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Determining the antimicrobial activity and permeabilizing effects of polyunsaturated fatty acids and piscidins on Vibrio parahaemolyticus and Vibrio vulnificus

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Determining the Antimicrobial Activity and Permeabilizing Effects of Polyunsaturated Fatty Acids and Piscidins on *Vibrio parahaemolyticus* and *Vibrio vulnificus*

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Abstract

Antimicrobial peptides (AMPs) are found in all organisms and have gained attention for their effectiveness against a variety of pathogens. Piscidins are AMPs that were discovered in mast cells of vertebrates, mostly within marine animals, and are an important part of the innate immune system due to their potent antimicrobial properties. Piscidin 1 (P1) and piscidin 3 (P3) are found within sea bass and have different antimicrobial activities. P1 exhibits strong membrane disruptive properties, while P3 goes through the membrane to the targeted DNA. These piscidins can experience metalation with copper (Cu^{2+}) which enhances their antimicrobial activity in vitro (P1-Cu^{2+} and P3-Cu^{2+}). *V. parahaemolyticus* is one of the leading foodborne pathogens in humans, causing acute gastrointestinal and immunological disorders. *V. vulnificus* has high mortality (18%) and economic burden ($320 million/year worldwide), causing necrotizing fasciitis and sepsis leading to approximately 100 deaths per year in the United States. The purpose of this study was to investigate the activity of metalated and nonmetallated P1 and P3 on *Vibrio parahaemolyticus* and *Vibrio vulnificus*, as the *Vibrio* genus possesses the ability to modify membrane permeability based on the available ambient fatty acids. To examine polyunsaturated fatty acid (PUFA) impacts on piscidin activity, we performed membrane permeability assays with the hydrophobic crystal violet and minimal inhibitory concentration (MIC) assays with linoleic (18:2), arachidonic (20:4) and docosahexaenoic (22:6) acids. In *V. parahaemolyticus*, PUFAS raised the MIC for P1 and P1-Cu^{2+} while the opposite effect was observed with P3 and P3-Cu^{2+}. The data highlights antimicrobial susceptibility differences dependent upon the piscidin isoform. Each PUFA caused a distinct change in membrane permeability, while the effects of each piscidin were variable. In *V. vulnificus*, at least one PUFA raised the MIC of each piscidin. All PUFAs lowered permeability, but significant piscidin-

dependent effects were observed with P1 only. The data herein sheds light on bacterial membrane dynamics regarding piscidin activity and PUFA-mediated effects which could potentially be used as future treatment for infectious diseases.

Introduction

Antibiotic resistance

In the 21st century, the lack of new antibiotic discovery and the rapid emergence of antibiotic-resistant bacteria has posed a significant threat to global public health. During the Golden Age of Antibiotics, in 1945, the discoverer of penicillin Alexander Fleming issued a public warning against the misuse of antibiotics [55]. By 1965, many doctors were prescribing antibiotics for common sicknesses like the common cold and influenza [55]. The extensive misuse of antibiotics in agriculture and treatment of human and animal infections over several decades are responsible for the irreversible emergence of antibiotic resistance among Gram-negative and Gram-positive pathogens [8,9,45]. Currently, extensive clinical research for new therapeutic options has been conducted and one of the most promising options is the naturally occurring class of antimicrobial peptides [9].

Antimicrobial Peptides

Antimicrobial peptides (AMPs) are a diverse group of innate immune activator molecules that are present in all organisms and recognized for their broad-spectrum activity against microorganisms [3,11,33]. AMPs are small polypeptide molecules, made up of 12 to 100 amino acids that are amphipathic, having both cationic and hydrophobic faces [4,40,45]. Along with their antimicrobial properties, these host defense peptides (HDPs) also have anti-inflammation,

antitumor, immune activation, and wound healing effects under physiological conditions [16,35]. The global spread of antibiotic-resistant bacteria imposes a serious threat to global health due to the rapid decline of new effective antibiotic discoveries, allowing new antimicrobial research to be conducted [9, 45]. Research on antimicrobial peptides has been rapidly gaining attention since the 1990s, showing astonishing chemical diversity in nature. Later research showed therapeutic potential by highlighting that AMPs function with bactericidal, fungicidal, virucidal, and tumoricidal activities [40]. Additionally, AMPs have great stability, low incidence of bacterial resistance, and low toxicity to the host which allows AMPs to be considered a potential drug alternative [33,40].

AMPs are classified into five different classes based on their secondary structure; α-helix, b-sheet, loop, extended coil, and cyclic peptides [40]. Depending on the dose, duration, target tissue, and host microbiota, AMP effectiveness differs [33]. AMPs do not induce resistance since they perform rapid germ-killing, target cell walls, and intracellular effects without targeting molecules and specific pathways, like protein binding sites [9,30,45]. For our study, we are focusing on AMPs with a cationic/amphiphilic α-helix structure. Cationic AMP antibacterial activity increases membrane permeability, cell membrane lysis, and intracellular content release [9]. For those actions to occur, the AMPs must pass through capsular polysaccharides and lipid polysaccharides (LPS) of Gram-negative bacteria to exert their antimicrobial activity. For AMPs to pass, they bind to the anionic lipid membrane and transform from its disordered structure to the amphiphilic α-helical structure. The amphiphilic α-helix structure is an advantageous modification for membrane binding since it matches the nature of phospholipids and enables both the hydrophilic and hydrophobic sides of the AMP to bind efficiently to the bilayer lipid membrane [30,40,43]. Although the antimicrobial peptide structure determines what activities

they will perform, the pore formations to penetrate the phospholipid layer with the peptide-lipid ratio also affect AMP interaction with the cellular membrane [9]. There are two pore form models and two non-pore forms; barrel-stave model, carpet model, toroidal-pore model, and aggregate model [9,45,58]. First, the barrel stave pore includes a peptide interacting with one another and forming a water channel through the phospholipid layer. Secondly, the toroidal pore results in a high concentration of AMP to bend, insert perpendicularly into the membrane, and deform the lipid molecules. Thirdly, the carpet model destroys the membrane from the high AMP concentration. Lastly, the aggregated model allows the translocation of AMPs across the membrane.

AMPs act as chemoattractant for the recruitment of innate immune cells to the infection site and initiate chemokine [33]. AMPs also have no toxic side effects on the human body [30]. In humans, AMPs are secreted in the gut, oral cavity, skin, and airways to promote a microbial and ecological balance within the body cells. Limitations and challenges do stand in the way for the majority of AMPs to be used in clinical trials. They have limited stability due to degradation by proteases, high extraction cost, short half-lives, cytotoxicity, and lack of specificity [37]. Solutions have been introduced; encapsulation, AMP synthesis, structural modification, and specific release targets.

Piscidins

Piscidins were the first AMPs discovered in the mast cells of vertebrates, revealing the critical role of AMPs and mast cells in the first line of defense against pathogenic diseases [20]. Piscidins are a part of the cationic α-helical AMP group, which is the most studied of the five

major AMP groups and is characterized by their abundance of histidine residue [12,37]. According to previous studies, piscidins contain up to 10 times as many histidines compared to other AMPs [27,40]. Marine animals produce potent AMPs that interact with harmful cells and diseases, such as cancer [12]. Piscidin 1 and piscidin 3 have highly similar sequences and α-helical structures that have been researched thoroughly due to their broad-spectrum antimicrobial properties on both Gram-negative and Gram-positive bacteria [40]. Piscidins eradicate bacteria both intracellularly by phagocytosis, extracellularly by degranulation, and salt-resilient [27].

The piscidin gene transcript has been present in European seabass, Hybrid Striped bass, Nile tilapia, Mandrian fish, and White bass [43]. Typically, in fish, piscidins are found in the granules of phagocytic mast cells and upregulated during pathogenic infections at the blood-brain barrier and near mucus-covered epithelial surface [27,40,43]. The major route for pathogenetic microorganisms is through the epithelial cells, gills, and gastrointestinal tract, allowing the fish's innate immune system to produce host defense peptides [43]. While piscidin 1 and piscidin 3 have been isolated from numerous fish, other organisms possess piscidins and other AMPs that could be used in research.

Piscidins 1 and 3

Piscidin 1 (FFHHIFRGIVHVGKTIHRLVTG-NH2) and piscidin 3 (FIHHIFRGIVHAGRSIGRFLTG-NH2) are the specific piscidins that we will focus on in our study that are extracted from hybrid striped seabass [32]. The Piscidin family is classified into three classes depending on their amino acid structure and antimicrobial activities [19]. Piscidin 1 and piscidin 3 are in Class I and they have the highest activity against gram-positive bacteria and

higher activity against prokaryotes and protozoans [19]. Piscidin 1 and piscidin 3 structures comprise 22 amino acid residues that have a high content of arginine, lysine, tryptophan, and histidine [20,36].

Figure 1: The NMR structure of antimicrobial piscidin 1 in aligned 4:1 phosphatidylcholine/cholesterol lipid bilayers

Compared with other piscidins, it is hypothesized that the unusually high level of histidine in the amino acid structure allows for translocation across the bacterial membrane [20]. Piscidin 1 and piscidin 3 are 68% homologous and highly potent but behave differently [12, 31, 44]. Piscidin 1 is more antimicrobial, hydrophobic, and targets the cellular membrane while piscidin 3 has more anti-inflammatory properties, higher specificity, more neutralizing, and goes through the cellular membrane to disrupt DNA [11,36,43]. Piscidin 1 is active against cancer and viruses such as HIV and coronavirus and neutralizes the septic effects of lipopolysaccharides [12,27]. Compared to piscidin 1, piscidin 3 shows a lower minimum inhibitory concentration and minimum bacterial concentration [43].

Piscidin Metalation

Both piscidin 1 (P1) and piscidin 3 (P3) contain an amino N-terminal copper and nickel bonding (ATCUN) motif that is utilized in binding to copper (Cu^{2+}) and nickel (Ni^{2+}) [27,32]. These piscidins can become metallated from their surrounding environment and adopt membrane-bound conformations that are favorable to survive disruptions [39]. The metalation of AMPs and its effects on the lipid membrane are not fully understood [36]. The addition of Cu^{2+} causes differences in binding efficiency; piscidin 1 experiencing decreased binding to heparin while piscidin 3 binding improves [38]. Recent studies of piscidin 1-Cu^{2+} (P1-Cu^{2+}) showed that the presence of oxidized lipids improves its membrane permeabilization effects and hypothesized that it was due to the loss of a positive charge and the oxidized lipids weaken the integrity of the membrane barrier [12]. Piscidin 3-Cu^{2+} (P3-Cu^{2+}) was observed to enhance cytotoxicity deeper membrane insertion, and greater lipid peroxidation than P3 in cancer cells. Another study found that P1-Cu^{2+} did not feature any prominent components but P3-Cu^{2+} features high specificity, strong DNA interactions, and fast nuclease activity in living cells [32]. This could result from the redox cycling of Cu^{2+} in the phagosome, forming reactive oxygen species (ROS) that chemically damage microbes and enhance the ability of P3-Cu^{2+} to nick DNA more efficiently [27,39].

Polyunsaturated Fatty Acids 18:2, 20:4, and 22:6

Polyunsaturated fatty acids (PUFAs) are associated with the cell membrane, incorporated into phospholipids, and regulate membrane fluidity and the function of membrane-bound enzymes and receptors [13,34,53,56]. The antimicrobial properties of fatty acids have been studied for decades to understand the effects on membrane composition. They are thought to exert four major immunomodulatory mechanisms that are not exclusive to certain PUFAs; membrane modulation, eicosanoid metabolism, gene expression, and cellular signaling [46].

PUFAs are classified as either omega-3 (n-3) or omega-6 (n-6) based on the location of the last double bond on the terminal methyl end of the molecule [56].

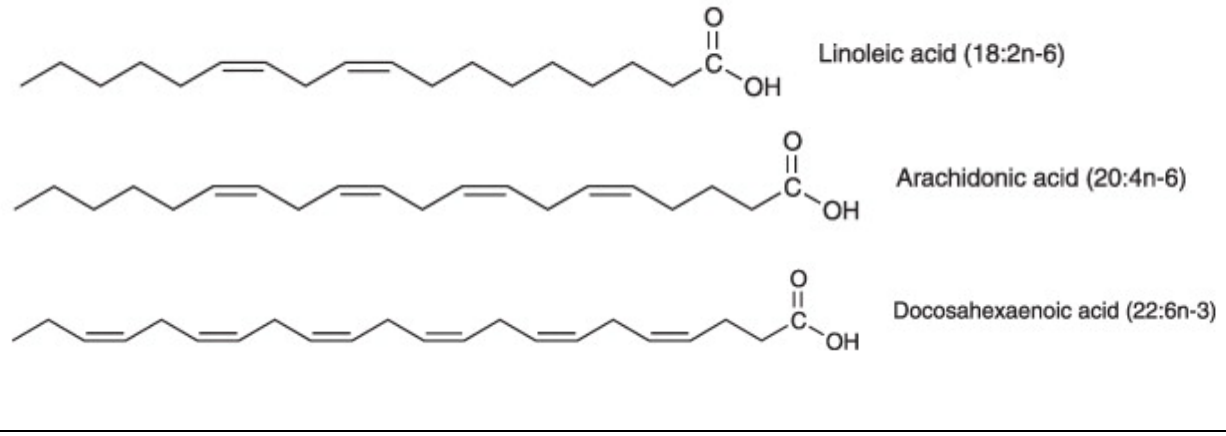

Figure 2: Chemical structure of PUFAS that are used with piscidin in this study- linoleic acid (18:2), arachidonic acid (20:4), and docosahexaenoic acid (22:6).

PUFAs provide beneficial health effects, n-6 which controls the acute pro-inflammatory response to protect the host against infection, and n-3 anti-inflammatory response and protection against chronic cardiovascular diseases (CVD) [46]. PUFAs are essential nutrients that cannot be synthesized endogenously in mammals and must be obtained by one's diet [19]. Many diets are not sufficient with both omegas and rely on solely one omega, like the Western world population that consumes mainly a 15:1 ratio of n-6 fatty acids to n-3 fatty acids [46,57]. A balanced intake is important for homeostasis, the development of fetuses and children, and the prevention of chronic diseases [1,46].

Linoleic acid and Arachidonic acid are omega-6 fatty acids that we used in our study. Linoleic acid (18:2) is an 18-carbon chain fatty acid and is the most abundant PUFA in the human diet [34,53]. 18:2 is synthesized in plants and widely distributed in food such as vegetable

oils, nuts, cereals, and dairy products. This fatty acid has a specific and unique role in the epidermis due to its essential constituent of ceramides [13]. The fluidity of the stratum corneum permeability barrier of the epidermis depends on the 18:2 content since no other fatty acid can substitute for 18:2. Arachidonic acid (20:4) is a 20-carbon chain fatty acid that is found in the brain [53]. Its four cis double bonds allow for flexibility, fluidity, and selective permeability to membranes, allowing specific membrane protein signaling and maintenance of the cells in neuron function. Previous studies have shown that 20:4 has tumoricidal potential in *vitro* and in *vivo* [42]. As well as being consumed, 20:4 can be converted from other PUFAs endogenously [19].

Docosahexaenoic acid is the only omega-3 fatty acid that we used in our study. Docosahexaenoic acid (22:6) is a 22-carbon chain, n-3 fatty acid that is rich in marine aquatic fish [1,56]. Deepwater fish, such as tuna and salmon, have the highest content of 22:6 since they store lipids in the flesh, while lean fish store lipids in the liver [56]. Humans have 22:6 found mostly in their brain by consuming seafood and their byproducts, like fish oil [1]. 22:6 is important in maintaining membrane fluidity of the retina and essential for proper cognitive and neurological functions of the brain [30, 46]. Once incorporated into the membrane, 22:6 is found in high concentrations due to its retainability at the expense of other fatty acids [57]. Numerous bacterial marine species, such as *Shewanella spp.* and *Photobacterium* have been shown to produce 22:6 and eicosatetraenoic acid (20:5) in unusual environments like low-temperature deep-sea and the intestines of fish [56]. The enrichment of these PUFAs has hypothesized that PUFA synthesis is an important adaption for countering the elevated hydrostatic pressure and low temperature on membrane fluidity [56]. Analysis of 22:6 with piscidins is of great importance since both are found within fish and mimic what would be found in the natural

environment. Fish produce 22:6 within the body and piscidins on their scales. Fish are susceptible to aquatic pathogens, such as *Aeromonas spp*. and *Vibrio spp.,* and allow transmission to human hosts if it is not properly cleaned and cooked. Piscidins act as the first line of defense for these fish but if infection persists, fish produce more 22:6 that elicits a proinflammatory response to promote the immune system.

Exogenous Fatty Acid Utilization of Bacteria

Recent studies of *Vibrio* have highlighted their ability to acquire fatty acids from the environment and use them in their cellular membrane [38]. *V. cholerae* was one of the first bacteria to be examined for exogenous PUFA uptake into the bacterial cell membrane [18]. Due to environmental changes, such as temperature and salinity, bacteria modify their membrane lipid composition and may adopt a phospholipid profile that mirrors the fatty acids that are present [18]. Later studies focused on exogenous PUFAs affecting multiple *Vibrio* species, including *Vibrio parahaemolyticus* and *Vibrio vulnificus*, and observing membrane permeability, biofilm formation, and resistance to polymyxin B [38]. These impacts were assessed using thin-layer chromatography, liquid chromatography-mass spectrometry, motility assay, biofilm assay minimal inhibitory concentration assays (MIC), and membrane permeability assays (crystal violet uptake) [18]. Phosphate buffered saline (PBS) is used for membrane permeability assays to discourage cell division while preventing cells from rupturing due to osmosis. When conducting these membrane permeability assays, they were in the presence of PBS and crystal violet for 21 minutes due to *Vibrio spp.* having a doubling time of 20-25 minutes. When treated with 20:4, *V. parahaemolyticus* experienced a decrease in permeability while *V. vulnificus* experienced a 30% decrease [38]. When treated with 22:6, *V. parahaemolyticus* was minimally affected while *V.*

vulnificus suffered. In the MIC assay, the study focused on resistance in *Vibrio spp*. if PUFAs were administered with an antimicrobial peptide called polymyxin B. *V. parahaemolyticus* had a modest decrease when treated with α-linolenic acid (18:3 α) and 20:4 while *V. vulnificus* conferred slight protective effects with four fatty acids (18:2, 20:3 [Dihomo-γ-linolenic acid], 20:4, and 22:6). Exogenous fatty acids linoleic acid (18:2) and arachidonic acid (20:4) stimulated biofilm formation in both bacteria [18]. The most recent study of *Vibrio alginolyticus* and *V. fischeri* demonstrated that PUFAs decreased membrane permeability in both species; the largest effects being a 20% decrease with 20:4 and 20:5 in *V. fischeri*. The MICs for both showed varied results; MICs that used cyclic peptide antibiotics polymyxin B and colistin decreased with some PUFA addition but a beta-lactam antibiotic had no effect, showing the differential efficacy of antibiotics that are membrane-active versus translocated via protein through the membrane [52]. The three studies confirmed that fatty acids play an important role in the *Vibrio* species, allowing fatty acids uptake from their environment and host niches [18,38,52].

Other than *Vibrio spp.*, more Gram-negative bacteria have exhibited the ability to assemble exogenous PUFAs for membrane remodeling. Studies with *Acinetobacter baumannii, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, and Aeromonas salmonicida,* confirmed the incorporation of PUFAs into the membrane and altered behavioral changes such as growth and motility rates [5,15,22,23,24]. Permeability assays performed on *K. pneumoniae, E. coli*, and *A. salmonicida* showed that 20:4 and 22:6 had significant increases in membrane permeability, allowing more hydrophobic crystal violet uptake [22,23,24]. Alternatively, *A. baumannii* and *P. aeruginosa* showed that 22:6 had a significant decrease in membrane permeability, causing resistance of the bacterial membrane [5,15]. All five bacterial membranes had no significant difference with the addition of 18:2. In the MIC studies, two

cyclic antimicrobial peptides, polymyxin B and colistin, were used. MICs performed on *K. pneumoniae* and *A. baumannii* showed polymyxin B and colistin resistance when all fatty acids were present [15,23]. Comparatively, *A. salmonicida* showed that PUFAs did not have much effect, only contributing to the increase with 18:2 and decrease with 22:6 [24]. *P. aeruginosa* and *E. coli* were shown that PUFAs increased resistance to polymyxin B and colistin [5,22]. In *P. aeruginosa*, incorporated polymyxin B with 22:6 increased by 4-fold while 20:4 increased by 8-fold. In *E. coli*, incorporated polymyxin B increased by 2-fold, 22:6 increased by 4-fold, and 20:4 by 8-fold.

Vibrio parahaemolyticus* and *Vibrio vulnificus

Vibrio parahaemolyticus and *Vibrio vulnificus* are Gram-negative, rod-shaped bacteria that belong to the family *Vibrionaceae*. Both are significant foodborne human pathogens that occur with the consumption of contaminated raw or undercooked fish and shellfish, especially oysters [14,21]. Vibriosis is the illness produced by *Vibrio* species and the symptoms include abdominal cramping, fever, nausea, vomiting, and watery diarrhea. *Vibrio parahaemolyticus* is one of the leading foodborne pathogens in humans, behind *Vibrio cholerae*, causing acute gastrointestinal and immunological disorders. However, most infections due to these bacteria are not reported since the clinical manifestations do not typically result in deadly sequelae, such as systemic shock unless the host is immunocompromised. Unlike *V. parahaemolyticus*, *Vibrio vulnificus* is an opportunistic pathogen and has a high mortality burden, causing gastroenteritis, necrotizing fasciitis, and sepsis. It has the highest case fatality rate (50%) of any food pathogen and is responsible for 95% of seafood-related deaths in the United States due to septicemia [14,17,44]. Both can arise from wound exposure to contaminated water or handling contaminated marine food [44, 48].

With the increasing global reported cases of these two microbes over the last two decades, two concerns have arisen. First, *Vibrio* optimally grows in warm water of 37ºC with a minimum growth temperature of 15^{O}C [35]. Their abundance in the natural environment mirrors their environmental temperatures that are increasing (>20^{O}C) [7,35]. With their rapid replication time, as little as 8-9 minutes, and warm environment in the ocean, *Vibrio* species are becoming more prevalent in coastal regions, allowing for more disease transmission [7]. Secondly, the excessive use of antibiotics has caused the development of antibiotic resistance in *Vibrio* species due to their high ability of horizontal gene transfer (HGT) between virulent and non-virulent strains [21, 47]. In *V. vulnificus*, a fibrous polysaccharide from scales of fish called chitins has been shown to help attach and exchange genetic information [6]. It is hypothesized that *V. parahaemolyticus* can undergo HGT since their pre-pandemic vs their pandemic strains reveal recombination. In a study from 2014, *V. parahaemolyticus* and *V. vulnificus* were recovered from Maryland Coastal Bays and tested against 26 antimicrobials [25,50]. Of this, *V. parahaemolyticus* resisted 15 of the 26 antibiotic treatments while *V. vulnificus* resisted 12 of the 26 antibiotic treatments.

Multiple proteins that are shared throughout each *Vibrio spp*. resemble acyltransferases, FadD, and FadL, supporting the idea that extra molecular machinery in the membrane allows for the production, uptake, and utilization of a wide range of exogenous fatty acids [18,37,47,54]. FadL is an outer membrane transporter that allows exogenous fatty acids to cross the cell. After getting through the outer membrane, the exogenous fatty acids travel through the periplasm and into the inner membrane to be activated by FadD. FadD has an addition of a coenzyme A group that allows the degradation of fatty acids to provide energy for the bacteria or the incorporation of fatty acids into phospholipids by acyltransferases. The acyltransferases consist of different

pathway functions that incorporate into the membrane. Possession of several homologues for each step in this fatty acid handling pathway likely allows *Vibrio spp*. to utilize many PUFAs that could affect susceptibility to piscidins [47].

Material and Methods

Media Preparation

For all assays with piscidins, we utilized CM9/HEPES (pH 7.4) media consisting of 100mM HEPES (pH 7.4), M9 5x Salts, 0.2% glucose, 0.2% casamino acids, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 3% NaCl. The overnight cultures were grown in Luria-Broth (LB) with 3% NaCl added.

Permeability Assays

V. parahaemolyticus and *V. vulnificus* from an overnight culture were grown in 3mL of CM9/HEPES (pH 7.4) in the presence and absence of the corresponding PUFAs (18:2, 20:4, and 22:6) at a starting OD_{600} of 0.2. The bacteria grew in the presence or absence of PUFA for 1 hour. After this time, a subinhibitory concentration of the desired piscidin (P1, P1-Cu^{2+}, P3, P3-Cu^{2+}) was added. All samples were then incubated for an additional 1 hour. These were pelleted using centrifugation, washed with phosphate-buffered saline (PBS), and resuspended in 600µL of PBS. The OD_{600} of each culture was captured and using the lowest of these OD_{600}, we calculated the allowable OD_{600} of the bacterial inoculum that would be added to PBS to achieve 2mLs. Once the calculated PBS and bacterial samples were prepared, we added 2µL of crystal violet to each

sample (5µg/ml). Then, every 7 minutes for 21 minutes, 650µL of the culture was pelleted for 1 minute at 13,3000 xg, and 600µL of the cultured supernatant was placed in a cuvette for analysis of absorbance at 570nm to determine the amount of crystal violet not taken up by the bacteria.

Minimum Inhibitory Concentration Assays

V. parahaemolyticus and *V. vulnificus* overnight cultures were pelleted and washed with CM9/HEPES (pH 7.4). The OD_{600} was captured using a spectrophotometer to calculate the volume of each bacterium to add to 3mL of CM9/HEPES (pH 7.4) for a starting OD_{600} of 0.2. Then, the bacteria were grown in either the presence or absence of PUFAs for two hours to the logarithmic phase. Following that, the cultures were pelleted and resuspended in CM9. 30µL of two-fold concentrations of each piscidin was placed on a microtiter plate. 170µL of the appropriate bacterial inoculum, without or with the presence of fatty acids, was added to the respective wells containing the 30µL of piscidin for a total volume of 200µL. The finished plate was incubated for 20 hours at 200 rpm at 37ºC. The finished incubated plate absorbance was then read at OD_{600} using a Biotek Synergy microplate reader.

Results

Permeability with *V. parahaemolyticus*

Our first goal was to investigate the piscidin-mediated permeability impact of linoleic acid, arachidonic acid, and docosahexaenoic acid cultures of *V. parahaemolyticus* and *V. vulnificus* and the effects of each piscidin with membrane activity. Observing membrane

permeability is important for the bacteria's survivability since it determines the passage of molecules between the cell and its environment. Bacteria have mechanisms to recognize changes to permeability to prevent damage that could lead to lysis. By measuring bacterial uptake of the hydrophobic compound crystal violet, the individual and combinatorial effects of PUFAs and piscidin on membrane permeability were assessed. Due to logistical challenges for including replicates within permeability assays, several biological replicates must be performed. Reliability of the data is gauged by the consistency of the baseline controls.

In **Figure 3**, the addition of P1 alone significantly increased membrane susceptibility, allowing more crystal violet uptake. The presence 20:4 and 22:6 significantly decreases membrane permeability when P1 is administered. Treatment with 18:2 had no significant to the membrane.

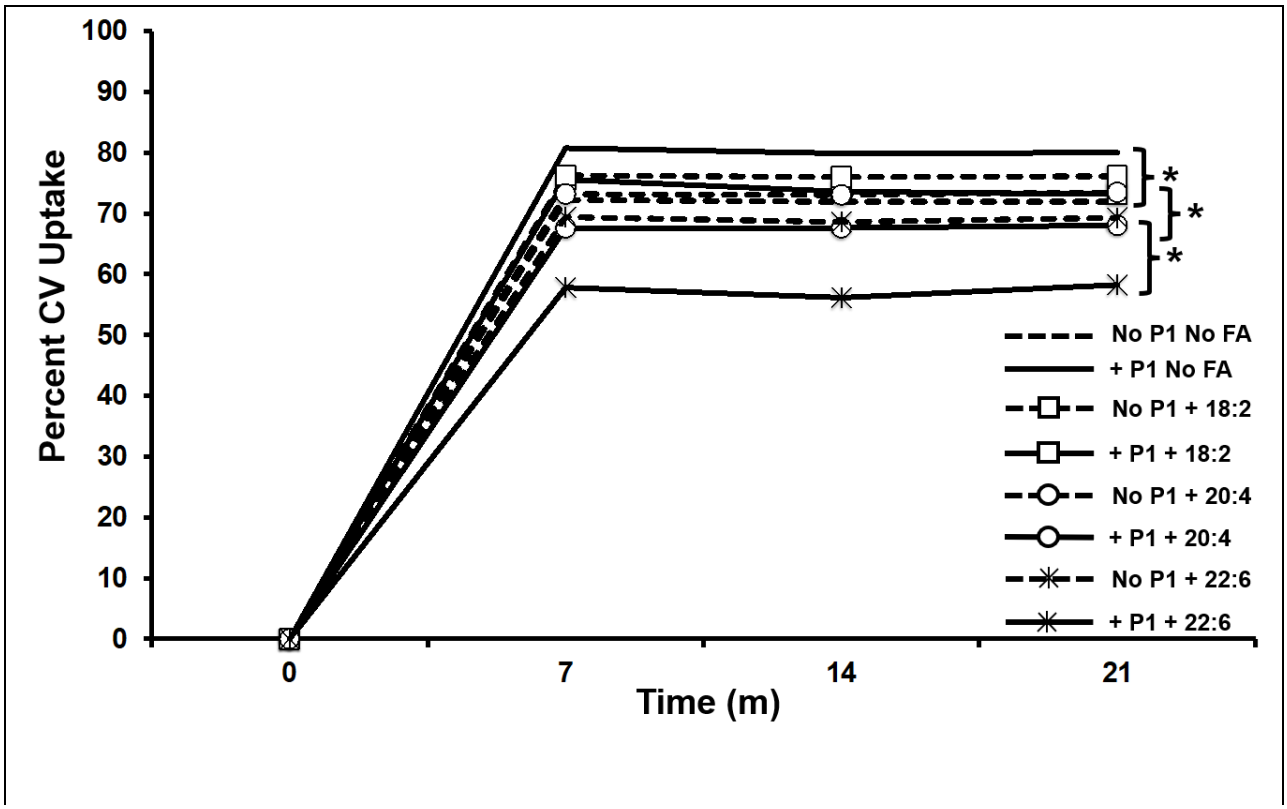

Figure 3: The effects of PUFAs (18:2, 20:4, and 22:6) and piscidin 1 on membrane permeability of *V. parahaemolyticus*. Asterisks (*) indicate significance (P <0.002) determined using the t-test for the three measured values compared to each other. A representative of three biological replicates is shown.

F**igure 4** shows that with addition of P1-$Cu2^{+}$ alone had no significance to membrane permeability. The presence of 20:4 with P1-Cu^{2+} decreased the permeability *of V. parahaemolyticus's* membrane, allowing less uptake of the crystal violet.

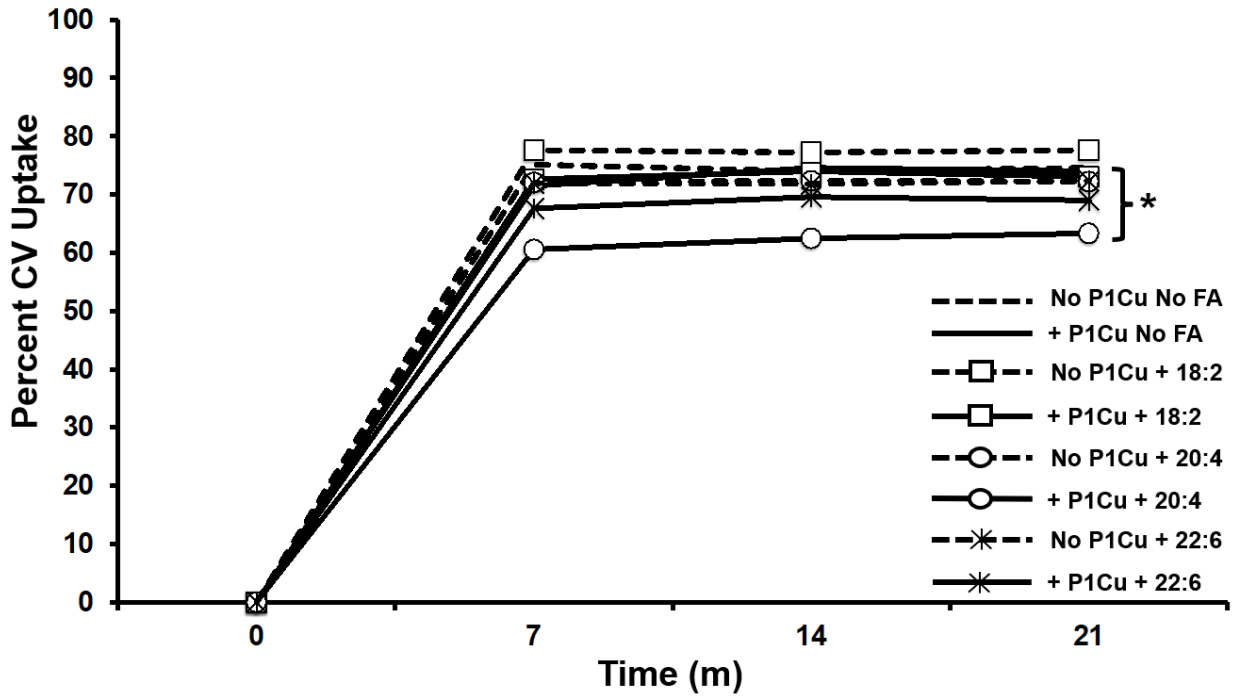

Figure 4: The effects of PUFAs (18:2, 20:4, and 22:6) and piscidin 1-Cu^{2+} on membrane permeability of *V. parahaemolyticus*. Asterisks (*) indicate significance (P <0.002) determined using the t-test for the three measured values compared to each other. A representative of three biological replicates is shown.

In **Figure 5**, permeability of *V. parahaemolyticus's* membrane was increased with addition of only P3 and decreased with the presence of 22:6 when treated with P3.

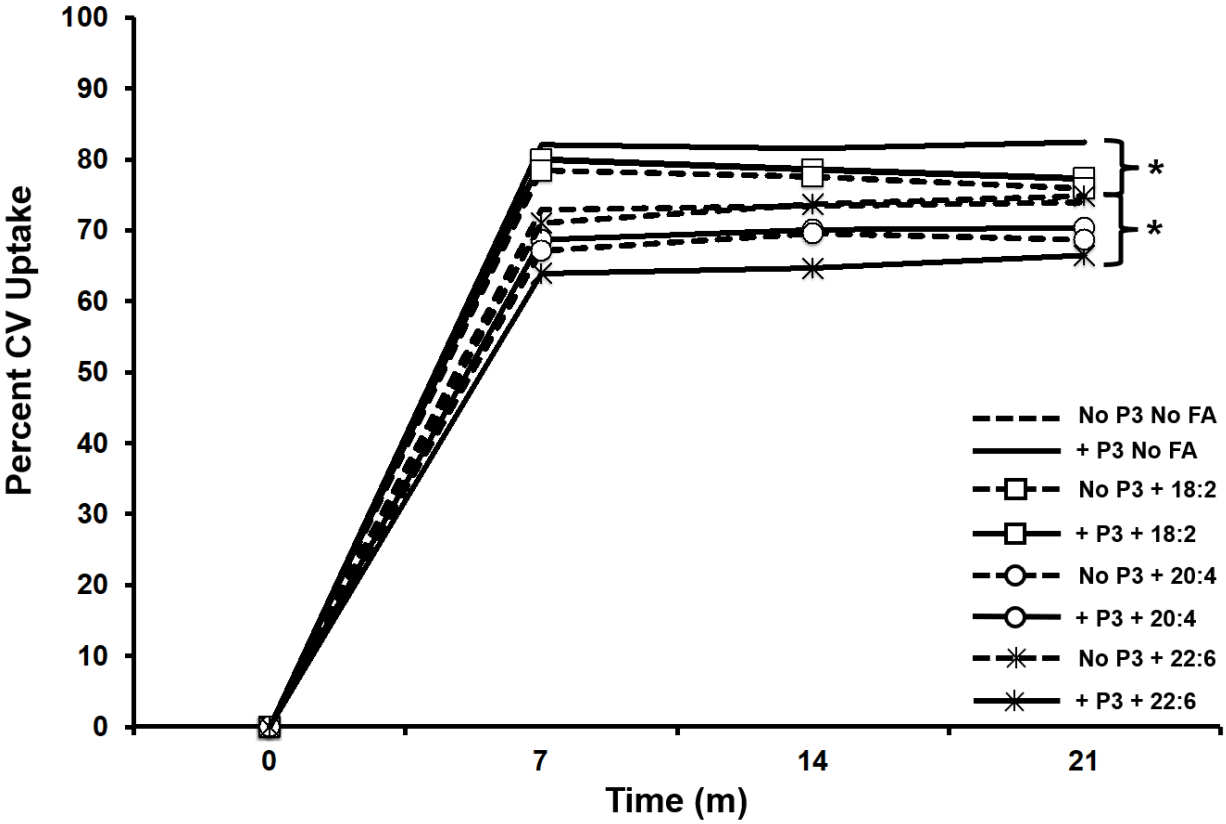

Figure 5: The effects of PUFAs (18:2, 20:4, and 22:6) and piscidin 3 on membrane permeability of *V. parahaemolyticus*. Asterisks (*) indicate significance ($P < 0.002$) determined using the t-test for the three measured values compared to each other. A representation of three biological replicate is shown.

Figure 6 shows that the presence of P3-Cu^{2+} with no fatty acids caused significant increases in *V. parahaemolyticus* membrane permeability. The addition of P3-Cu^{2+} allows for the

percent uptake of crystal violet to be ~95%, the highest out of all the conducted permeability experiments. In this study, the largest permeability significance is shown with a 20% difference between the baseline control and addition of P3-Cu^{2+} only. The addition of P3-Cu^{2+} and 18:2 also caused the increase in membrane permeability.

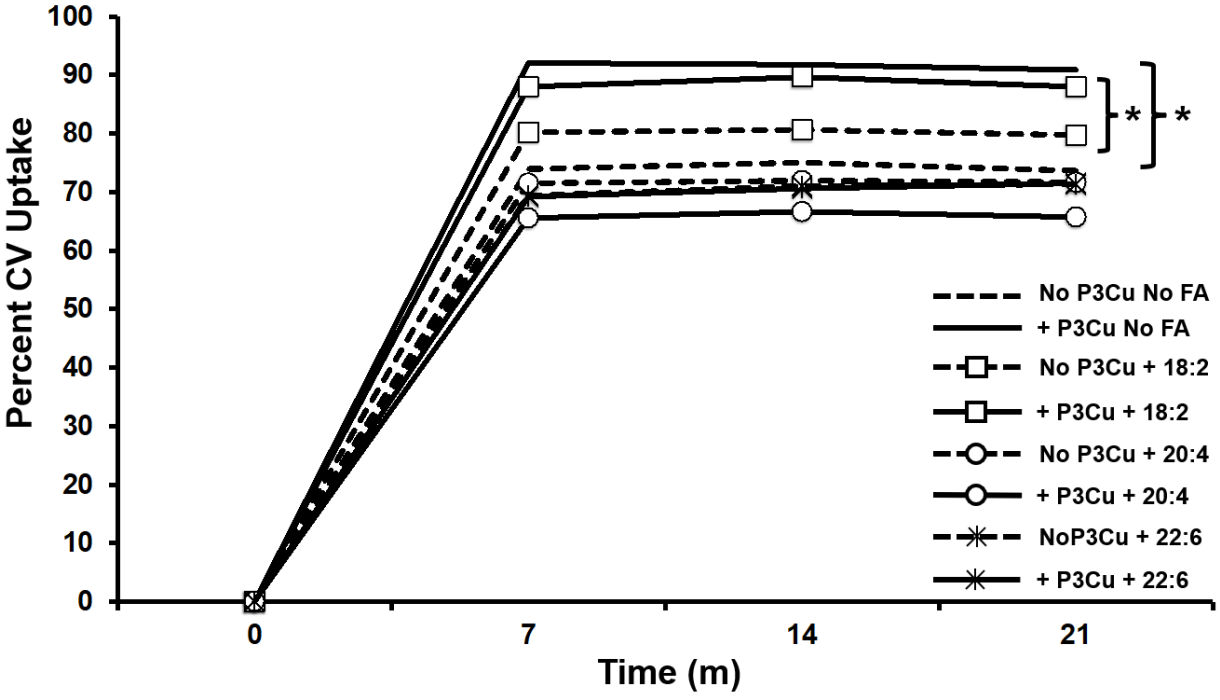

Figure 6: The effects of PUFAs (18:2, 20:4, and 22:6) and piscidin 3-Cu^{2+} on membrane permeability of *V. parahaemolyticus*. Asterisks (*) indicate significance (P <0.002) determined using the t-test for the three measured values compared to each other. A representation of three biological replicate is shown.

Permeability of *Vibrio vulnificus*

As *V. parahaemolyticus*, we investigated the same effects in *V. vulnificus*. Throughout all of the *V. vulnificus* data, the addition of only piscidin resulted in no significance.. **Figure 7** shows

that all three fatty acids had either a positive or negative effect on the membrane. 18:2 had a negative effect on the membrane, allowing more crystal violet uptake while 20:4 and 22:6 provided more resistance to the membrane.

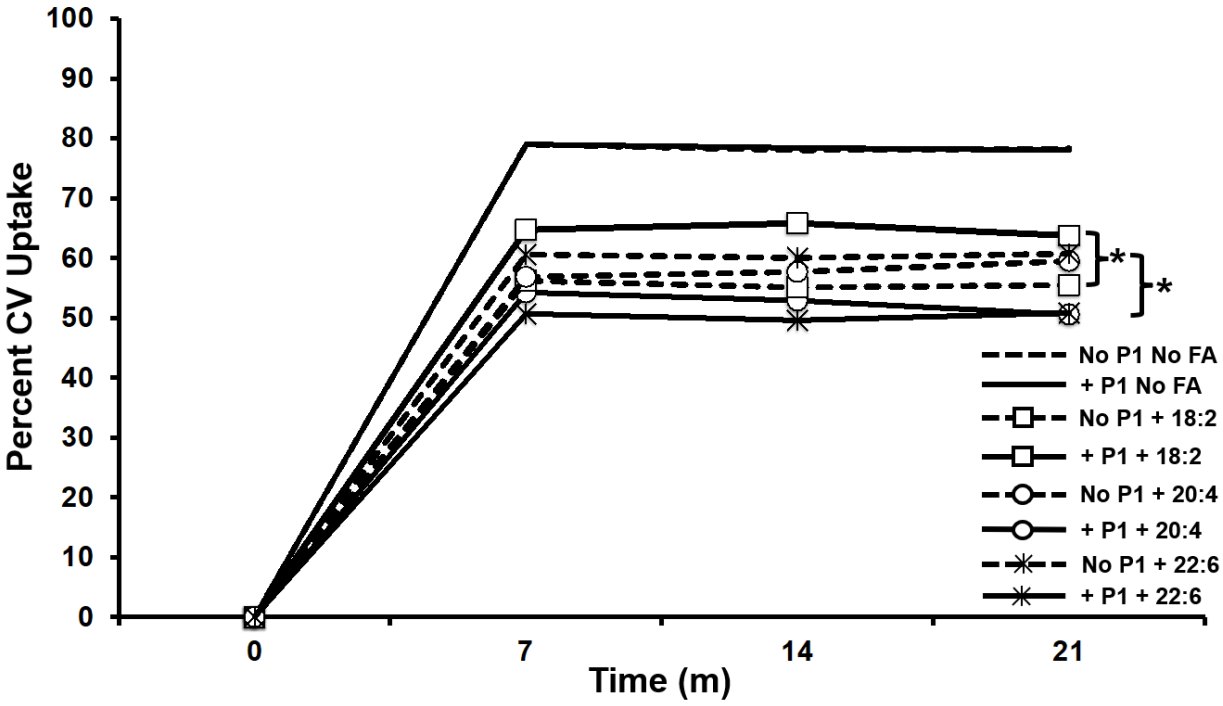

Figure 7: The effects of PUFAs (18:2, 20:4, and 22:6) and piscidin 1 on membrane permeability of *V. vulnificus*. Asterisks (*) indicate significance (P <0.002) determined using the t-test for the three measured values compared to each other. A representation of three biological replicate is shown.

In **Figure 8**, P1-Cu^{2+} administered in addition with 20:4 experienced a significant increase in membrane permeability.

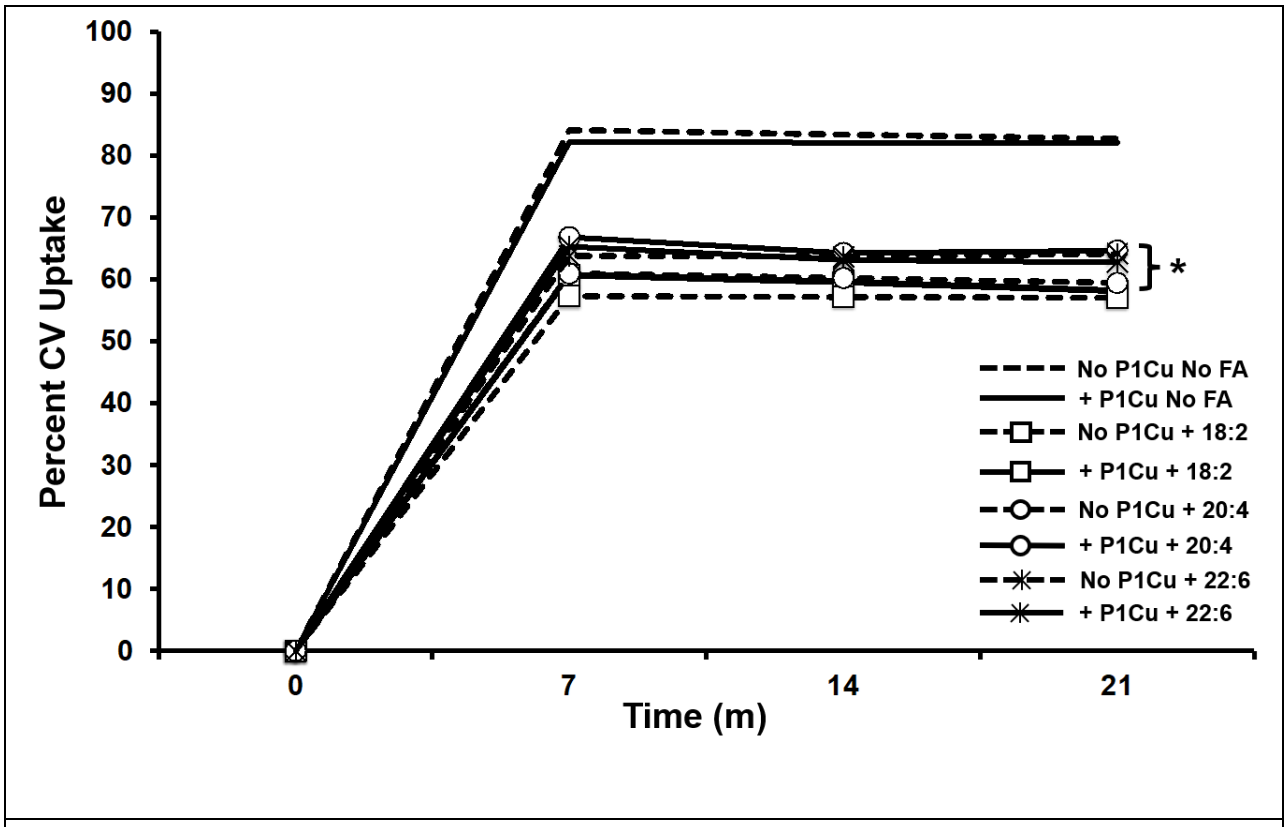

Figure 8: The effects of PUFAs (18:2, 20:4, and 22:6) and piscidin 1-Cu^{2+} on membrane permeability of *V. vulnificus*. Asterisks (*) indicate significance (P <0.002) determined using the t-test for the three measured values compared to each other. A representation of three biological replicate is shown.

Unlike the other permeability assays of both bacteria, **Figure 9** shows no significant changes when P3-Cu^{2+} is administered only and in addition with PUFAs.

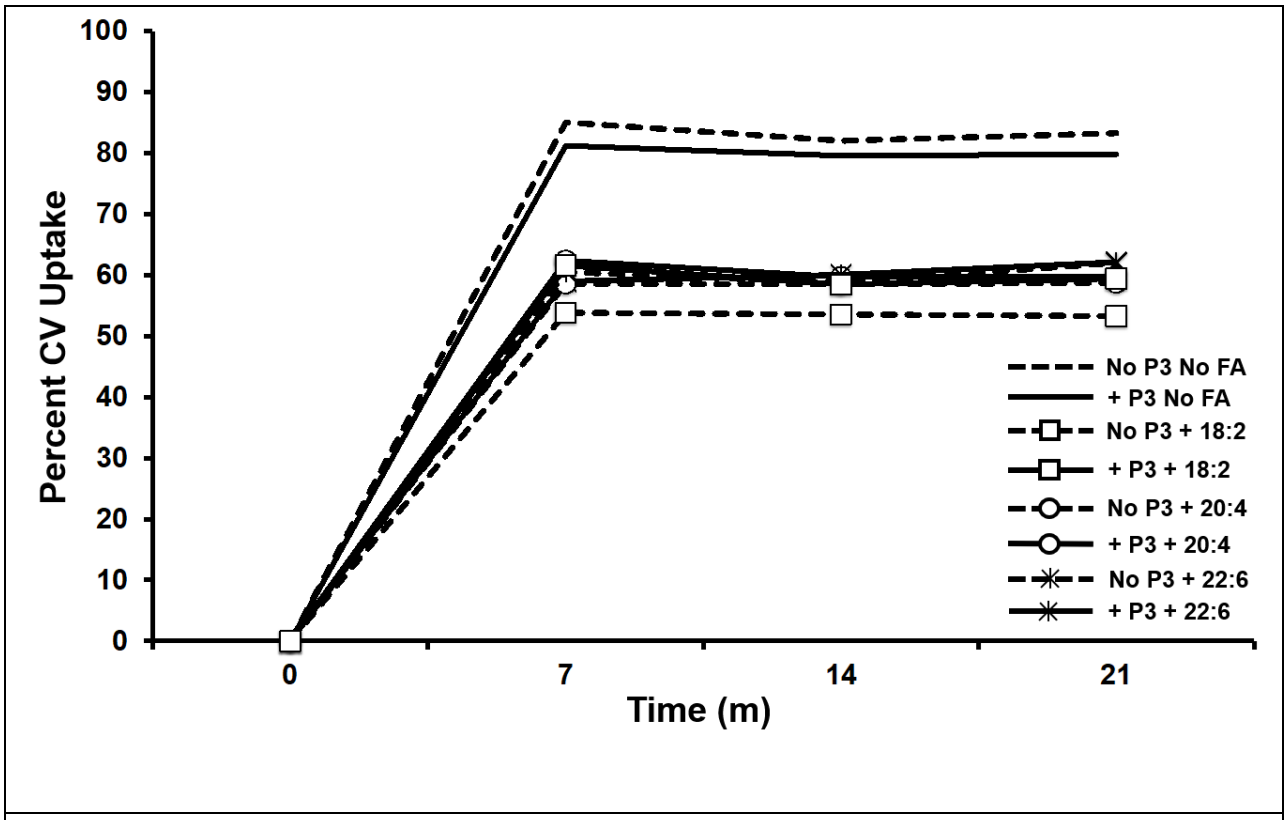

Figure 9: The effects of PUFAs (18:2, 20:4, and 22:6) and piscidin 3 on membrane permeability of *V. vulnificus*. Asterisks (*) indicate significance (P <0.002) determined using the t-test for the three measured values compared to each other. A representation of three biological replicate is shown.

Figure 10 shows that only 18:2 had a significant increase in membrane permeability, decreasing the protective effects. No fatty acid, 20:4, and 22:6 have no significant disruption to the membrane.

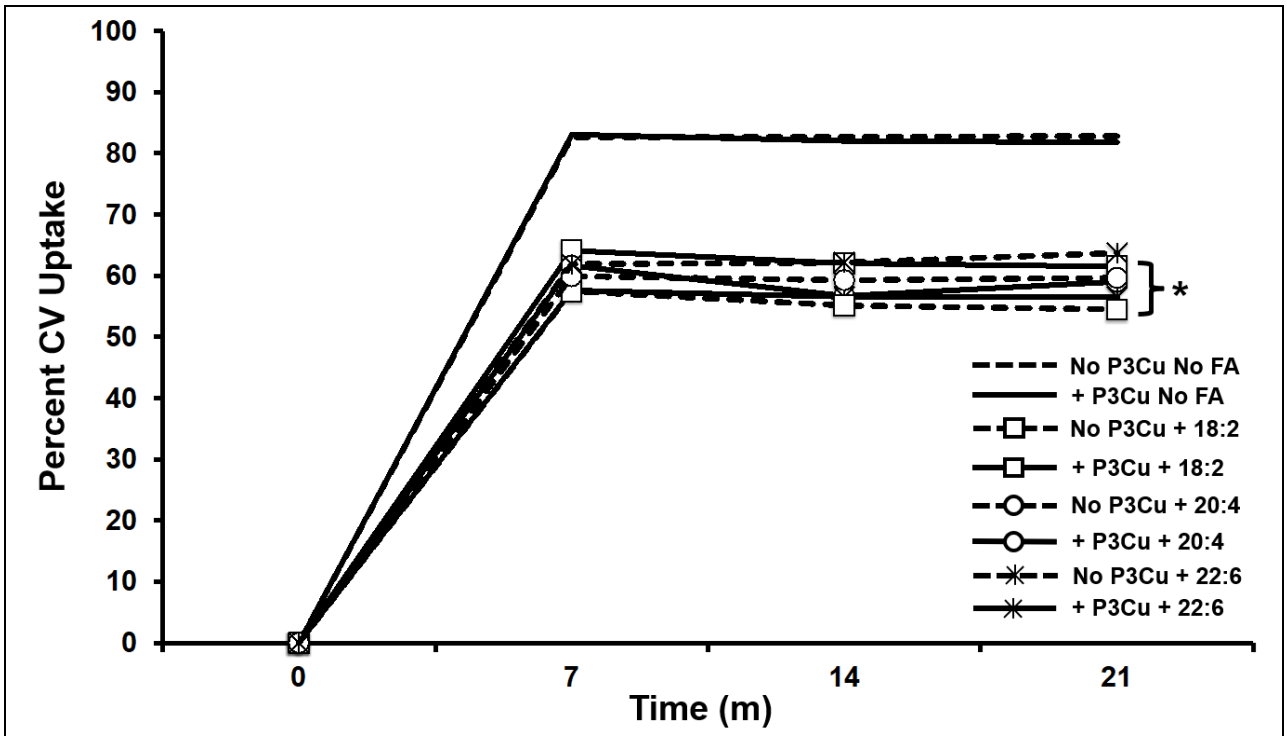

Figure 10: The effects of PUFAs (18:2, 20:4, and 22:6) and piscidin 3-Cu^{2+} on membrane permeability of *V. vulnificus*. Asterisks (*) indicate significance (P <0.002) determined using the t-test for the three measured values compared to each other. A representation of three biological replicate is shown.

Minimum Inhibitory Concentrations (MIC) of Piscidins

Our next goal was to determine the impact that exogenous fatty acid had on *Vibrio parahaemolyticus* and *Vibrio vulnificus* minimal inhibitory concentration (MIC) of the various piscidins. MIC results show the lowest concentration of the desired piscidin in the presence or absence of PUFAs at which the bacteria growth is completely inhibited [27]. There is the control, which has no presence of any fatty acids while the other three solid lines represent the three fatty acids; 18:2, 20:4, and 22:6. The first MIC shows that all three exogenous fatty acids did not

provide *V. parahaemolyticus* protection from the antimicrobial abilities of the piscidin. **Figure 11** outlines the results from the MIC assay with P1 when acting on *V. parahaemolyticus* either grown in the presence or absence of 18:2, 20:4, or 22:6. The uptake of the control and the three samples (18:2, 20:4, and 22:6) does not identify any impact on the survival of *V. parahaemolyticus* when treated with P1. All four samples were inhibited at a concentration of 8μM of P1.

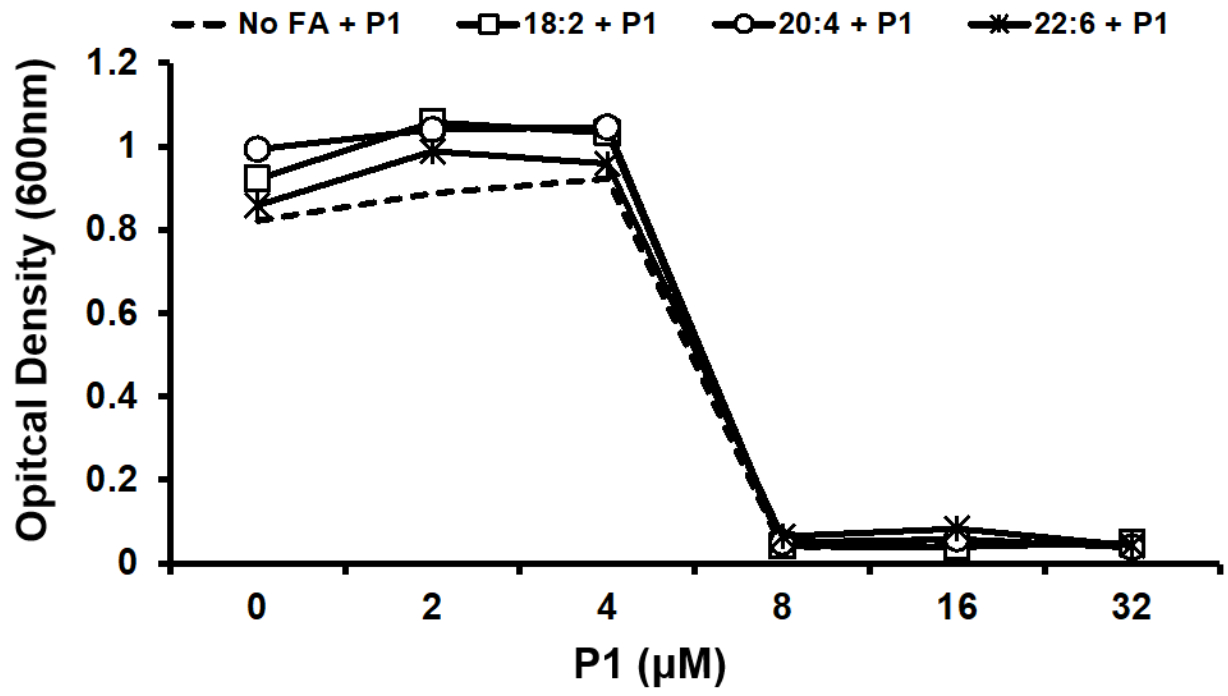

Figure 11: Determination of the minimum inhibitory concentration of *Vibrio parahaemolyticus* grown in the presence of 18:2, 20:4, or 22:6 when treated with a 2-fold concentration increase of piscidin 1. The symbols circled by a dotted line indicate significance ($P<0.02$) as determined by a t-test performed for the triplicate values at each concentration versus control. All standard deviations were less than 0.03. A minimum of 3 biological replicates were performed.

Figure 12 shows the MIC for 20:4 and 22:6, and the control is inhibited at 4µM of P1-Cu^{2+}. However, the MIC increased by two-fold (8µM) for 18:2, showing a slight impact on the MIC with P1-Cu^{2+}.

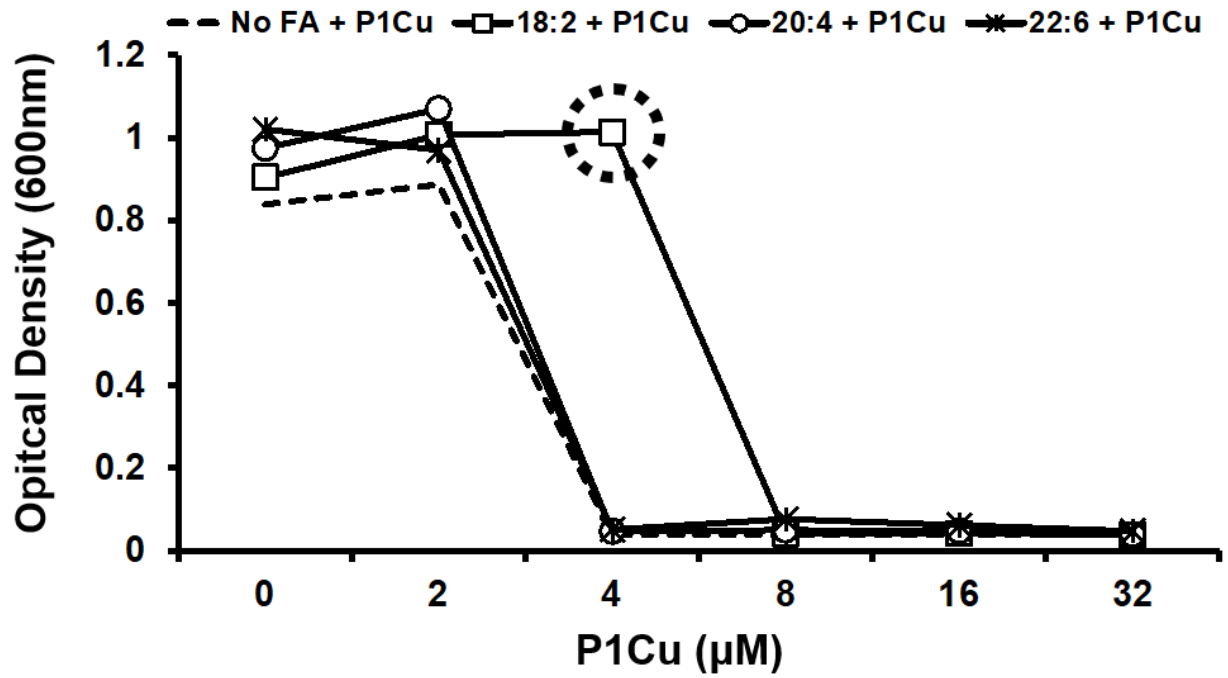

Figure 12: Determination of the minimum inhibitory concentration of *Vibrio parahaemolyticus* grown in the presence of 18:2, 20:4, or 22:6 when treated with a 2-fold concentration increase of piscidin 1-Cu^{2+}. The symbols circled by a dotted line indicate significance ($P<0.02$) as determined by a t-test performed for the triplicate values at each concentration versus control. All standard deviations were less than 0.03. A minimum of 3 biological replicates were performed.

Unlike the previous MIC assays, the results of the MIC with P3 show that the uptake of exogenous fatty acids with P3 has an extremely negative impact on the survival of *V. parahaemolyticus*. **Figure 13** shows that, in all cases, fatty acid availability decreased the survival of *V. parahaemolyticus* when treated with P3. All three samples were inhibited at 8µM while the control surpassed 32µM of P3. This is the most significant experiment of the 8 conducted MICs.

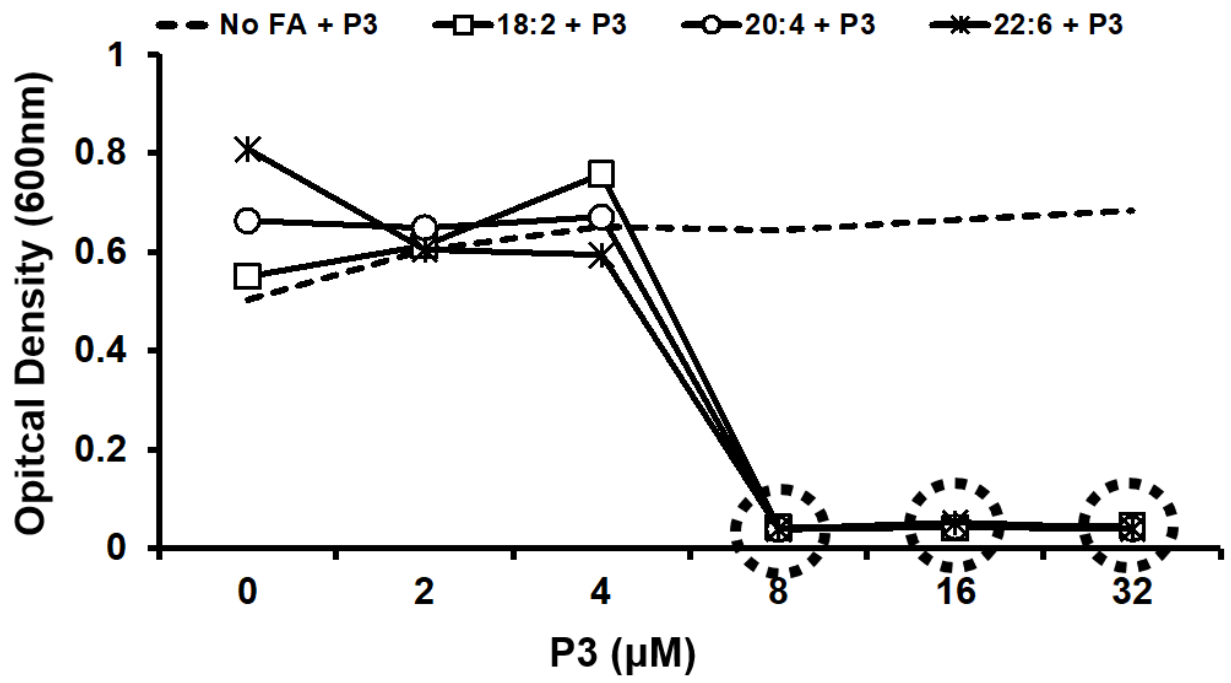

Figure 13: Determination of the minimum inhibitory concentration of *Vibrio parahaemolyticus* grown in the presence of 18:2, 20:4, or 22:6 when treated with a 2-fold concentration increase of piscidin 3. The symbols circled by a dotted line indicate significance ($P<0.02$) as determined by a t-test performed for the triplicate values at each concentration versus control. All standard deviations were less than 0.03. A minimum of 3 biological replicates were performed.

Figure 14 shows that fatty acid availability of 18:2 and 20:4 decreased the survival of *V. parahaemolyticus* while 22:6 had no impact when treated with P3-Cu^{2+}. Both the control and the 22:6 sample had an MIC of 16μM of P3-Cu^{2+}. The 18:2 and 20:4 samples experienced a two-fold MIC decrease to 8μM.

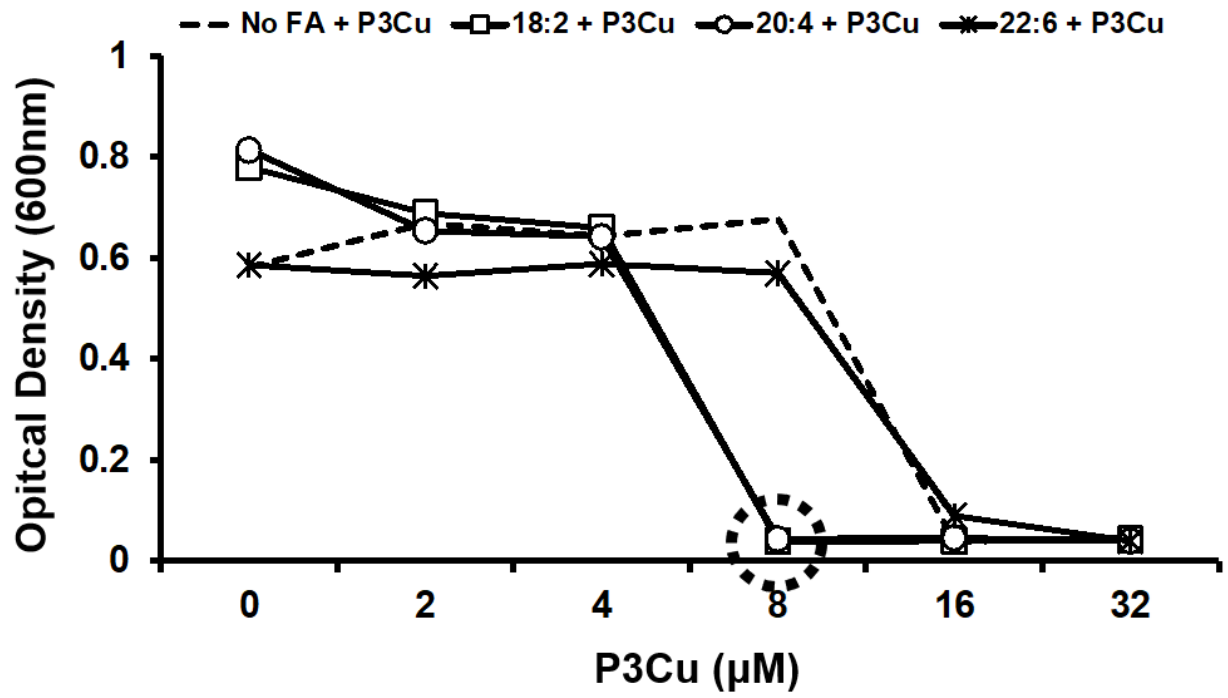

Figure 14: Determination of the minimum inhibitory concentration of *Vibrio parahaemolyticus* grown in the presence of 18:2, 20:4, or 22:6 when treated with a 2-fold concentration increase of piscidin 3-Cu^{2+}. The symbols circled by a dotted line indicate significance ($P<0.02$) as determined by a t-test performed for the triplicate values at each concentration versus control. All standard deviations were less than 0.03. A minimum of 3 biological replicates were performed.

In **Figure 15**, MIC assays for *V. vulnificus* with P1 identified only 20:4 as changing the antimicrobial activity resistance, increasing the MIC by two-fold (8μM). The control, the 18:2 sample, and the 22:6 sample were all inhibited at 4μM of P1.

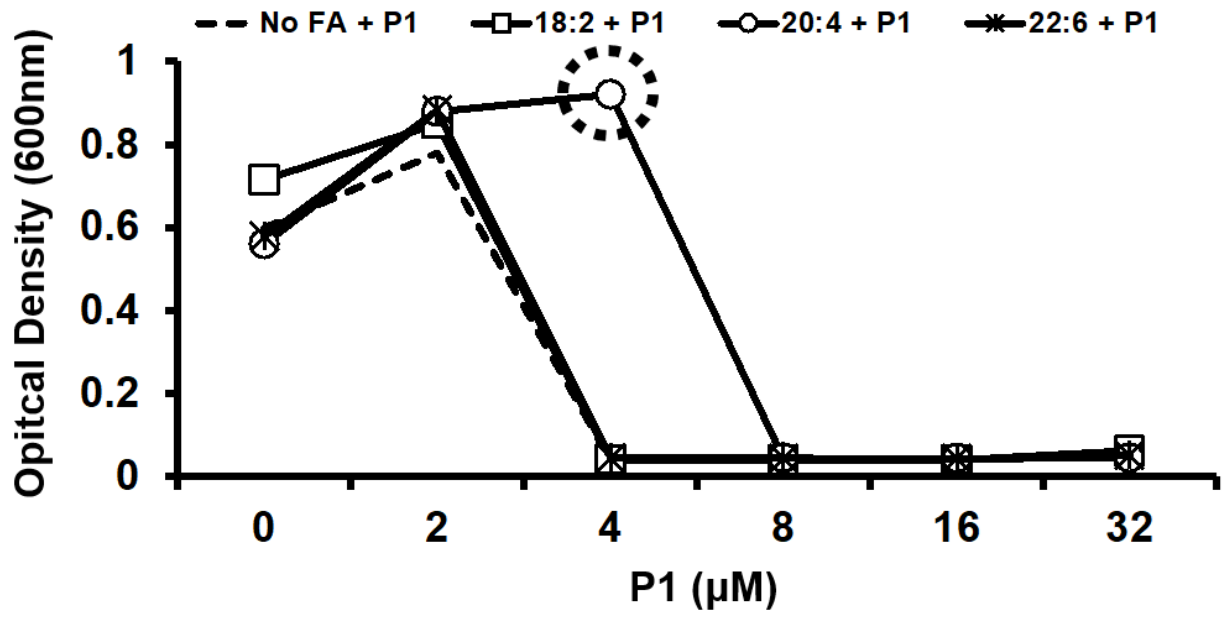

Figure 15: Determination of the minimum inhibitory concentration of *Vibrio vulnificus* grown in the presence of 18:2, 20:4, or 22:6 when treated with a 2-fold concentration increase of piscidin 1. The symbols circled by a dotted line indicate significance ($P<0.02$) as determined by a t-test performed for the triplicate values at each concentration versus control. All standard deviations were less than 0.03. A minimum of 3 biological replicates were performed.

Figure 16 shows that all the fatty acid samples increased the survival of *V. vulnificus* when treated with P1-Cu^{2+}. The control has a MIC of 4μM of P1-Cu^{2+} while the 18:2 sample, the 20:4 sample, and the 22:6 sample experienced a two-fold increase to 8μM.

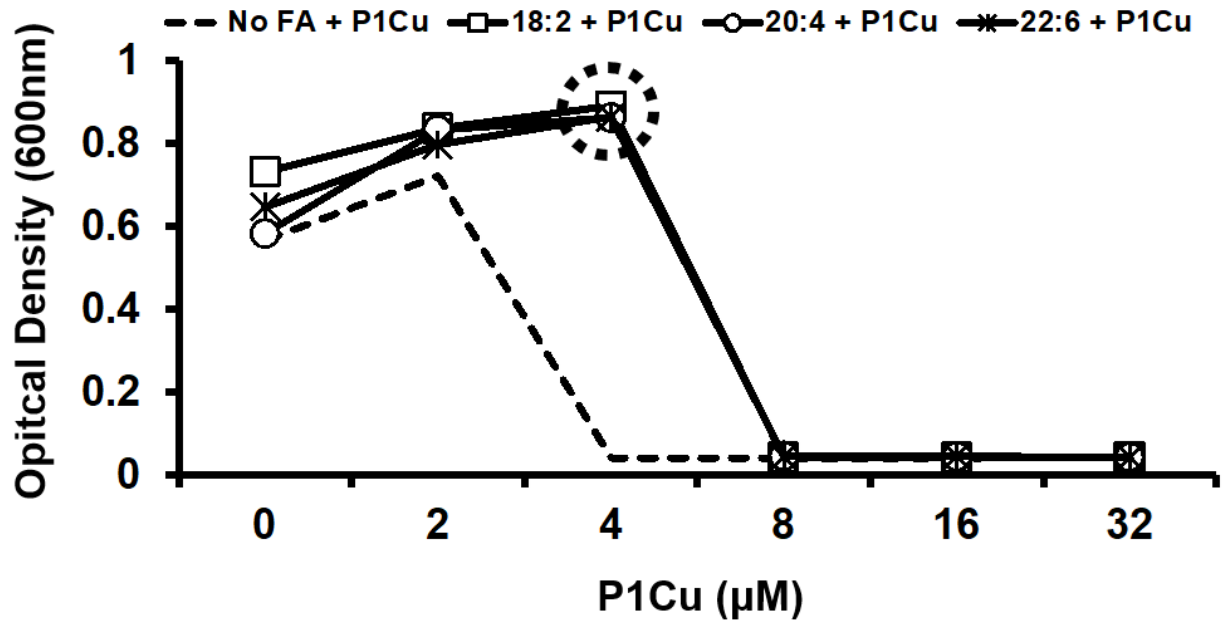

Figure 16: Determination of the minimum inhibitory concentration of *Vibrio vulnificus* grown in the presence of 18:2, 20:4, or 22:6 when treated with a 2-fold concentration increase of piscidin 1-Cu^{2+}. The symbols circled by a dotted line indicate significance ($P<0.02$) as determined by a t-test performed for the triplicate values at each concentration versus control. All standard deviations were less than 0.03. A minimum of 3 biological replicates were performed.

Figure 17 identifies the 20:4 sample and the 22:6 sample as changing the antimicrobial activity resistance to P3 in *V. vulnificus*, increasing the MIC two-fold (16µM). The control and the 18:2 sample were inhibited at 8µM of P3.

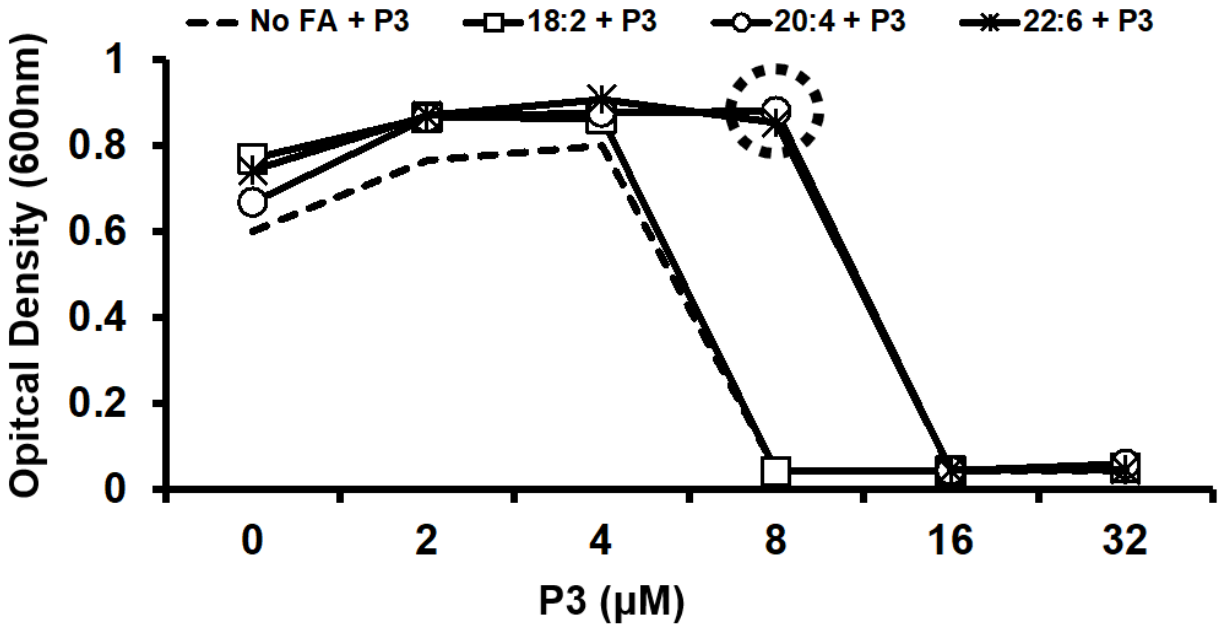

Figure 17: Determination of the minimum inhibitory concentration of *Vibrio vulnificus* grown in the presence of 18:2, 20:4, or 22:6 when treated with a 2-fold concentration increase of piscidin 3. The symbols circled by a dotted line indicate significance ($P<0.02$) as determined by a t-test performed for the triplicate values at each concentration versus control. All standard deviations were less than 0.03. A minimum of 3 biological replicates were performed.

In **Figure 18**, it shows that the 20:4 sample and the 22:6 sample increase the survival of V. vulnificus by increasing the MIC of P3-Cu^{2+} by two-fold (8µM). Both the 18:2 samples had no impact with growth occurring at 4µM.

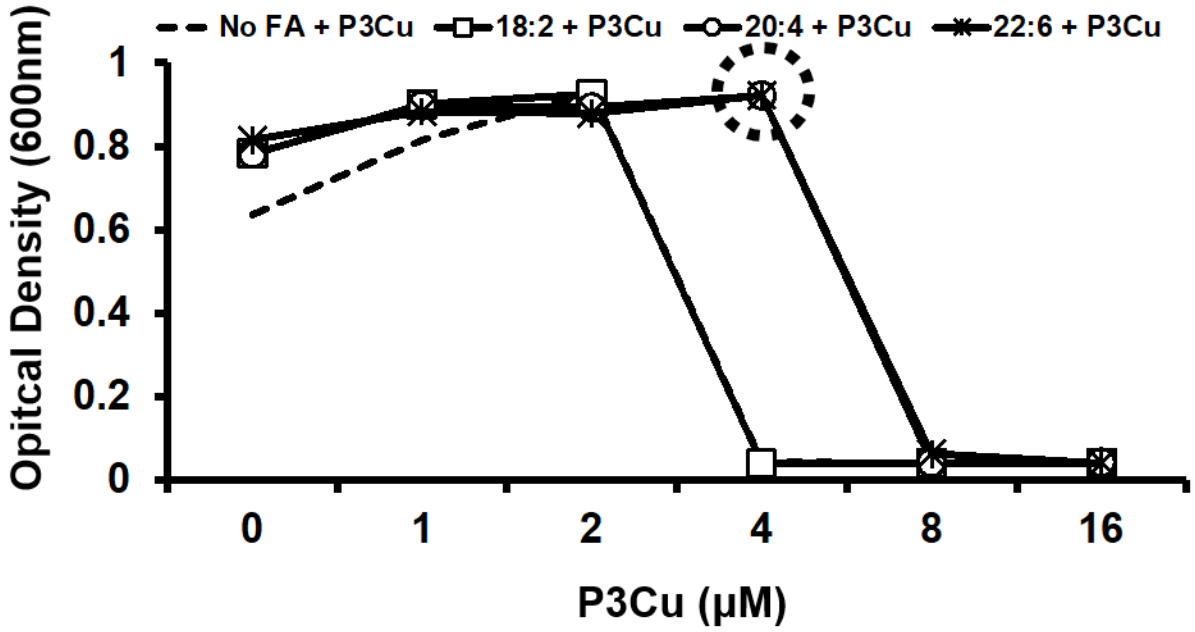

Figure 18: Determination of the minimum inhibitory concentration of *Vibrio vulnificus* grown in the presence of 18:2, 20:4, or 22:6 when treated with a 2-fold concentration increase of piscidin 3-Cu^{2+}. The symbols circled by a dotted line indicate significance ($P<0.001$) as determined by a t-test performed for the triplicate values at each concentration versus control. All standard deviations were less than 0.03. A minimum of 3 biological replicates were performed.

Discussion

The devastating increase in antibiotic-resistant bacteria has posed a global threat to public health. With the substantial decrease in the antibiotic pipeline and access, research of new therapeutic options is on the rise. Antimicrobial peptides provide a great promise for the future of medical treatment options. With their ability to change conformationally to have a broad

spectrum of activities and aversion of bacterial membrane resistance strategies. More than 3000 AMPs have been discovered but only seven have been approved by the FDA for consumer use; Gramicidin, Daptomycin, Colistin, Vancomycin, Oritavancin, Dalbavancin, and Telavancin [10]. Besides Colistin, six of the seven approved AMPs for clinical usage are only for Gram-positive bacteria. Piscidin was the first HDP discovered in mast cells of vertebrates and affects a broad spectrum of bacteria [31]. P1 is more membrane-active on model lipid bilayers and cellular membranes [27]. P3 requires entering the cells, going through the membrane, and disrupting the DNA [26]. Metalation of piscidins is thought to enhance their antimicrobial activities [12,31].

Vibrio parahaemolyticus and *Vibrio vulnificus* are gram-negative, halophilic bacteria found in mainly contaminated oysters. Despite these two bacteria resembling one another in their history of infection, other than *V. vulnificus's* necrotizing fasciitis, their virulence factors are particular to each other. [6]. *V. parahaemolyticus's* virulence factors are hard to determine due to the multiple strains that have evolved [6,25]. When *V. parahaemolyticus* was first studied in the 1980s, it was thought that the thermostable direct hemolysin (TDH) gene was only present in pathogenic strains. Although that is not the case, it is hypothesized that the bacterial fitness of the strains that contain the TDH genes played a significant role. Some strains contained cloned homologous TDH-related hemolysin (TRH) and thermolabile hemolysin (TLH) genes that had similar activity but were distinctive in the genome. It is hypothesized that the three hemolysins along with two type III secretion systems (T3SS1 and T3SS2) help *V. parahaemolyticus's* virulence factors since T3SS2 has been apparent in most pathogenic strains [6,25,29]. One study with *V. parahaemolyticus* infecting rabbits did show an increase of fatty acid in the intestinal tract from the TDH gene enterotoxicity [37]. *V. vulnificus* is an opportunistic pathogen that has little research on its membrane and what causes such rapidly fatal infections. The virulence

factors that have been demonstrated are the ability to acquire iron, the *V. vulnificus* hemolysin gene (VVH), and capsular polysaccharides (CPS) of the bacterium [6,25,35,37]. Non-capsulated bacteria are phagocytized by macrophages. Specific virulence-related functions such as resistance to human serum and antiphagocytic abilities are associated with CPS [41]. This study extends exogenous fatty acid-mediated phospholipid remodeling to other *Vibrio* species and the impact of antimicrobial peptides. We investigate this by conducting permeability assays and MICs.

Membrane Permeability Assays

We investigated the membrane permeability effects of *V. parahaemolyticus* with the addition of piscidins and PUFAs first. After gathering out data, there were no significant trends that were similar in all four treatments. As shown in **Figure 3**, the incorporation of 20:4 and 22:6 within *V. parahaemolyticus's* membrane provided more of a protective impact with the addition of P1. In this case, the presence of 20:4 and 22:6 significantly decreased membrane permeability, allowing for major resistance to P1. The addition of P1 only increased the crystal violet uptake, resulting in the membrane becoming less tightly packed, becoming weaker and more fluid. The sample with the addition of 18:2 did not create any significant differences in permeability with P1.

In **Figure 4**, the incorporation of 20:4 with P1-Cu^{2+} provided more resistance to the *V. parahaemolyticus's* membrane. Compared to **Figure 3**, 20:4 has a significant effect on the membrane with both P1 and P1-Cu^{2+}. This represents that 20:4 has a protective effect over *V. parahaemolyticus's* membrane and does not allow much uptake into the membrane as the other PUFAs. The samples with the addition of 18:2 and 22:6 did not create any significant differences in permeability with P1-Cu^{2+}, meaning no disruptions to the membrane. P1 may be more

antimicrobial and membrane disruptive, and both P1 and P1-Cu^{2+} with the incorporation of 20:4 and 22:6 for P1 strengthen vulnerable spots of the membrane.

Unlike P1-Cu^{2+}, **Figure 5** shows the addition of P3 with no fatty acids significantly increased membrane permeability, disrupting the membrane and allowing increased crystal violet incorporation. Resembling the data from P1, the incorporation of 22:6 with P3 provided more resistance to the *V. parahaemolyticus* membrane. Since both P1 and P3 had significantly decreased membrane permeability, both metallated forms (P1-Cu^{2+} and P3-Cu^{2+}) did not suggest that Cu^{2+} has a repulsive effect with 22:6 on the membrane. Nonmetallated piscidins with 22:6 is causing an intramembrane interaction that could be caused with both present. When looking at 22:6 interacting with piscidins, two things might occur within the *V. parahaemolyticus* membrane. First, 22:6 interacts with P1 or P3 and makes them stuck in the membrane. Since Gram-negative bacteria membrane consists of negatively charged lipopolysaccharides and piscidins are positively charged, their attraction causes them to tightly form together when 22:6 is present, promoting decreased permeability. Secondly, 22:6 with P1 or P3 triggers the membrane immune response. Bacterial cells can change their surface fluidity depending on the environmental conditions. Production of 22:6 is done in fish and with piscidins being found on fish skin, they might have coevolved and better the immune system together. However, when exposed to this combination, the bacterial membrane sees this as a threat to its immune system and triggers stress proteins to strengthen the membrane to avoid damage. This can include alteration of length, fatty acid composition, or phospholipid composition.

Similarly to the previous permeability assay with P3, **Figure 6** shows the incorporation of P3-Cu^{2+} with no exogenous fatty acids significantly increased membrane permeability. This increase in susceptibility is more significant with P3-Cu^{2+} than P3 and could be due to the

metalation with Copper (Cu^{2+}). Metalation with P3 appears to have a much more dramatic effect than metalation with P1 in terms of peptides getting redistributed in the bilayer center of the membrane [10]. Observing **Figure 4** and **Figure 6**, the control of 22:6 with no metalated piscidin compared to the solid lines of 22:6 with metalated piscidin shows almost complete overlap in both experiments. From a previous study with *V. parahaemolyticus* and other *Vibrio* species, 22:6 was shown to have minimal effects on *V. parahaemolyticus* membrane, proposing that 22:6 has no significant impact on LPS and its virulence factors [37]. The baseline control (no FA and no piscidin) in this experiment is close to overlapping both the presence and absence of 22:6 with metalated piscidin. Cu^{2+} is important for enzyme function, hemolysis, and ROS formation within bacteria, and interaction with 22:6 in the bacterial membrane could reduce one, or more, of its primary functions it would pose otherwise [32]. In previous studies, *V. parahaemolyticus* has shown heavy metal resistance (83% with copper) found in oysters and clams in Korea [27]. With the coordination of Cu^{2+}, heavy metal resistance produced with the addition of 22:6 could allow Cu^{2+} effects to be eliminated from the bacterial membrane.

We investigated the same effects in *V. vulnificus* and the membrane permeability assays conducted did not resemble *V. parahaemolyticus* outcomes. *V. vulnificus* shows that the addition of only the desired piscidin resulted in no significance, showing the baseline control and the treatment with only piscidin overlapping throughout. However, administration of PUFAs caused significant decreases in permeability, and treatment with piscidins did not recover the permeability.

Figure 7 shows that there were significant differences in the bacterial membrane with the addition of two fatty acids (18:2 and 22:6) with P1. *V. vulnificus's* membrane with the addition of 18:2 with P1 was weaker, allowing increased uptake of the hydrophobic crystal violet. With the

addition of 22:6 with P1, the membrane was stronger, preventing major uptake of crystal violet. In **Figure 8**, assimilation of 20:4 with P1-Cu^{2+} caused *V. vulnificus* membrane permeability to decrease, preventing uptake of crystal violet. P1 is more active, hemolytic, and targets the bacterial membrane while P1-Cu^{2+} enhances these effects. As suggested previously with *V. parahaemolyticus* with P1 and P3, *V. vulnificus* in addition to 22:6 with P1 and 20:4 with P1-Cu^{2+} could get stuck in the membrane or cause a membrane stress response that allows for protection of the bacteria.

With **Figure 9**, it does not express any significance in the bacterial membrane permeability of *V. vulnificus* with the addition of P3. All the fatty acid lines (control and non-control) are overlapping, showing little to no differences that are irrelevant to membrane permeability. Like **Figure 7**, **Figure 10** expresses a significant increase in membrane permeability of *V. vulnificus* with the incorporation of 18:2 with P3-Cu^{2+}. No fatty acid, 20:4, and 22:6 in addition to P3-Cu^{2+} resulted in no significance. This is an unusual pattern for piscidins because the two piscidins that resemble each other with 18:2 making the membrane more permeable, P1 and P3-Cu^{2+}, are the most dissimilar. P1 is more membrane-active and fast-acting while P3-Cu^{2+} binds more efficiently and attacks/nicks the DNA instead of the membrane [27]. With *V. vulnificus*, virulent strains have capsular LPS (CPS) production to protect the bacteria and are more resistant to phagocytosis [40]. Since P1 is fast-acting, it might have been able to target the membrane and bind to the lipid bilayer with 20:4 before the CPS could inhibit. For P1-Cu^{2+}, its ROS formation is slow and the CPS could have prevented it from interacting with the membrane with other PUFAs other than 20:4 [38]. The heparin structure of P1-Cu^{2+} does not affect bacterial killing as P3-Cu^{2+} does, and with the addition of 20:4, it got to the target site and created a stress response by CPS. P3 attacks the DNA but with it being slower than P1 and

having to get through the bacterial membrane, PUFAs would not provide any advantage to get through the membrane. P3 relies heavily on Cu^{2+} for potency and for ROS to nick DNA. Possibly, since P3-Cu^{2+} is even stronger than P3 and creates a heparin structure at the N-terminal that increases the efficacy of receptor binding [27]. With this increase in binding efficiency and incorporation with 18:2, it is always the perfect amount of time to bind and cause an increase in membrane permeability.

Conducting membrane permeability assays is relatively challenging since one must perform several biological replicates and compare the negative controls to prove correct results. For the experiment itself, usage of different dyes, such as ethidium bromide (EtBr) and propidium iodide (PI), could be used to measure bacterial membrane uptake. For this study, we used hydrophobic crystal violet that has a positive charge. Although we used crystal violet, it could misrepresent permeability due to its differential binding with the membrane, such as LPS, other glycolipids, and proteins. Future studies could use additional measures of membrane permeability to confirm the accuracy of the dyes and compare the results.

Minimal Inhibitory Concentration (MIC)

In the MIC assays of *V. parahaemolyticus*, the concentration of piscidin required to inhibit the growth of this bacteria varied greatly. In **Figure 11**, the assay with P1 was unique with the exogenous fatty acids introduced resulting in no resistance or susceptibility to the membrane. In contrast with P1, in **Figure 12,** the metalated form P1-Cu^{2+} with the introduction of 18:2 increased *V. parahaemolyticus* resistance, allowing survival of the bacteria for another two-fold (8μM). With the addition of P1-Cu^{2+}, 18:2 was the most desirable fatty acid since with *V. parahaemolyticus*, it strengthened the resistance to the peptide.

The MIC with P3 was unique from P1 and P1-Cu^{2+} because all exogenous fatty acids greatly increased susceptibility greatly. **Figure 13** shows that P3 in addition to PUFAs significantly made the membrane weaker, allowing for bacterial death for all three to occur at 8-fold. The control, no fatty acid in addition to P3, survived and kept growing past 32-fold, which is the largest concentration of piscidin done throughout this study. The concentration of P3 was not potent enough to conduct further growth with *V. parahaemolyticus* and P3. This result is our most significant result out of the 16 figures present. P3 is less antimicrobial, less hemolytic, and disrupts DNA instead of the membrane [11,27]. The effects of all fatty acids in our study with P3 had a desirable incorporation effect, allowing the concentration of P3 to enter the membrane with fatty acid before it could be without fatty acids. In **Figure 14**, P3-Cu^{2+} with the incorporation of both 18:2 and 20:4 of *V. parahaemolyticus* resulted in increased susceptibility while 22:6 provided no impact on the MIC. As stated before, P3 targets the DNA. With P3-Cu^{2+}, 20:4 and 18:2 inhibited the bacterial membrane and allowed it to be more susceptible to the piscidin effects, getting to the DNA faster and attacking it at a lower concentration.

In the MIC assays with *V. vulnificus*, the concentration of piscidin required to inhibit the growth of *V. vulnificus* did not vary as greatly. In **Figures 15, 16, 17,** and **18**, all four piscidins assimilated with 20:4 strengthened *V. vulnificus's* resistance, requiring double the concentration of each piscidin to inhibit the bacteria. Except for P1, 22:6 also strengthened the membrane for P1-Cu^{2+}, P3, and P3-Cu^{2+} and 18:2 only strengthened the membrane for P1-Cu^{2+}. P1, P3, and P3-Cu^{2+} did not have any inhibited growth with 18:2. From a previous study with *V. vulnificus* and antimicrobial peptide Polymyxin B, all three fatty acids (18:2, 20:4, and 22:6) provided resistance and allowed the inhibition of bacterial death, especially with 22:6 [37]. Since *V. vulnificus* has a CPS, the fatty acids and piscidin used in this study were incorporated within the

CPS and allowed for more protective effects against the piscidin concentrations, especially with the addition of P1-Cu^{2+}.

A 2021 published study examined the effects of PUFAs with piscidins in *Vibrio cholerae*, which causes acute diarrheal illness from ingestion of contaminated water and food. For membrane permeability, they only used different concentrations of 18:2 but an unpublished paper conducted in 2022 in the same laboratory examined 20:4 and 22:6 effects on membrane permeability. The addition of all three PUFAs with P1 caused decreased membrane permeability while P1-Cu^{2+} caused a decrease in membrane permeability except for 18:2, which increased membrane permeability [2]. P3 and P3-Cu^{2+} with PUFAs significantly increased membrane permeability except for 20:4 with P3-Cu^{2+} which caused a decrease. In the conducted MICs at least one PUFA increased the resistance to bacterial death except for P3 (P1 with 18:2 and 22:6, P1-Cu^{2+} with 20:4 and 22:6, and P3-Cu^{2+} with 20:4). P3 had a significant decrease with 18:2 and 20:4, causing a weaker membrane with the PUFA addition. When comparing the results from our study, *V. parahaemolyticus* data did not significantly relate to what occurred with *V. cholerae*. *V. vulnificus* resembled some trends from the *V. cholerae* permeability membrane assay and MIC data. *V. cholerae* membrane permeability had more extreme differences compared to the control and the addition of the desired piscidin than *V. vulnificus* while their MICs provided some resistance with each piscidin but with different PUFAs except for P3-Cu^{2+}, which was the same. These studies confirm the ability of enhanced activity of PUFAs and piscidins within *Vibrio spp*. bacterial membrane.

Vibrio parahaemolyticus and *Vibrio vulnificus* are two halophilic bacteria that are found in coastal and estuarine environments. In humans, *V. parahaemolyticus* causes acute gastroenteritis from the consumption of contaminated seafood with *V. vulnificus* also causing

necrotizing fasciitis directly to exposed wounds. Both are found in seafood, primarily in contaminated shellfish such as oysters and shrimp and can infect aquatic animals with Vibriosis. *Vibrio spp*. incorporate exogenous fatty acids in their desired aquatic revisor and human host. In this study, we investigated the effects of PUFA incorporation along with piscidin broad activity on the bacterial membrane. Most experimental runs with the desired fatty acids and piscidins resulted in the enhancement of resistance or susceptibility to membrane penetration. *V. parahaemolyticus* showed that only piscidins increased the membrane permeability almost throughout while the addition of PUFAs decreased membrane permeability. With the conducted MICs, all four piscidins with PUFAs showed varied results, with one showing increased resistance and two showing a drastic decrease in inhibition of bacterial death. *V. vulnificus* showed that the addition of only piscidin had no significance but the addition of piscidin and PUFAs allowed for a significant decrease in membrane permeability. With the MICs, at least one PUFA with the incorporation of all piscidins increased protection against bacterial death.

PUFAs can be acquired from various environments or produced within the body while piscidins are only associated with fish. PUFAs change the ability of piscidins to interact with the membrane and therefore exert and enhance their antimicrobial effects. Piscidins have the potential to be used in therapeutic alternatives, and this study reveals the potential combinatorial use of PUFAs and piscidins, along with other membrane-active AMPs.

Conclusions and Future Directions

Piscidins are the most studied α-helical antimicrobial peptide but there is still debate about the mechanism of each nonmetalated and metalated piscidin. As seen above, the behavior

of these piscidins on *V. parahaemolyticus* and *V. vulnificus* bacterial membranes had diverse results, making it difficult to find a linkage between the conducted experiments. To further examine PUFA-mediate effects on piscidin treatment, other methodologies could supplement our findings. Membrane permeability can be measured in various ways such as dyes and fluorescent probes that can distinguish between the inner versus outer membrane permeability. Other PUFAS such as α-linolenic acid, γ-linolenic acid, eicosatrienoic acid, and eicosapentaenoic acid could elicit stronger reactions from antimicrobial treatment. Usage of other piscidins, such as piscidin 2, piscidin 4, and piscidin 5 could compare the effects of all three Class I piscidins, Class II, and Class III piscidins. Other fish AMPs that have not been as thoroughly researched, such as β-defensins and hepcidins, could promote broader antimicrobial and bacterial membrane activity that has not been explored. Lastly, temperature changes for aquatic bacterial cultures and growths could reveal new outcomes since this experiment resembled the human body temperature (37ºC). *Vibrio spp.* that resides in fish encounters cooler temperatures and studying the effects on fish could open new results. This study contributed valuable data for piscidin research while supporting further investigation of piscidins and exogenous fatty acids on Gram-negative bacteria for potential use for future therapeutic options.

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