Exogenous polyunsaturated fatty acid (PUFA) scavenging affects phospholipid profiles and virulence phenotypes in Vibrio cholerae and Vibrio parahaemolyticus

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Exogenous Polyunsaturated Fatty Acid (PUFA) Scavenging Affects Phospholipid Profiles and Virulence Phenotypes in *Vibrio cholerae* and *Vibrio parahaemolyticus*

by

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Departmental Honors Thesis

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**ABSTRACT**

**AIM:** This research had two primary objectives. First, this study sought to determine whether or not exposing *Vibrio cholerae* and *Vibrio parahaemolyticus* to polyunsaturated fatty acids (PUFAs) would lead to changes in their respective phospholipid profiles. Secondly, if membrane remodeling occurred, this study sought to determine the impact of membrane modifications on bacterial fitness by evaluating their effect on membrane permeability, biofilm formation, and response to stresses.

**METHODS:** The Bligh and Dyer method was used to extract bacterial lipids, which were subsequently analyzed via thin-layer chromatography (TLC). The effect of fatty acids on bacterial fitness was assessed using stress, biofilm, and crystal violet (CV) assays. Stresses used include: lactic acid, hydrogen peroxide, and the antimicrobial peptide polymyxin B (PMB).

**RESULTS:** TLC of isolated phospholipids from *V. cholerae* and *V. parahaemolyticus* grown in the presence of PUFAs indicated structural changes, suggesting incorporation of exogenous fatty acids into membrane lipids. CV uptake tests revealed fatty acid-dependent changes in permeability of up to 30% in *V. cholerae* and 20% in *V. parahaemolyticus*. *V. parahaemolyticus* displayed increased sensitivity to oxidative stress for all PUFAs, while exposure to docosahexaenoic acid (22:6) enhanced its resilience. The susceptibility of both *V. cholerae* and *V. parahaemolyticus* to PMB was increased. Following exposure to multiple PUFAs, biofilm assays revealed a reduced capacity for biofilm formation in *V. cholerae* for all fatty acids except 22:6. Furthermore, *V. parahaemolyticus* exhibited an increase of over 30% in biofilm activity. Significant exceptions to this behavior were 22:6 and 18:3γ which decreased biofilm production in *V. parahaemolyticus* by 30% and 70%, respectively. These results implicate fatty acids as important resources for bacterial membrane remodeling that may affect environmental persistence and host virulence in *Vibrio* species.
INTRODUCTION

In the wake of the devastating 2010 earthquake, the worst epidemic of cholera in modern times ravaged through Haiti and neighboring nations, Cuba and the Dominican Republic.\(^1\) It was reported that since this outbreak *Vibrio cholerae* has killed approximately 8,231 Haitians, hospitalizing thousands more.\(^2\) When ingested from contaminated water, enough bacteria are able to survive the extremely acidic environment of the stomach and pass into the small intestine, where they target the epithelia for colonization.\(^3\) Upon colonization, *V. cholerae* multiplies and eventually begins to express cholera toxin (CT), a virulence factor responsible for initiating the onset of vomiting and acute gastroenteritis, which are hallmark symptoms of the cholera infection it produces in human hosts. Other strains of *Vibrio* lacking the cholera toxin, like *Vibrio parahaemolyticus*, produce similar symptoms in the human reservoir although to a lesser degree. Despite the rigorous conditions of the human gastrointestinal tract, *Vibrio* species are able to survive and colonize, reflective of their advanced bacterial fitness and aptitude for engaging in keen homeoviscous adaptations.

V. CHOLERAЕ

*V. cholerae* is a motile, Gram-negative curved rod within the *Vibrionaceae* family that naturally inhabits aquatic ecosystems.\(^4,5-8\) Due to its ability to proliferate outside the human host, *V. cholerae* is classified as a facultative human pathogen.\(^4\) Intestinal and extraintestinal infections are primarily associated with O1 and O139 serogroups.\(^4,9-12\) Non-O1 and –O139 serogroups as well as non-toxigenic O1 strains have seldom been found to produce these infections.\(^4,9-12\) Within the marine environment, *V. cholerae* adheres to surfaces provided by surrounding crustaceans, insects, copepods, filamentous
green algae, and plants.\textsuperscript{4,6,13} Environmental non-O1 and non-O139 \textit{V. cholerae} strains have also been isolated from chironomid egg masses, suggesting environmental association only with these seemingly non-virulent strains.\textsuperscript{14} O1 El Tor (C6706) and O139 strains have been found to express genes promoting the assembly of a mannose-sensitive hemagglutinin (MSHA) type IV pili, allowing these strains to bind to the chitin of zooplankton.\textsuperscript{15} Association with zooplankton and egg masses not only protects \textit{Vibrio} from harsh environmental conditions but also provides surfaces prime for biofilm formation, which increases resilience its stress.\textsuperscript{4} The primary difference between non-toxigenic environmental strains and virulent O1 and O139 strains is the presence of the CT-encoding phage (CTX\textsuperscript{a}) integrated within the chromosome of the O1 and O139 strains.\textsuperscript{16} However, phage transduction of CTX\textsuperscript{a} has been used to convert environmental strains to toxigenic strains.\textsuperscript{16} Another major virulence factor of toxigenic serogroups is the toxin-coregulated pilus (TCP), which is a type IV pilus that promotes colonization within the intestine.\textsuperscript{17} Accessory virulence factors, like adhesins, assist CT and TCP in developing the cholera infection.\textsuperscript{17-19} \textit{V. cholerae} possesses the type VI secretion system, T6SS, that enables it to evade the extracellular environment and inject its bacterial effector proteins directly into the membrane and cytoplasm of host cells.\textsuperscript{20,21} This study used \textit{Vibrio cholerae} O1 El Tor for all experiments.

\textbf{\textit{V. PARAHAEOMLYTICUS}}

Like \textit{V. cholerae}, \textit{V. parahaemolyticus} is a Gram-negative halophile typically found motile within aquatic ecosystems, such as estuaries or marine and costal environments.\textsuperscript{20-26} However, \textit{V. parahaemolyticus} can adhere to inert or animate surfaces provided by zooplankton, fish, shell-fish, or other underwater matter via biofilm
formation (although not as extensively as *V. cholerae*).\textsuperscript{20, 27} Virulent strains of *V. parahaemolyticus* are primarily transmitted through consumption of raw or undercooked seafood and produce acute gastroenteritis within their human hosts.\textsuperscript{20, 28, 29} *V. parahaemolyticus* has been recognized as the source of over half of all bacterial associated food-poisonings, and in the United States alone, it is the leading cause of seafood associated human gastroenteritis.\textsuperscript{20, 28, 30, 31} The primary difference between pathogenic and non-pathogenic, environmental strains of *V. parahaemolyticus* are the genes for thermostable direct hemolysin (*tdh*) and TDH related hemolysin (*trh*) found in pathogenic strains.\textsuperscript{20, 32-34} These genes are the primary source of illness to human and marine animals in *V. parahaemolyticus* infections.\textsuperscript{20, 32-34} Like *V. cholerae*, *V. parahaemolyticus* strains have several different virulence factors including adhesins but possess two type III secretion systems, T3SS1 and T3SS2.\textsuperscript{20, 35} While all strains of *V. parahaemolyticus* have the T3SS1, the T3SS2 is unique to pathogenic strains.\textsuperscript{20, 36} Similar to *V. cholerae*, pathogenic strains of *V. parahaemolyticus* also possess type VI secretion systems, the T6SS1 and T6SS2.\textsuperscript{20, 37, 38} Because the T3SS2 and the T6SS2 co-exist, it is believed they work in tandem to promote an infection with T6SS2 initiating the first step of adhesion and T3SS2 continuing the infection by inducing cytotoxicity via endotoxin release.\textsuperscript{20, 39, 40} In addition to these secretion systems, *V. parahaemolyticus* also has two different types of flagella with distinct functions for swimming, swarming, and capsule production.\textsuperscript{20, 41}
**VIBRIO: ADAPTATION AND SURVIVAL IN UNFAVORABLE ENVIRONMENTS**

*Vibrio’s* ability to colonize within the small intestine suggests advanced adaptations to environmental stressors. From its aquatic reservoir to the human host, *Vibrio* is exposed to significant changes in pH, temperature, and osmolarity. From its aquatic reservoir to the human host, *Vibrio* is exposed to significant changes in pH, temperature, and osmolarity. Within the intestinal environment alone, *Vibrio* continues to proliferate despite the prominence of native growth inhibitory substances, like bile and organic acids, as well as the defense mechanisms initiated by the innate immune system, such as complement proteins secreted by intestinal epithelial cells and defensins secreted by Paneth cells. Recognition of foreign *Vibrio* cells triggers proteases to cleave specific complement proteins, which release cytokines and consequently activate the complement cascade. This cascade is the primary effector pathway of the humoral component of the innate immune system and promotes phagocytosis and cytolysis of foreign bacterial cells. Simultaneously, upon recognition of foreign pathogens, such as bacteria, viruses, or fungi, cationic, antimicrobial proteins called defensins are secreted by host Paneth cells. These proteins bind to the membranes of *Vibrio* cells, increasing their membrane permeability and consequently inducing an efflux of essential nutrients and ions out of the cell. In spite of this harsh environment, *Vibrio* is able to colonize and express virulence factors. Although there are approximately 200 known serogroups of the O strain of *V. cholerae*, only two, O1 and O139, have been linked with severe outbreaks of disease, bringing to question what makes these strains especially virulent.
THE BIPHASIC LIFESTYLE OF VIBRIO

Despite expression of virulence phenotypes within the human reservoir, *Vibrio* does not seem to express these phenotypes within its natural reservoirs. The biphasic lifestyles of *Vibrio* species are believed to involve an environmental association beneficial for survival and resistance as well as a host association that is relatively short-lived and harmful, as seen in the human host. Unique to *V. cholerae*, in response to unfavorable conditions, the pathogen can enter into a dormant, viable but non-culturable (VNC) state. Recent studies have found evidence suggesting *V. cholerae* uses this dormant state as a skillful survival strategy, remaining in its VNC state when exposed to unfavorable conditions and reverting to its active, culturable state when exposed to favorable growth conditions. The mechanism of this conversion is still under investigation. Although a VNC state has not been found in *V. parahaemolyticus*, other species of *Vibrio*, such as *V. vulnificus*, have been found to possess this ability.

VIBRIO: ENHANCED HOMEOVISCIOUS ADAPTATIONS AND PROPENSITY FOR MEMBRANE REMODELING

*Vibrio*’s ability to survive extremely harsh conditions and the unique capability of some strains to convert between an active and dormant, VNC state reflects advanced bacterial fitness, which is defined according to a bacterium’s ability to adjust its membrane lipid composition to maintain internal homeostasis when exposed to stress or unfavorable conditions. Adjustments in the fatty acid composition of the membrane made to maintain homeostasis are known as homeoviscous adaptations. These adaptations can lead to changes in membrane curvature, known as membrane
remodeling.\textsuperscript{58} \textit{Vibrio} species have numerous physiological advantages that enhance their aptitude for homeoviscous adaptations and hence membrane remodeling.\textsuperscript{3} In \textit{Vibrio} and other gram-negative bacteria, exogenous fatty acids are brought into the cell via the fatty acid transport pathway.\textsuperscript{3,59} Outer membrane transporter protein, FadL, recognizes exogenous fatty acids and initiates bacterial acquisition and utilization through an incompletely known mechanism.\textsuperscript{3,59} Once at the cytosolic face of the inner membrane, the acyl-CoA synthetase FadD generates acyl-CoA thioesters that can enter several pathways, one being membrane remodeling.\textsuperscript{3,59} Unique to \textit{Vibrio} are three homologues encoding for FadL and three encoding FadD.\textsuperscript{3} Moreover, the \textit{Vibrio} genome encodes several predicted acyltransferases.\textsuperscript{3} This additional cellular machinery provides \textit{Vibrio} with broader uptake capabilities than other bacterial species.\textsuperscript{3} Complementing this advantage, \textit{Vibrio} species also express several different lipases that can aid in liberation of fatty acids from a variety of different lipid sources.\textsuperscript{57} Exploiting this advantage, \textit{V. cholerae} has a surface-exposed lipoprotein that can act as a lysophospholipase on lysophosphatidylcholine (LPC), excising fatty acid residues from these molecules to be ferried into the cell via the fatty acid transport pathway.\textsuperscript{57} This protein provides \textit{V. cholerae} a distinct advantage over other \textit{Vibrio} species, enabling it to utilize both exogenous fatty acids as well as alternative sources of fatty acids, such as LPC.\textsuperscript{57} Thus, \textit{Vibrio}’s unique cellular machinery enhances its ability for uptake and utilization of fatty acids from a wider repertoire of lipid species.\textsuperscript{3,57}

**PRIOR RESEARCH AND OUR STUDY**

Several studies have investigated the potential advantages that \textit{Vibrio}’s unique physiology may impart. In response to conditions simulating both aquatic and human GI
 niches, *V. cholerae* was found to uptake exogenous fatty acids from its environment and re-incorporate them into its membrane as phospholipids.\textsuperscript{60} This supports the hypothesis that membrane remodeling may alter virulence phenotypes, allowing *Vibrio* to adapt and persist following exposure to stress. Additionally, biofilm formation has been shown to play a critical role in *Vibrio*’s ability to survive amongst harsh environmental conditions. Prior research strongly supports biofilm formation as a primary mechanism of colonization upon abiotic surfaces.\textsuperscript{4, 60-63} Thus, not only do *Vibrio* species have the physiological machinery to use membrane remodeling to their advantage, but research has also shown how these advantages may alter virulence phenotypes and hence persistence amongst unfavorable conditions.

The positive effects of PUFAs on human health and development have been well-documented and continue to grow.\textsuperscript{64-67} In humans, consumption of more than 2g of eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6) a day has shown to elicit an anti-inflammatory response.\textsuperscript{64} Moreover, these fatty acids have proved beneficial against rheumatoid arthritis (RA), inflammatory bowel disease (IBD), and asthma in animal models.\textsuperscript{64} The anti-inflammatory, anti-arrhythmic, and hypolipemic properties of PUFAs have been found to promote cardiovascular health and consumption, and several government agencies have suggested increased consumption of fatty fish or ω-3-PUFAs to decrease risk of cardiovascular disease (CVD).\textsuperscript{65} Studies have also shown long-chain polyunsaturated fatty acids (LCPUFA), specifically ω-3-PUFAs such as 20:5 and 22:6, promote bone health and turnover.\textsuperscript{66} LCPUFAs, specifically 22:6, have even been found essential for complete visual and neural development in pre-term infants.\textsuperscript{67} With this growing body of research supporting their biological benefits to eukaryotic organisms,
this study seeks to discover whether PUFAs can also benefit prokaryotic organisms, such as Vibrio. Considering *Vibrio* is especially equipped to utilize these macromolecules, it is hypothesized that PUFA-induced membrane remodeling will confer *Vibrio* with some form of competitive advantage and enhance its bacterial fitness. This study seeks to further expand upon previous research and demonstrate the assimilation and effect of exogenous PUFAs on virulence phenotypes within *V. cholerae* and *V. parahaemolyticus* when exposed to stresses, such as hydrogen peroxide (H$_2$O$_2$), lactic acid, and the peptide antimicrobial polymyxin B (PMB). The effect of selected PUFAs on biofilm formation capabilities and membrane permeability to crystal violet (CV) will also be explored.
METHODOLOGY

MATERIALS

The most physiologically relevant fatty acids, linoleic acid (18:2), α-linolenic acid (18:3α), γ-linolenic acid (18:3γ), dihomo-γ-linolenic acid (20:3), arachidonic acid (20:4), eicosapentaenoic acid (20:5), docosatrienoic acid (22:3), docosapentaenoic acid (22:5), and docosahexaenoic acid (22:6) were purchased commercially (Cayman Chemicals) along with *Vibrio cholerae* (O1 El Torr) and *Vibrio parahaemolyticus* (VP Tor clinical isolate). A table of fatty acids used can be found in Table 1 of the Appendix. Luria Burtani broth (LB) served as a nutritionally rich media, while G-56 (0.2% glucose), consisting 0.45 mM Hepes (pH 7.5), 0.3 mM KH$_2$PO$_4$, 10 mM KCl, 10 mM (NH$_4$)$_2$SO$_4$, 0.2% glucose, 0.03 mM FeSO$_4$, and 0.075mM thiamine, served as a minimal media. The media was also supplemented with 1mM CaCl$_2$ and MgSO$_4$ as well as 0.4% casamino acids. This recipe for G-56 was obtained from Dr. David K. Giles post-graduate work in another lab. G-56 was prepared the day of usage and filter-sterilized into a sterile glass bottle. Test tubes used to prepare media or cultures were autoclaved before use. All work was done using aseptic technique.

PREPARATION OF BACTERIAL CULTURES

Overnight cultures were produced by transferring isolated colonies from a viable plate to one or two 7mL LB samples using a sterile wooden applicator and shaking at 37 degrees Celsius. Following 24 hours of incubation, initial optical density (OD) readings for each culture were performed at 600nm.
LIPID EXTRACTION AND THIN LAYER CHROMATOGRAPHY

The volume of overnight culture required to start 7mL cultures in G-56 (pH 7.5) at an OD$_{600}$ of 0.1 was determined and subsequently added. 300µM of each fatty acid was added to a single culture with the control receiving no fatty acids. All cultures of *V. parahaemolyticus* received 300mM NaCl in addition to the following PUFAs: negative (control), 18:2, 18:3α, 18:3γ, 20:3, 20:4, 20:5, 22:3, 22:5, and 22:6. All samples were grown shaking at 37 degrees Celsius to an OD$_{600}$ of 0.8-1.0 and thereafter pelleted down via centrifugation. The supernatant was discarded and the remaining pellet was washed and re-suspended in solution using G-56.

The long-standing lipid extraction method of Bligh and Dyer was used to extract the lipids. First, 5mL of G-56 was added to wash each sample of impurities. Cultures were then centrifuged at 4000rpm for 10 minutes, and the top, buffer layer was poured off. Next, pellets were re-suspended in 5mL of single phase Bligh/Dyer chloroform/methanol/water mixture (1:2:0.8v/v) to extract lipids from the cell. Each sample was incubated at room temperature for ~20 minutes and vortexed at 5 minute intervals. Subsequent centrifugation at 4000rpm for 10 minutes isolated phospholipids and sugar nucleotides within the supernatant, concentrating lipid A in the solid pellet. The liquid supernatant was then extracted and transferred into a clean, glass tube, while the pellet was discarded. Adding 1.3mL of both chloroform and water to the conserved supernatant, a two-phase solution was created. This solution was vortexed and centrifuged as previously conducted for 10 minutes; afterwards, the lower phase was extracted and transferred into a new, smaller glass tube. 2.6mL of chloroform was added to the remaining upper phase, which was then vortexed and centrifuged as previously
conducted for another 10 minutes. Once again, the lower phase was extracted. The lower phases were pooled and dried down under a stream of nitrogen gas. Dried lipids were stored at -20 degrees Celsius until thin layer chromatography (TLC) was performed.68, 69

Dried lipids were re-suspended in a 4:1 chloroform/methanol solution and spotted onto a silica gel-coated TLC plate. To develop a lipid profile using thin layer chromatography (TLC), a 65:25:10 chloroform/methanol/acetic acid solvent system was used to drive lipids up the plate according to their hydrophobicity. Before adding the plates, the TLC tank was allowed to equilibrate for approximately 2 hours. Afterwards, the spotted TLC plate was placed inside the tank and left to develop for an hour and 40 minutes. Following TLC, charring was used to develop the plate and produce a visual representation of the lipids found within the membrane. The TLC plates were sprayed with 10% sulfuric acid in 100% ethanol and heated to 200 °C for 5 minutes to enable visualization of lipid profiles.

CRYSTAL VIOLET UPTAKE ASSAY

Changes in membrane permeability induced by incubation with PUFAs were determined using crystal violet uptake assays, which measured the percent of crystal violet (CV) taken up by bacterial cells. The volume of overnight culture required to start 5mL cultures in PBS at an OD$_{590}$ of 0.1 was determined and subsequently added. To a single culture, 300μM of each fatty acid was added with the control receiving no fatty acids. All cultures of *V. parahaemolyticus* received 300mM NaCl in addition to PUFAs. Cultures grown and used in this assay as inocula were: negative (control), 18:2, 18:3α, 18:3γ, 20:3, 20:4, 20:5, 22:3, 22:5, and 22:6. Once at an OD of 0.8-1.0, all cultures were washed with 1mL of PBS and centrifuged at 4000rpm for 5 minutes. The supernatant was
poured off and the conserved pellet was re-suspended in 1mL of PBS. OD’s of each culture were then taken at 600nm. The lowest OD measured determined the starting OD of all the inocula. Appropriate volumes of each culture, PBS, and PUFA were added to clean plastic tubes to produce a 5mL sample with the same OD as the lowest measured inoculum OD. CV was added to a final concentration of 5μg/mL. All samples were then placed on a rocker and left to rock at room temperature for 5 minutes. After 5 minutes, the samples were removed from the rocker, and 800μL of each sample was transferred to a new Eppendorf, which were then centrifuged in a microcentrifuge at 17xg for 1 minute for \textit{V. cholerae} and 2 minutes for \textit{V. parahaemolyticus}. After centrifugation, the supernatant was drawn off and transferred into a cuvette. ODs at 590nm were subsequently taken. This process was repeated at 5 minute intervals for 25 minutes.

**LACTIC ACID STRESS ASSAY**

Lactic acid stress assays were performed to determine whether homeoviscous adaptations incorporating exogenous PUFAs influenced cellular resistance to acid stress. The volume of overnight culture required to start 5mL cultures of inocula in G-56 (pH 7.5) at an OD$_{600}$ of 0.1 was determined and subsequently added. To a single culture, 300μM of each PUFA was added with the control receiving no fatty acids. All cultures of \textit{V. parahaemolyticus} received 300mM NaCl in addition to PUFAs. Cultures grown and used in this assay as inocula were: negative (control), 18:3γ, 20:3, 20:4, 20:5, and 22:6. Once at an OD of 0.8-1.0, inocula were washed with 1mL of G-56 and centrifuged for 5 minutes. Afterwards, the supernatant was poured off and the conserved pellet was re-suspended in 1mL of G-56. OD’s of each inoculum were taken at 600nm. Using an 11.98M stock, a series of dilutions were performed to produce 1.2mL Eppendorf tubes of
desired lactic acid concentrations. Minimum inhibitory concentration (MIC) microtiter plate lactic acid stress assays were performed in duplicate. Under the hood, using a multichannel pipette, 30\(\mu\)L of each concentration of lactic acid and then 170\(\mu\)L of each inoculum were added to appropriate wells in the microtiter plate. Plates were then left shaking in the incubator at 37 degrees Celsius for 24 hours. The next day, the optical densities of each well were measured at 600nm using a Biotek Synergy Microplate reader accompanied by Gensys software.

**HYDROGEN PEROXIDE STRESS ASSAY**

Hydrogen peroxide (H\(_2\)O\(_2\)) stress assays were performed to test bacterial resilience to hydrogen peroxide stress following PUFA-induced membrane remodeling. Overnight cultures were used to prepare the inocula as described for the lactic acid stress assay. Cultures grown and used in this assay as inocula were: negative (control), 18:3\(\gamma\), 20:3, 20:4, 20:5, and 22:6. Using a 9.79M stock, a series of dilutions were performed to achieve 1.2mL Eppendorf tubes of desired H\(_2\)O\(_2\) concentrations. Microtiter plates were arranged in quadruplicate for both organisms, and MIC hydrogen peroxide stress assays were performed as detailed for the lactic acid stress assay. Plates were read at 600nm using the same instruments mentioned in the lactic acid stress assay protocol.

**POLYMYXIN B STRESS ASSAY**

Polymyxin B (PMB) stress assays were performed to test bacterial resistance to antibiotic stress following incorporation of PUFAs into Vibrio cell membranes. Overnight cultures were used to prepare the inocula as described for the lactic acid stress assay. Cultures grown and used in this assay as inocula were: negative (control), 18:2, 18:3\(\alpha\), 18:3\(\gamma\), 20:3, 20:4, 20:5, and 22:6. Using a 5mg/mL stock, a series of dilutions
were performed to achieve 1.2mL Eppendorf tubes of desired PMB concentrations. Microtiter plates were arranged in quadruplicate. MIC microtiter plate PMB stress assays were performed as detailed for the lactic acid stress assay, and plates were read at 600nm using the same instruments.

**BIOFILM ASSAY**

Biofilm stress assays were performed to determine whether membrane remodeling via PUFA incorporation influenced biofilm formation. Overnight cultures were pelleted, washed, and re-suspended in LB for *V. cholerae* and G-56 (pH 7.5) for *V. parahaemolyticus*. Optical density readings were subsequently performed at 600nm. These cultures were used to prepare the inoculum in LB for *V. cholerae* and G-56 for *V. parahaemolyticus* at an OD of 0.1. To produce a solution of PUFA-supplemented media, 300μM of each fatty acid was added to LB for *V. cholerae* and G-56 for *V. parahaemolyticus*. 90µL of this PUFA-supplemented media was distributed into microtiter plate wells. Once this was done for all fatty acids, 10µL of inocula was added to every well. The plate was then left shaking in the incubator at 37 degrees Celsius for approximately 24 hours. The next day, the plate was turned over and shook, dumping the cells out of the plate. To remove unattached cells and media components, the plate was then submerged in a tub of diH₂O and once again turned and shook. This was performed twice. After the second repetition, 125µL of a 0.1% CV solution was added to each well using a multichannel pipette, and the plate was allowed to incubate at room temperature for 15 minutes. The microtiter plate was then rinsed with diH₂O 3-4 times as previously described. After the final rinse, to remove any remaining excess cells or dye, the plate was turned upside down and blotted vigorously on a stack of paper towels. It was then
left upside down to dry overnight. After 24 hours, 125μL of 30% acetic acid was added to all wells, solubilizing remaining CV. Upon addition of acetic acid, the plate was incubated at room temperature for 10-15 minutes and subsequently read at 600nm using a Biotek Synergy Microplate paired with Gensys software.
RESULTS

Growth of Vibrio cholerae and Vibrio parahaemolyticus in the presence of various fatty acids modified phospholipid profiles.

To evaluate the effect of PUFAs on Vibrio’s lipid profiles, bacteria were grown in the presence and absence of micromolar concentrations of each fatty acid in minimal media. Bacterial phospholipids were extracted and visualized via TLC. As shown in Figure 1, each of the three major phospholipids Vibrio produces (phosphatidylethanolamine [PE], phosphatidylglycerol [PG], and cardiolipin [CL]) exhibited upward migrational shifts, indicating phospholipid species became more hydrophobic as carbon number and degree of unsaturation increased. Figure 2 depicts a representative plate of 3 biological replicates. The structures of the three major phospholipid species produced by Vibrio and other Gram-negative bacteria are shown in Figure 2.
**Figure 1:** TLC phospholipid profiles for *V. cholerae* and *V. parahaemolyticus* following exposure to exogenous PUFAS. Bacteria were grown to log phase (OD 0.8-1.0) in G56 minimal media (pH 7.5) at 37°C with or without 300μM fatty acid (and with 300mM NaCl for *V. parahaemolyticus*) prior to Bligh and Dyer extraction of phospholipids (PLs) and separation by TLC. For visualization of lipids, the plate was sprayed with 10% sulfuric acid in 100% ethanol and charred at 200°C. 22:5 was removed from the TLC plate for *V. parahaemolyticus* because the lipid migration was heavily disturbed. The plates shown above are representative plates from a series of 3 repetitions.

**Figure 2:** The structures of phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL), the primary phospholipid species produced by *Vibrio* and other Gram-negative bacteria.
Exogenous fatty acids significantly altered the membrane permeability of *V. cholerae* and *V. parahaemolyticus* to hydrophobic compounds

Following TLC data suggestive of PUFA-induced membrane remodeling, CV uptake assays were performed to determine whether or not membrane remodeling influenced permeability of the cellular membrane. As shown in Figure 3, 18:3\(\gamma\) and 22:5 increased membrane permeability of *V. cholerae* to CV. Of these fatty acids, 22:5 had the most noticeable effect, increasing membrane permeability by approximately 30% compared to the control. Although 18:2, 18:3\(\alpha\), 20:3, and 20:4 had relatively no effect, 20:5, 22:3, and 22:6 decreased membrane permeability of *V. cholerae* to hydrophobic compounds, resulting in reduced CV uptake. Among these fatty acids, 22:6 decreased permeability the most, reducing CV uptake by almost 30% over time. As shown in Figure 4, all PUFAs except for 22:6 decreased membrane permeability of *V. parahaemolyticus* to CV by more than 10%. The greatest decrease in permeability was elicited by 20:4, which reduced CV uptake by approximately 20%.
V. cholerae

Optical Density (590 nm)

Control
Linoleic Acid (18:2)
α-Linolenic Acid (18:3ω)
γ-Linolenic Acid (18:3γ)
Dihomo-γ-Linolenic Acid (20:3)
Arachidonic Acid (20:4)
Eicosapentaenoic Acid (20:5)
Docosatrienoic Acid (22:3)
Docosapentaenoic Acid (22:5)
Docosahexaenoic Acid (22:6)

Time (minutes)
V. parahaemolyticus

- Control
- Linoleic Acid (18:2)
- α-Linolenic Acid (18:3α)
- γ-Linolenic Acid (18:3γ)
- Dihomo-γ-Linolenic Acid (20:3)
- Arachidonic Acid (20:4)
- Eicosapentaenoic Acid (20:5)
- Docosatrienoic Acid (22:3)
- Docosapentaenoic Acid (22:5)
- Docosahexaenoic Acid (22:6)
Exposure to PUFAs produces no effect upon *V. cholerae* and *V. parahaemolyticus*’s resistance to lactic acid stress.

Lactic acid stress assays were performed to determine if PUFA-induced membrane remodeling influenced the resilience of *Vibrio* to acid stress. No clear trend was able to be determined for *V. cholerae* since growth for all PUFAs was not eliminated or nearly eliminated (OD 0.1-0) at the highest concentration of lactic acid. *V. parahaemolyticus* grown amongst fatty acids displayed no significant deviations from the behavior of the control. Lactic acid stress assays performed with *V. cholerae* and *V. parahaemolyticus* can be seen in Figures 5 and 6, respectively.

![Figure 5: MIC assay for lactic acid stress on V. cholerae grown shaking at 37 degrees Celsius for 24 hours in G-56 (pH 7.5) with and without 300μM of the indicated fatty acids. The assay was one of three replicates and was performed in duplicate with OD measurements read at 600nm. All standard deviations were less than or equal to 0.08.](image-url)
Figure 6: MIC assay for lactic acid stress on V. parahaemolyticus grown shaking at 37 degrees Celsius for 24 hours in G-56 (pH 7.5) with and without 300μM of the indicated fatty acids. The assay was one of three replicates and was performed in duplicate with OD measurements read at 600nm. All standard deviations were less than or equal to 0.08.
PUFAs enhance the resistance of *V. cholerae* but increase the susceptibility of *V. parahaemolyticus* to hydrogen peroxide (*H₂O₂*) stress.

Hydrogen peroxide assays were performed to evaluate the influence of PUFAs on *Vibrio*’s resilience to oxidative stress. As shown in Figure 7, while all other PUFAs behaved nearly identical to the control, 20:5 and 22:6 increased *V. cholerae*’s resistance to oxidative stress, respectively withstanding 2 and 4 times as concentrated *H₂O₂* as the control. Unlike *V. cholerae*, all fatty acids decreased the resistance of *V. parahaemolyticus* to hydrogen peroxide stress. 22:6 exhibited the earliest decline in growth for all PUFAs. These results can be seen in Figure 8.

**Figure 7**: Hydrogen peroxide MIC stress assay for *V. cholerae* grown shaking at 37 degrees Celsius for 24 hours in G-56 (pH 7.5) with and without 300μM of indicated PUFAs. The assay was one of three replicates and was performed in quadruplicate with optical density measurements read at 600nm. All standard deviations were less than or equal to 0.01.
Figure 8: Hydrogen peroxide MIC stress assay for *V. parahaemolyticus* grown shaking at 37 degrees Celsius for 24 hours in G-56 (pH 7.5) with and without 30μM of indicate fatty acids. The assay was one of three replicates and was performed in quadruplicate, and OD measurements were read at 600nm. All standard deviations were less than or equal to 0.07.
With the exception of 20:3 for *V. parahaemolyticus*, all PUFAs increase the susceptibility of both *Vibrio* species to PMB.

As shown in Figure 9, despite initial variations, all PUFAs clearly increase *V. cholerae*’s susceptibility to PMB, as best shown at concentrations greater than or equal to 100μg/mL. Although 22:6 does present more growth than the control at 100μg/mL, this growth steadily declines to significantly less than that of the control at higher concentrations. Of all fatty acids, 18:3α seems to increase the susceptibility of *V. cholerae* to PMB the most, presenting over 10% less growth than the control for all concentrations except 25μg/mL. Similarly, all PUFAs except for 20:3 increased the susceptibility of *V. parahaemolyticus* to antibiotic stress, as best observed between 200-400μg/mL. Notably, 18:3γ, 20:4, and 22:6 initially increased the resistance of *V. parahaemolyticus* to PMB, although as Figure 10 reflects, growth for these fatty acids significantly declined between 100-200μg/mL. All PUFAs except for 20:3 presented minimal growth at concentrations nearly half as concentrated as those eliciting the same effect from the control. Although 20:3 appeared to slightly increase *V. parahaemolyticus* resistance to PMB between 100-200μg/mL, its growth eventually declined and became nearly identical to that of the control at concentrations greater than or equal to 400μg/mL, reflecting 20:3 had no effect on the MIC of *V. parahaemolyticus* exposed to antibiotic stress, as shown in Figure 10.
Figure 9: PMB MIC stress assay for *V. cholerae* grown shaking at 37 degrees Celsius for 24 hrs in G-56 (pH 7.5) with and without 300μM of indicated fatty acids. The assay was one of three replicates and was performed in quadruplicate with optical density measurements recorded at 600nm. All standard deviations were less than or equal to 0.08.
Figure 10: PMB MIC stress assay for *V. parahaemolyticus* grown shaking at 37 degrees Celsius for 24 hours in G-56 (pH 7.5) with and without 300μM of indicated fatty acids. The assay was one of three replicates and was performed in quadruplicate with optical density measurements read at 600nm. All standard deviations were less than or equal to 0.1.
Incubation with exogenous fatty acids significantly altered biofilm formation in *V. cholerae* and *V. parahaemolyticus*.

Biofilm assays were performed to determine the effect of PUFA-induced membrane remodeling upon biofilm formation. Optimal conditions determined by prior experiments were used. These experiments can be seen in Appendix Figures A-3 and A-4 for *V. cholerae* and *V. parahaemolyticus* respectively. All PUFAs except 22:6 decreased biofilm formation in *V. cholerae* with 22:6 increasing biofilm activity by approximately 25.5%. All asterisked fatty acids indicate data points reflecting statistically significant differences from the control, as determined by the p-value, which was less than or equal to 0.02. Remarkably, all fatty acids except 18:3\(\gamma\), 22:5, and 22:6 increased biofilm formation in *V. parahaemolyticus* by over 30%. Of these PUFAs, 22:5 decreased biofilm activity the least, while 22:6 and 18:3\(\gamma\) produced 30% and 70% reductions, respectively. As for *V. cholerae*, all asterisked fatty acids indicate data points reflecting statistically significant differences from the control, as determined by the p-value, which was less than or equal to 0.03 for *V. parahaemolyticus*. These findings can be seen in Figures 11 and 12 for *V. cholerae* and *V. parahaemolyticus*, respectively.
Figure 11: Biofilm activity of *V. cholerae* grown shaking at 37 degrees Celsius for 24 hours in LB with and without 300μM of indicated fatty acids. The assay was one of three replicates and was performed in octuplet with optical density measurements read at 600nm. All standard deviations were less than or equal to 0.2. P values representing statistically significant differences from the control were determined to be less than or equal to 0.02 for all asterisked fatty acids.
Figure 12: Biofilm activity of *V. parahaemolyticus* grown shaking at 37 degrees Celsius for 24 hours in G-56 (pH 7.5) with and without 300μM of indicated fatty acids. The assay was one of three replicates and was performed in octuplet with optical density measurements read at 600nm. All standard deviations were less than 0.08. The asterisk marks indicate data points reflecting statistically significant differences from the control, as determined by p-values less than or equal to 0.03.
DISCUSSION

ORIGINAL APPROACH

Initially, this project was approached from a different angle. As previously mentioned, within its environmental reservoirs, such as oysters and chironomid egg masses, *Vibrio* does not seem to produce any of the destructive effects seen within the human reservoir. It was believed *Vibrio* must express different virulence phenotypes within its environmental reservoirs than expressed within the human host, explaining this dramatic difference in host associations. Thus, it was originally hypothesized that PUFAs found within *Vibrio*’s natural surroundings were taken up and incorporated into the membrane to induce the expression of seemingly non-virulent phenotypes within these reservoirs. To determine whether *Vibrio* used exogenous lipids to modify its phospholipid profiles, this study had intended to grow *Vibrio* amongst oyster meat and juice, using TLC to visualize lipid profiles. Oyster meat and two types of meat juice (one being squeezed from the meat itself and the other secreted from the oyster upon shucking) were pelleted down, re-suspended using marine broth (MB) and LB for two different sets of samples, and then filter-sterilized. MB was used to simulate environmental conditions as similar to *Vibrio*’s natural marine environment as possible, while LB was chosen to prevent cell death in case the oyster cells lacked essential nutrients once removed from the oyster. A mortar and pestle, centrifuge, and microcentrifuge were all used to homogenize oyster components but each method had little success. Although the highest possible speed settings within reason on the centrifuge were used, oyster components still were not homogenized well enough to yield the tight pellets necessary for efficient lipid extraction. Consequently, cellular debris contaminated extracted lipids, and the heavy
lipid loads of samples spotted onto our TLC plate caused excessive streaking with a few intersecting lanes. Due to these setbacks, this study was modified to focus more on the implications for overall fitness membrane remodeling may hold, considering fatty acids can be used to serve several purposes other than membrane remodeling; furthermore, it was believed that there must be some explanation as to why Vibrio choose to use these precious resources for phospholipid remodeling instead of other uses.3, 59

OBJECTIVES AND HYPOTHESES

Although this project sought to determine whether or not fatty acids were being incorporated into existing Vibrio phospholipid profiles, previous research provided ample support this did indeed occur.3, 57, 70 Ability to endure unfavorable environmental conditions has long been recognized as an indicator of advanced fitness; hence, it was hypothesized that phospholipid remodeling would modify virulence phenotypes to enhance overall fitness, increasing resistance to stress as well as cellular defense mechanisms, such as biofilm formation and cellular membrane impermeability.4,55-57, 71, 72 Thus, the primary purpose of this experiment was to determine whether or not this had occurred. Stress, biofilm, and CV assays were performed to assess if membrane remodeling had affected virulence phenotypes; and if so, to distinguish the affect these new phenotypes had upon bacterial fitness. Since V. cholerae and V. parahaemolyticus belong to the same family, it was believed that modifications of lipid species would affect these organisms in a similar manner, altering their membranes in a comparable fashion to produce parallel virulence phenotypes that would elicit similar responses to stress.
PHYSIOLOGICAL RELEVANCE OF EXPERIMENTAL CONDITIONS

Influenced by the original project’s focus on the biphasic lifestyle of *Vibrio*, all experimental conditions were tailored to be as physiologically relevant as possible. Although the primary focus was the human reservoir, conditions prevalent in *Vibrio*’s natural marine environments were also considered. Simulating normal, resting body temperature, all cultures were incubated at 37 degrees Celsius. The pH of all G-56 minimal media was adjusted to be 7.5, which was optimal for growth and as close to human physiological pH (pH= 7.2-7.4) as possible given growth restrictions. G-56 minimal media was used for most experiments to exclude fatty acid contributions found in complex media, such as LB and tryptic soy broth (TSB). However, media other than G-56 was used for biofilm experiments to generate species-specific conditions optimal for biofilm formation as determined by initial biofilm assay experiments. See Figures A-3 and A-4 in the appendix for further detail.

LIPID VISUALIZATION VIA TLC

TLC was used to evaluate whether *Vibrio* had utilized its cellular machinery to take up exogenous PUFAs and incorporated them into membrane. Upon comparison to the control, visualization of both species’ lipid profiles revealed significant upward migrations of all three major phospholipid species produced by *Vibrio* and other Gram-negative bacteria for all fatty acids, as shown in Figure 1. The structures of these species can be found in Figure 2. In TLC, upward migrations reflect increasing hydrophobicity, which can be caused by expanding the hydrocarbon chain and/or the addition of new degrees of unsaturation. Thus, these shifts suggested *Vibrio* was not only taking up
extracellular PUFAs but also incorporating them into its membrane. Considering these results, stress, biofilm, and CV assays were subsequently performed to determine whether any implications existed for these membrane modifications.

CV ASSAYS

Considering the vital role of the cell membrane in homeostasis, the influence of PUFAs upon Vibrio’s membrane permeability to hydrophobic compounds, such as CV, could have significant implications for bacterial fitness. CV assays revealed phospholipid remodeling had altered membrane permeability to hydrophobic compounds for both species. All PUFAs decreased V. cholerae’s membrane permeability to CV except for 18:3γ, 20:3, and 22:5. 22:6 evoked the most significant decrease in membrane permeability of V. cholerae, decreasing permeability by almost 30% compared to the control. 20:3 produced the least significant increase in permeability while 18:3γ and 22:5 increased membrane permeability by approximately 10% and 30%, respectively. Unlike V. cholerae, every PUFA decreased membrane permeability of V. parahaemolyticus to hydrophobic compounds with 20:4 producing the greatest decrease in permeability (~30%). Considering the hydrophobic properties of several antibiotics, decreased permeability to hydrophobic compounds could provide a competitive advantage against these antimicrobials. While the increased permeability to hydrophobic compounds evoked by 18:3γ, 20:3, and 22:5 does not seem likely to enhance V. cholerae’s fitness, these findings do possess significant implications for increasing the effectiveness of antimicrobials. Before a discussion of these implications, antimicrobial peptides’ mechanism of action will briefly be addressed.
ANTIMICROBIAL PEPTIDES: POLYMYXIN B

Antimicrobial peptides exploit their amphipathic structures to disrupt the cell membrane of target organisms. The hydrophilic segments and positive net charge of these peptides allow them to electrostatically interact with the negatively charged cell membrane and subsequently insert their hydrophobic cores, which engage in noncovalent interactions with the inner leaflet of the outer membrane. Polymyxin B, for example, utilizes its cationic properties and hydrophilic segments to engage in electrostatic interactions with negatively charged lipopolysaccharides (LPSs) anchored to the Gram-negative outer cell membrane. This interaction brings polymyxin B’s fatty acyl tail closer to the outer membrane while displacing the calcium and magnesium ions stabilizing LPS and the outer membrane. Together, the hydrophobic fatty acyl tail and amino acid residues of the cyclic moiety destabilize the outer membrane via insertion of their hydrophobic components into the membrane, engaging in hydrophobic interactions with the core polysaccharide of the LPS molecule. These noncovalent interactions instigate and stabilize the formation of a complex, expanding and eventually disintegrating the outer membrane. By disrupting the outer membrane, polymyxin B increases the susceptibility of Gram-negative bacteria to other hydrophobic antibiotics whose antibacterial mechanisms would otherwise be impeded. This process as well as the structure of polymyxin B can be seen in Figures A-1 and A-2 of the appendix, respectively.
THE IMPLICATIONS OF OUR CV STUDIES TO ANTIBIOTIC DEVELOPMENT

Although traditionally effective, antibiotic abuse has spurred the development of drug resistant strains of bacteria resilient to this mechanism.\textsuperscript{78} Initial studies found the addition of a fatty acid tail to polymyxin enhanced its antimicrobial activity and selectivity.\textsuperscript{78, 85-87} If the tail was removed, the antimicrobial activity of polymyxin was significantly decreased, indicating a strong correlation between peptide potency and the presence of a fatty acid chain.\textsuperscript{78, 85-87} Although the exact reason for this relationship is unclear, it is believed conjugated fatty acids may enhance the ability of antimicrobial peptides to form secondary structures.\textsuperscript{78} Recent studies strongly support fatty acids serve as an effective tool for amplifying antimicrobial activity.\textsuperscript{78, 85-87} Among these studies, Shai and Oren (2001) found fatty acid conjugation enhanced antibacterial and/or antifungal and/or anticancer properties of peptides previously weak or inactive by 2-20 fold depending on the protein.\textsuperscript{79, 88, 89} Malina and Shai (2005) obtained similar results conjugating different aliphatic acids to a biologically inactive cationic protein, although they noted a decrease in antibacterial activity with increasing fatty acid chain length.\textsuperscript{78, 90} Regardless of their effect on antimicrobial properties, all findings clearly implicate fatty acid conjugations can be used to effectively manipulate antibacterial potency and consequently toxicity to eukaryotic cells.\textsuperscript{78, 85-87} Researchers have exploited these findings to produce custom-made antimicrobials.\textsuperscript{78}

Considering CV assays revealed 22:5 and 18:3\textgreek{y} increased \textit{V. cholerae}'s permeability to hydrophobic compounds by 30\% and 10\%, respectively, the effects of conjugating these fatty acids to antimicrobials should be further investigated, especially
since antimicrobials, like polymyxin B, ultimately serve to increase Gram-negative susceptibility to hydrophobic antibiotics. Moreover, further studies should investigate the effect of membrane remodeling with 22:5 and 18:3γ on antibiotics. Given the results of CV assays, the influence of these fatty acids on *V. cholerae*’s membrane permeability could potentially amplify the effectiveness of antibiotics. As previously mentioned, several species of bacteria have developed resistance against common antibiotics. In fact, several Gram-negative bacteria, including *Vibrio*, have gained resistance to polymyxins via modifications to lipid A molecules.91, 92 Thus, exploiting natural mechanisms for increasing the susceptibility of bacterial to antibiotics could prove extremely useful against resistance strains.

**LACTIC ACID STRESS ASSAYS**

Acid stress was chosen considering its relevance to the human host, where *Vibrio* somehow survives the harshly acidic conditions of stomach to colonize in the small intestine. Although lactic acid is physiologically relevant to the human reservoir, hydrochloric acid (HCL), the primary acid found within the stomach, is more relevant to this study.93 It is possible a clear effect may have been observed for *V. cholerae* if this more physiologically relevant acid was used.

Contrary to the initial hypothesis, altered virulence phenotypes did not seem to enhance bacterial resistance to acid stress for either species. Unfortunately, a clear response for *V. cholerae* was indiscernible. Although 20:3, 20:5, and 22:6 presented greater growth than the control for concentrations of lactic acid between 50-100mM, this growth soon declined and was nearly identical to the controls’ at 200mM. Why minimal growth occurred at 200mM but steadily increased with increasing concentration of lactic
acid was unclear. One explanation could be that *V. cholerae* was somehow metabolizing lactic acid at these higher concentrations; however, there was no support for this theory found in the literature. PUFAs did not seem to have any effect on *V. parahaemolyticus*, which behaved nearly identical to the control as reflected in Figure 6.

**OXIDATIVE STRESS ASSAYS**

Oxidative stress via hydrogen peroxide was chosen as one of the stress assays due to its high physiological relevance.\(^{94-97}\) Within the human reservoir, studies have shown the presence of invading microorganisms triggers the expression of the dual oxidase 2 (DUOX2) gene in the large and small intestines.\(^{94,95}\) The expression of this gene induces epithelial cells to secrete the enzyme DUOX2, which primarily serves as a regulatory producer of reactive oxygen species (ROS).\(^{94-96}\) ROS triggers biological defenses mechanisms against pathogens, serving as initiators for lactoperoxidase-mediated killing.\(^{94-96}\) In one study performed on bovine LPO, high antibacterial activity was detected against several different pathogens, including *V. cholerae*-non01 strains, when 100mM H\(_2\)O\(_2\) was present within the media.\(^{97}\) These studies all suggest and support ROS-induced lactoperoxidase-mediated killing as a defense mechanism employed by eukaryotic cells within both human and animal reservoirs.\(^{94-97}\)

Oxidative stress is also present in *Vibrio*’s aquatic environments.\(^{3,98}\) Within the ocean, the adsorption of solar radiation by dissolved organic matter induces photochemical production of H\(_2\)O\(_2\) and other ROS.\(^{3,98}\) Because these species are relatively stable in seawater, they are able to pass through biological membranes relatively easily.\(^{3,98}\) Thus, considering it is a stress predominant in both human and aquatic reservoirs, this study aimed to determine whether PUFA-induced membrane
remodeling could provide *Vibrio* with enhanced resistance to oxidative stress. Hydrogen peroxide was chosen considering its relevance to marine environments. Although it is an ROS, nitric oxide (NO) has been known to be produced during human *V. cholerae* infection, making it more physiologically relevant to the human reservoir than H$_2$O$_2$. To best simulate oxidative stress found in both reservoirs, H$_2$O$_2$ as well as NO should have been used to perform oxidative stress assays. Differences in *Vibrio*’s response to these ROS would have been interesting to compare, and future work stemming from this project should consider performing oxidative stress assays using both ROS.

Despite initial hypotheses that PUFAs would increase *Vibrio*’s resistance to oxidative stress, hydrogen peroxide assays revealed all fatty acids for *V. cholerae* behaved nearly identical to the control except for 20:5 and 22:6. *V. cholerae* grown amongst 20:5 was able to withstand H$_2$O$_2$ concentrations nearly two times greater than the control, while 22:6 withstood concentrations approximately four times greater than the control. Notably, 20:5 and 22:6 both belong to the ω-3 fatty acid family, which are native to marine environments and found primarily in aquatic organisms, such as algae, phytoplankton, and fish consuming algae.$^{3,99}$ It is intriguing that other ω-3 fatty acids, 20:3 and 20:4, did not produce effects similar to 20:5 and 22:6. A table of all PUFAs within the ω-3 fatty acid family can be found in Appendix Table A-2.$^{100}$ Given the prominence of 20:5 and 22:6 within aquatic ecosystems, *V. cholerae* may gain enhanced resilience to oxidative stress by taking up these ubiquitous fatty acids and utilizing them for membrane remodeling, subsequently altering its ability to persistence amongst this stress.$^{3,99}$
Contrary to initial hypotheses, all PUFAs made *V. parahaemolyticus* more susceptible to oxidative stress. Of all fatty acids tested, 22:6 increased the susceptibility of *V. parahaemolyticus* to oxidative stress the most with 20:5 following. Notably, 20:5 and 22:6 significantly increased the resistance of *V. cholerae* to oxidative stress, but for *V. parahaemolyticus* these same fatty acids significantly decreased resistance. Why these opposing effects occur is particularly intriguing and warrants further study.

**PMB ASSAYS**

As previously discussed, antimicrobial stress was highly relevant to this project. PUFA remodeling was expected to enhance *Vibrio*’s resistance to PMB. Notwithstanding initial variations, all PUFAs clearly increased *V. cholerae*’s susceptibility to PMB, as best shown at concentrations greater than or equal to 100μg/mL. Although 22:6 presented more growth than the control at 100μg/mL, this growth steadily declined to significantly less than that of the control at higher concentrations. Of all fatty acids, 18:3α appeared to increase the susceptibility of *V. cholerae* to PMB the most, presenting over 10% less growth than the control for all concentrations except 25μg/mL. Likewise, PUFA-induced membrane remodeling clearly increased the susceptibility of *V. parahaemolyticus* to PMB for all fatty acids except 20:3, as best observed between 200-400μg/mL. Within this concentration range, all fatty acids except for 20:3 clearly enhanced the susceptibility of *V. parahaemolyticus* to PMB yielding minimal growth long before the control and 20:3, which continued to present growth until concentrations greater than or equal to 400μg/mL. Although between 100-200μg/mL 20:3 appeared to slightly increase *V. parahaemolyticus* resistance to PMB, its growth eventually declined and was nearly identical to that of the control at concentrations greater than or equal to 400μg/mL.
Notably, although PUFAs were able to increase the susceptibility of *V. parahaemolyticus* to PMB, they were also found to decrease its membrane permeability to hydrophobic compounds. Considering the mechanism of PMB, these results merit further investigation.

**BIOFILM ASSAYS**

As previously mentioned, several studies note *Vibrio*’s acute ability to form biofilms has helped it persist amongst extremely unfavorable conditions.\(^4,60-63\) Original biofilm assays were performed to determine optimal conditions for biofilm activity. Within these studies, *Vibrio*’s biphasic lifestyles were considered and consequently several physiologically relevant variables, including temperature, salinity, and media, were altered. Representatives of these original assays most pertinent to this study can be seen in Appendix Figures 3-1 and 3-2. From these assays, optimal biofilm growth conditions were determined to be shaking at 37 degrees Celsius for both species. However, LB appeared to be optimal media for *V. cholerae* while G-56 appeared to be optimal for *V. parahaemolyticus*. These respective media were used for biofilm assays shown in Figures 10 and 11.

Contrary to initial hypotheses, for *V. cholerae*, all PUFAs decreased biofilm formation except 22:6, which increased biofilm activity by roughly 25.5%. Notably, CV assays revealed 22:6 also produced a substantial decrease in membrane permeability of *V. cholerae* to hydrophobic compounds. These results suggest membrane remodeling utilizing exogenous 22:6 is quite advantageous for *V. cholerae*, especially considering it appears to enhance two primary defense mechanisms of bacteria, cellular rigidity and biofilm formation. Further studies should be performed with 22:6 to determine whether
this PUFA enhances other survival mechanisms of *V. cholerae*. Also significant, both 18:3γ and 22:5 not only increased the membrane permeability of *V. cholerae* to hydrophobic compounds but also elicited the substantial reductions in biofilm formation. Considering these two fatty acids seem to significantly impair two of *Vibrio cholerae’s* primary defense mechanisms, further investigation is merited.

All fatty acids except for 18:3γ, 22:5, and 22:6 increased biofilm formation of *V. parahaemolyticus*. 22:5 decreased biofilm activity of *V. parahaemolyticus* the least, while 22:6 and 18:3γ produced 30% and 70% reductions, respectively. Although 22:5 and 22:6 both belong to the ω-3 fatty acid family, 18:3γ does not and is of much shorter chain length with fewer degrees of unsaturation. Considering the remarkable structural differences between these PUFAs, we have no explanation as to why 18:3γ, 22:5, and 22:6 behaved similarly.

**CONCLUSION**

As this study reflects, *Vibrio* species utilize exogenous fatty acids found within their environments to modify their lipid profiles. While stress assays did not all support initial hypotheses, they did yield several intriguing findings. CV assays support membrane remodeling with PUFAs significantly alters the membrane permeability of both *V. cholerae* and *V. parahaemolyticus* to hydrophobic compounds. Oxidative stress assays revealed PUFA modifications increased the susceptibility of *V. parahaemolyticus*, while 20:5 and 22:6 increased the resistance of *V. cholerae*. PUFAs increased the susceptibility of both species to PMB despite decreased membrane permeability to hydrophobic compounds conferred to *V. cholerae* by all fatty acids but 18:3γ, 20:3, and 22:4 and to *V. parahaemolyticus* by all fatty acids. Moreover, biofilm assays revealed all
PUFAs but 22:6 decreased biofilm formation for *V. cholerae*, while all fatty acids except 18:3γ, 22:5, and 22:6 increased biofilm formation for *V. parahaemolyticus*. As these findings suggest, PUFA membrane modifications are reflective of *Vibrio*’s propensity for keen homeoviscous adaptations but may also be exploited to decrease or increase virulence.
**APPENDIX**

<table>
<thead>
<tr>
<th>Common Name of Fatty Acid</th>
<th>Lipid Name</th>
<th>ω-3 or ω-6 Family*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linoleic acid</td>
<td>18:2 (n-6)</td>
<td>ω-6</td>
</tr>
<tr>
<td>α-Linolenic acid (ALA)</td>
<td>18:3α (n-3)</td>
<td>ω-3</td>
</tr>
<tr>
<td>γ-Linolenic acid</td>
<td>18:3γ (n-6)</td>
<td>ω-6</td>
</tr>
<tr>
<td>Dihomo-γ-linolenic acid</td>
<td>20:3 (n-6)</td>
<td>ω-6</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>20:4 (n-6)</td>
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<tr>
<td>Eicosapentaenoic acid (EPA)</td>
<td>20:5 (n-3)</td>
<td>ω-3</td>
</tr>
<tr>
<td>Docosatrienoic acid</td>
<td>22:3 (n-3)</td>
<td>ω-3</td>
</tr>
<tr>
<td>Docosapentaenoic acid (DPA)</td>
<td>22:5 (n-3)</td>
<td>ω-3</td>
</tr>
<tr>
<td>Docosahexaenoic acid (DHA)</td>
<td>22:6 (n-3)</td>
<td>ω-3</td>
</tr>
</tbody>
</table>

*All ω-3 and ω-6 fatty acids are physiologically relevant in both aquatic and human reservoirs. They are primarily found within fish and fish oils. Microalgae and brown algae serve as additional sources of DHA and EPA respectively.*
Figure A-1-1: The outer membrane of Gram-negative bacteria is a phospholipid monolayer with anchored LPS molecules. LPS is composed of an O-antigen, lipid A component, and R-core. Ca\(^{2+}\) and Mg\(^{2+}\) electrostatically bind to the negatively charged lipid A component of each LPS molecule.\(^{73, 83}\)

Figure A-1-2: Electrostatic interactions between the negatively charged lipid A and positively charged polymyxin molecule displace Ca\(^{2+}\) and Mg\(^{2+}\) ions while bringing polymyxin’s fatty acyl chain closer to the outer membrane.\(^{73, 83}\)
Figure A-1-3: The hydrophobic amino acid residues of the cyclic moiety and fatty acyl tail are inserted into the outer membrane and consequently interact with the core polysaccharide of the LPS molecule via hydrophobic interactions. These interactions produce and stabilize the formation of a complex, expanding and eventually disintegrating the outer membrane, which will ultimately instigate cell lysis.\textsuperscript{73, 83}

Figure A-2: The structure of peptide antimicrobial polymyxin B. A family of pentacationic polypeptides, polymyxins have three general components: a cyclic
Figure A-3: Original biofilm experiments *V. cholerae* used to determine optimal conditions for biofilm formation. For the inoculum, overnight cultures were pelleted, washed, and re-suspended in LB for 4-1 and G-56 (pH 7.5) for 4-2 at ODs of 1. Biofilm assay allowed 24 hours of incubation shaking at 37 degrees Celsius and was performed in octuple. ODs were read at 600nm. All standard deviations were less than or equal to 0.08 for A-3-1 and less than or equal to 0.04 for A-3-2. The asterisk marks indicate data points reflecting statistically significant differences from the no fatty acid control, as determined by p-values less than or equal to 0.01.
Figure A-4: Original biofilm experiments with *V. parahaemolyticus* used to determine optimal conditions for biofilm formation. For the inoculum, overnight cultures were pelleted, washed, and re-suspended in LB for 4-1 and G-56 (pH 7.5) for 4-2 at ODs of 1. Biofilm assay allowed 24 hours of incubation shaking at 37 degrees Celsius and was performed in octuplet. ODs were read at 600nm. All standard deviations were less than or equal to 0.01 for 4-1 and less than or equal to 0.09 for 4-2. The asterisk marks indicate data points reflecting statistically significant differences from the no fatty acid control, as determined by p-values less than or equal to 0.001.
<table>
<thead>
<tr>
<th>Common Name of Fatty Acid</th>
<th>Lipid Name</th>
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<tbody>
<tr>
<td>Hexadecatrienoic acid (HTA)</td>
<td>16:3 (n-3)</td>
</tr>
<tr>
<td>α-Linolenic acid (ALA)</td>
<td>18:3 (n-3)</td>
</tr>
<tr>
<td>Stearidonic acid (SDA)</td>
<td>18:4 (n-3)</td>
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<td>Eicosatrienoic acid (ETE)</td>
<td>20:3 (n-3)</td>
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<tr>
<td>Eicosapentaenoic acid (EPA)</td>
<td>20:5 (n-3)</td>
</tr>
<tr>
<td>Heneicosapentaenoic acid (HPA)</td>
<td>21:5 (n-3)</td>
</tr>
<tr>
<td>Docosapentaenoic acid (DPA)</td>
<td>22:5 (n-3)</td>
</tr>
<tr>
<td>Docosahexaenoic acid (DHA)</td>
<td>22:6 (n-3)</td>
</tr>
<tr>
<td>Tetracosapentaenoic acid</td>
<td>24:5 (n-3)</td>
</tr>
</tbody>
</table>
REFERENCES


aquentic environment by fluorescent-mono-clonal antibody and culture methods. 


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