect on the human body, using them to strengthen the sensitivity to drugs such as colistin and polymyxin B is quite possible in

the future of medicine. It is also important to note the contributions of LPS to gram-negative bacterial resistance to cationic antimicrobial peptides. A key strategy is modification of lipid A with positively charged moieties, which serve to repel cationic antimicrobials.<sup>31</sup>

The effectiveness of  $\beta$ -lactam antibiotics like imipenem has a hypothesized correlation to biofilm formation. It is believed that the strains of bacteria that can make their own biofilms have less resistance to these antibiotics, as evidenced in our results.<sup>32</sup> Strains that cannot form biofilms must rely on antimicrobial resistance because there are no biofilms present to provide protection and virulence.

While *A. baumannii* is only one of 23 described species in the genus *Acinetobacter*, its role as an opportunistic pathogen in human health and disease has exponentially increased especially in the last two decades, now as a major threat in the United States as well as many parts of Europe and Asia.<sup>33</sup> One of the biggest problems we face in healthcare is the increasingly arbitrary use of broad-spectrum antibiotics that have caused an explosion of antibiotic-resistant bacteria, leaving doctors and scientists with their last lines of defense against them.<sup>34</sup> New drugs and medicines cannot keep up with the rate of resistance in bacteria. That being said, many of the fatty acids used in our experiments are known to have important functions in the body. For example, cell growth, inflammation, and the central nervous system are controlled by the arachidonic acid cascade, which is composed of over twenty signaling pathways. The pathway is begun by arachidonic acid (20:4) and also requires 18:3, 20:3, and 20:5 to properly proceed.<sup>35</sup> Knowing this information, it is perhaps feasible that we could incorporate fatty acids into new strains of medication that can inhibit inflammatory responses from the body in a more efficient manner. This is just one example of many to illustrate the



important potential that this research could have in the field of microbial and clinical medicine.

Data from UPLC and MS reported various phospholipid species present in *A. baumannii* when grown with various fatty acids. The outer membrane of gram-negative cells provides an extra, unique layer of protection to the cells. The asymmetrical outer membrane is composed of a lipopolysaccharide outer leaflet and a phospholipid inner leaflet. 95% of the inner membrane is made up of phosphatidylethanolamine and phosphatidylglycerol head groups, which are the groups verified through UPLC-MS.<sup>36</sup> Considering how many phospholipids make up the cell envelope of gram-negative bacteria, the assimilation of exogenous fatty acids would be expected to significantly alter membrane properties. Additionally, it is important to note that all of the fatty acids used in this study contained cis bonds. It is unknown whether or not *A. baumannii* possesses a cis-trans isomerase that can alter the fatty acid double bond conformation in the membrane. These studies not only identify ramifications of exogenous fatty acids on permeability and phospholipid composition, but also reveal phenotypic consequences that may affect survival and pathogenesis of *A. baumannii*.

## **VII. FURTHER STUDIES**

Research shows that vaporized hydrogen peroxide (VHP) is used to treat multidrug-resistant bacteria such as *A. baumannii*.<sup>3</sup> Using VHP has proven to dramatically decrease the number of bacterial cultures in the intensive care unit of hospitals.<sup>37</sup> Having learned this information, it would be of interest to revisit the hydrogen peroxide assay and test the disinfecting abilities it may exhibit towards the bacteria in the presence and absence of various fatty acids.

One assay whose modification we could potentially benefit from is the growth curve assay. A long-term growth curve that goes to completion would show a better overall image of the points at which the bacteria ultimately dies off. Although our growth curve showed a progression of over 24 hours, a longer run could show us more. The fatty acids used were not included in every assay that we conducted, but if they had been then we would have seen significant increases in the effects measured and observed. It would

be worthwhile to replicate all of the assays in this project with direct incorporation of the fatty acids to gain a better understanding of their function in bacterial cells.

Since biofilms are capable of forming on hydrophilic and hydrophobic surfaces, and our studies were done on plastic microtiter plates, it would be interesting to see how results would differ if the biofilm assay was performed on a glass surface. Also, using a different mode of incubation aside from the microtiter plates would give insight to whether or not our particular strain (ATCC 17978) forms greater biofilms at the liquid-air border as opposed to the plastic walls of the microtiter wells.

Targeted MIC assays with various antibiotics to explore therapeutic potential of fatty acids would be beneficial for further levels of studies. This could potentially help determine if, how, and which fatty acids in conjunction with antibiotics or antimicrobial peptides can diminish infectious pathogens like *A. baumannii* without affecting the body's natural microflora. Similar studies to this have been done with strains of *Streptococcus mutans* using specifically targeted antimicrobial peptides (STAMPs).<sup>38</sup> Due to time and budget constraints, only selected fatty acids were used for the environmental stress and even biofilm assays in comparison with the full spectrum used in the growth curve. It would be beneficial to try all of the fatty acids and see if there is any consistency of any particular ones throughout the various experiments in this project.

In addition, results warranting further study and analysis include the chromatograms from the UPLC/MS. There is a significant amount of information pertaining in the UPLC/MS data that has to be thoroughly analyzed in order to understand the potential shifts in phospholipids among varying polyunsaturated fatty acids. MS/MS analyses have already been planned for the lipid samples used in this study. This approach determines whether the exogenous fatty acids are incorporated at

the sn-1 or sn-2 position. Furthermore, the fatty acids used in this study contained cis bonds. In order to determine the conformation of these fatty acids extracted from the bacteria, fatty acid methyl esters could be generated and analyzed by gas chromatography with appropriate standards.

While the extent of this project has been mainly with *A. baumannii*, many of the mechanisms and functions among other gram-negative, antibiotic-resistant bacteria are fairly similar. These bacteria include *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. It would be worthwhile to see the similarities and differences if the experiments from this project were conducted on any of these other bacteria. By doing so, we would be able to paint a broader picture of the role that fatty acids play in the membrane and how this could affect the potential methods of treating and preventing diseases caused by them on a global scale.

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