Lipid modification in Aeromonas salmonicida through exogenous polyunsaturated fatty acid incorporation

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Lipid Modification in *Aeromonas salmonicida* through Exogenous Polyunsaturated Fatty Acid Incorporation

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Departmental Honors Thesis
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Abstract

*Aeromonas salmonicida* is a Gram-negative bacterium that can infect a wide host range of fish populations, including salmonids and non-salmonids as well as freshwater and marine life. *A. salmonicida* causes the disease furunculosis, which can cause lethargy, intestinal inflammation, ulcers, hemorrhaging, and death. The infection is spread through fish-to-fish contact, and the presence of infection can have devastating effects on cultivated fish populations. The purpose of this study was to explore the ability of *A. salmonicida* to incorporate polyunsaturated fatty acids (PUFAs) into its lipid profile and test the phenotypic effects thereof. *A. salmonicida* cultures were grown in minimal media in the presence or absence of one of seven PUFAs. Lipids were extracted from these cultures and analyzed for lipid modification by thin-layer chromatography and ultra performance liquid chromatography and tandem mass spectrometry, showing *A. salmonicida* capable of incorporating all seven of the PUFAs studied. Phenotypic effects were determined through the use of assays that tested for biofilm formation, membrane permeability, and antimicrobial peptide susceptibility. It was found that the PUFAs 18:2 and 22:6 caused significant (*P* < 0.01) decrease in biofilm formation. The PUFAs 18:3γ, 20:4, and 22:6 showed significant (*P* < 0.001) increases in membrane permeability as tested by the uptake of the hydrophobic compound crystal violet. Additionally, the PUFAs 18:3γ, 20:5, and 22:6 showed significant (*P* < 0.001) protection against the antimicrobial peptide polymyxin B. The prevalence of 22:6 in significant phenotypic effects could indicate a connection between the ability of *A. salmonicida* to utilize the long-chain PUFA and its role as a fish pathogen.
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Chapter 1: Introduction

1.1 Gram-Negative Membranes

Gram-negative bacteria are distinguished from their Gram-positive counterparts by the fact that they do not retain crystal violet (CV) dye following a staining technique developed by Hans Christian Gram. Gram-positive bacteria retain CV, appearing purple after staining, due to a thick layer of peptidoglycan found on the outside of the membrane, which is a single lipid bilayer. Peptidoglycan can also be found in Gram-negative bacteria—in a much smaller quantity that does not retain CV as well—as a thin layer in the periplasmic space between two membranes as shown in Figure 1.1.

![Figure 1.1 Structural model of a Gram-negative cell envelope](image)

The presence of two membranes makes Gram-negative bacteria particularly good subjects for this project’s focus on membrane remodeling. The two membranes are lipid
bilayers, denoted as the inner membrane, which has an interface with the cytosol, and the outer membrane, which has an interface with extracellular fluids. Lipids are a broad classification of largely nonpolar bioorganic molecules. They encompass a diverse range of functions, including energy storage, signaling, and serving as the structural matrix of membranes. The lipids that comprise biological membranes consist of polar head groups and nonpolar tail groups. This amphiphilic property causes a hydrophobic effect that drives the formation of bilayers, as the nonpolar tail groups favorably group together, with the polar head groups aligned together against aqueous interfaces, such as the periplasm, cytosol, and extracellular fluids.

The specific lipids that comprise the membranes of Gram-negative bacteria are phospholipids (PLs) and lipopolysaccharides (LPSs). The entirety of the inner membrane is comprised of phospholipids, and the inner leaflet of the outer membrane (the lipid layer that interfaces the periplasm) is comprised of phospholipids, while the outer leaflet of the outer membrane (the lipid layer that interfaces extracellular fluids) is comprised of LPSs. LPSs are largely believed to contribute to many Gram-negative bacteria's pathogenic nature. However, the focus on this project was the modification of PLs, as they are more abundant in the membrane.

1.2 Phospholipids and Fatty Acids

Phospholipids are a specific subcategory of lipids, characterized as such by the presence of a phosphate group in the polar head group. Glycerophospholipids are specifically of interest, due to their natural prevalence in all biological membranes. Glycerophospholipids consist of a glycerol backbone, with a phosphate group at the sn-3 position and fatty acyl chains at the sn-1 and sn-2 positions, as shown in Figure 1.2. The
phosphate group is also attached to a more polar head group, which is the first means of identifying a glycerophospholipid. The most common head groups of Gram-negative species are ethanolamine, denoted as phosphatidylethanolamine (PE), and glycerol, denoted phosphatidylglycerol (PG). Figure 1.2 shows an example of a PE and Figure 1.3 shows an example of a PG.
Figure 1.2. Phosphatidylethanolamine 16:0/18:2. The sn-1, sn-2, and sn-3 carbons of the glycerol backbone are labeled.

Figure 1.3. Phosphatidylglycerol 16:0/18:2. The sn-1, sn-2, and sn-3 carbons of the glycerol backbone are labeled.
The PL is subsequently identified according to the characterization of the sn-1 and sn-2 chains, which are esterified fatty acids (FAs). FAs are carboxylic acids with long hydrocarbon side chains. These FAs are characterized by length, or number of carbons, and degree of unsaturation, or number of double bonds. A FA can be either saturated, monounsaturated, or polyunsaturated, meaning it contains no double bonds, a single double bond, or more than one double bond, respectively. For example, a polyunsaturated fatty acid (PUFA) with twenty carbons and three double bonds would be denoted as 20:3. Additional classification includes Δ-notation, which is used to specify the location of any double bonds. An example of a specific 20:3 FA is dihomo-γ-linolenic acid, which can also be specified as 20:3 (Δ⁸,¹¹,¹⁴), showing that the three double bonds occur between C8 and C9, C11 and C12, and C14 and C15, counting from the carbonyl carbon. An alternative method of identifying the location of the double bonds in a PUFA is by specifying whether it is a ω-3 or a ω-6 FA, which denotes the location of the last double bond in the chain, counting from the terminal methyl group. The classification of ω-3 or ω-6, combined with the fact that the double bonds in most PUFAs tend to be three carbons apart, can also be used to know the exact locations of double bonds. The PUFAs used in this study were 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), and docosahexaenoic acid (22:6). The structures of these PUFAs are shown in Figures 1.4 through 1.10. The length and degree of unsaturation of a fatty acid affect many of the molecule's physical properties, such as polarity and melting point.³
Figure 1.4 Linoleic acid (18:2)

Figure 1.5 α-Linolenic acid (18:3α)
Figure 1.6 γ-Linolenic acid (18:3γ)

Figure 1.7 Dihomo-γ-linolenic acid (20:3)
Figure 1.8 Arachidonic acid (20:4)

Figure 1.9 Eicosapentaenoic acid (20:5)
Figure 1.10 Docosahexaenoic acid (22:6)
1.3 Previous Research

Most naturally occurring fatty acids in Gram-negative bacteria are either fully saturated or monounsaturated, with few, if any, PUFAs occurring natively. This also extends to the acyl chain identities of native phospholipids. Because the additional double bonds present in PUFAs affect properties such as melting point and polarity, the incorporation of PUFAs into the phospholipid profile of Gram-negative bacteria would be expected to cause alterations to biological properties of the membrane (e.g. membrane fluidity and membrane lipid homeostasis) and therefore affect functions carried out by the membrane. Previous research has shown that membrane modification via PUFA incorporation in certain Gram-negative species can result in alteration to biological functions such as motility, permeability, biofilm formation, and susceptibility to antimicrobial peptides.

The mechanism by which Gram-negative bacteria utilize exogenous fatty acids in lipid modification has been studied using the model organism Escherichia coli. The process begins with the FadL gene, which encodes the outer membrane protein (FadL) that acts as a transporter of long-chain fatty acids. The fatty acid is then converted to an acyl-CoA thioester by the acyl-coenzyme A (CoA) synthetase, FadD. At this point, the acyl-CoA can either be β-oxidized, or it can be used for phospholipid synthesis via the acyltransferases PlsB and PlsC. The presence of FadL, FadD, PlsB, and PlsC homologs in other Gram-negative species can indicate ability to utilize exogenous PUFAs in lipid modification rather than β-oxidation.

Previous work has also shown the efficacy of ultra performance liquid chromatography with tandem mass spectrometry (UPLC-MS) in detection and identification of lipids. Reversed-phase liquid chromatography is a powerful means of separating mixtures of
lipids, which are all hydrophobic molecules. Following this separation of lipids of interest, mass spectrometry is utilized to detect mass:charge (m/z) ratios of the molecules. Specifically, electrospray ionization (ESI) is widely used in the study of phospholipids\textsuperscript{7, 16-18, 21}, as many classes of phospholipids can acquire either a positive or negative charge in the mass spectrometer source. When analyzed using negative-mode ionization (ESI(-)), phospholipids molecular ions appear as negative ions after single deprotonation of the phosphate group, notated as [M-H]-. ESI(-) can also cause distinctive fragmentation of phospholipids, such as the loss of the acyl chains from the sn-1 and sn-2 positions as fully intact carboxylate ions.\textsuperscript{16, 17, 21} The cleaved acyl chains are negatively charged and can therefore be detected in ESI (-) mode, allowing for a richer analysis of the lipids of interest.

1.4 \textit{Aeromonas salmonicida}

\textit{Aeromonas salmonicida} is a Gram-negative bacteria that was discovered by Emmerich and Weibel in 1894 at a Bavarian brown trout hatchery.\textsuperscript{22} It is a widespread fish pathogen that was initially believed to solely infect the salmonids, such as Atlantic salmon, brown trout, and sea trout, but the host range has since been expanded to include freshwater non-salmonids, such as American eel, catfish, northern pike, and smallmouth bass, as well as marine non-salmonids, such as Atlantic cod and sea bream.\textsuperscript{22, 23} Infection by \textit{A. salmonicida} is referred to as furunculosis, and symptoms can vary depending on the classification of the infection. Paracute furunculosis occurs in young fish and usually lacks any telltale markers because it results in rapid death. Similarly, acute furunculosis is typically fatal within 2 to 3 days, but infected fish can display skin lesions. Chronic furunculosis occurs in older fish and has a lower rate of mortality than paracute and acute
infections. Symptoms of chronic furunculosis include lethargy, intestinal inflammation, hemorrhages in muscle and liver, as well as the furuncles for which the disease is named.

* A. salmonicida* is a non-motile bacterium with recorded temperatures of 18°C to 26°C being optimal for growth. It was widely accepted that *A. salmonicida* could not grow at 37°C and therefore did not pose a risk to humans. However, a recent case study has reported the first known case of isolation of *A. salmonicida* from a human blood sample. While this was an isolated incident, and *A. salmonicida* does not pose significant risk to humans directly, infections can have devastating effects on fish populations cultivated as food sources. The disease spreads from contact with infected fish. While the pathogenesis of *A. salmonicida* is not especially well understood, it is largely contributed to two factors: cell-surface structures and extracellular products excreted by the cell.

Current treatment of furunculosis infections includes vaccination, selective breeding, and antibiotic use. However, each of these methods of disease control is limited. For example, vaccination has been used successfully in salmonid populations, but has not been explored in non-salmonids. Therefore, there is still need for exploration of novel treatment methods. This project sought to determine the ability of *A. salmonicida* to incorporate exogenous PUFAs into its lipid profile and biological effects thereof, including susceptibility to an antimicrobial peptide, which could potentially affect future therapeutic efforts.
Chapter 2: Materials and Methods

2.1 Growth procedure for UPLC-MS and TLC analysis

*Aeromonas salmonicida subsp. salmonicida* (ATCC® 33658™) was obtained from American Type Culture Collection and was used in this and all subsequent experiments. Overnight cultures of *A. salmonicida* were prepared by aseptically transferring colonies of *A. salmonicida* from a Luria agar plate to sterile glass tubes containing 4-mL Luria broth (LB). These cultures grew at room temperature with shaking on a New Brunswick Scientific Innova 2100 platform shaker at 200 rotations per minute (rpm) and were transferred to 15-mL polypropylene conicals and centrifuged in a Corning LSE compact centrifuge at 4250 rpm for 10 minutes the following morning. The supernatant was discarded, and the pelleted bacteria were washed with 1-mL CM9 minimal media (0.4% glucose, 0.2% Casamino Acids) and centrifuged again at 4250 rpm for 5 minutes. Again, the supernatant was discarded and the washed pellet was gently resuspended by pipette in 1-mL CM9. The optical density (OD) of the resuspended cells was measured with a Thermo Scientific Genesys 10S UV-Vis Spectrophotometer at 600 nm (OD$_{600}$). This culture was then used to inoculate eight 20-mL CM9 cultures to a starting OD$_{600}$ of 0.1 in 50-mL polypropylene conicals. Seven of those cultures were supplemented with 300 μM of one of the seven PUFAs studied (18:2, 18:3α, 18:3γ, 20:3, 20:4, 20:5, or 22:6). All PUFAs were obtained from Cayman Chemical Company. These cultures were grown at 23°C in a Thermo Scientific MaxQ 4000 incubator with shaking at 200 rpm to an OD$_{600}$ of approximately 0.9. To cease growth, the 20-mL cultures were pelleted via centrifugation at 4250 rpm for 10 minutes, after which the supernatant was discarded. The pellet was then washed with 1-mL CM9
and centrifuged again at 4250 rpm for 5 minutes and the supernatant was discarded. Pelleted cultures were stored at -20°C until lipid extractions were performed.

Cultures grown for analysis by thin-layer chromatography (TLC) were grown identically to the above procedure, except 14-mL cultures were prepared as opposed to 20-mL cultures.

2.2 Bligh and Dyer Lipid Extractions

Lipid extractions for both TLC and UPLC-MS analysis were performed according to the procedure outlined by Bligh and Dyer. Stored bacterial pellets were resuspended in 5-mL single-phase Bligh/Dyer mixture (1:2:0.8 chloroform:methanol:water) and immediately transferred via glass Pasteur pipette to clean 10-mL glass tubes. These tubes were capped and vortexed, and the mixture sat for 20 minutes, with additional vortexing after 10 minutes. This process lysed the cells, allowing the lipids to be freed into solution. After lysing, the tubes were centrifuged for 10 minutes at 4000 rpm such that the supernatant contained the lipids of interest and cellular components that were not of interest were pelleted. The supernatant was removed and placed into clean 10-mL glass tubes to which 1.3-mL chloroform and 1.3-mL water were added to create a two-phase Bligh/Dyer mixture consisting of an aqueous upper phase and an organic lower phase. This mixture was homogenized by vortexing and then centrifuged at 4000 rpm for 10 minutes. The lower phase containing the lipids of interest partitioned into the chloroform was gently removed with a glass Pasteur pipette and placed in a clean 10-mL glass tube. An additional 2.6-mL aliquot of chloroform was added to the original tubes (now containing only the upper aqueous phase from the previous step) in order to reestablish a two-phase Bligh/Dyer mixture. This was then vortexed and centrifuged at 4000 rpm for 10 minutes, and the lower phase was extracted as before and pooled with the previous extraction. To
purify the extractions, the two-phase Bligh/Dyer mixture was reestablished with the pooled lower phases by adding 5.2-mL methanol and 4.68-mL water to them. This was then vortexed and centrifuged at 4000 rpm for five minutes. The lower phase was again extracted gently by glass Pasteur pipette and placed into clean 5-mL glass tubes. These constituted the final lipid extracts, which were dried down to remove all solvent under a gentle stream of nitrogen gas on an N-EVAP 111 Nitrogen Evaporator. Dried lipid extracts were stored at -20°C until weighing and transferring.

2.3 Thin-Layer Chromatography

Lipids extracted from the 14-mL cultures were spotted onto a silica gel 60 TLC plate. A solvent system of chloroform-methanol-acetic acid (65:25:10, volumetrically) was used to separate lipids in a closed chamber. The tank system containing the solvent was equilibrated for two hours prior to placing the TLC plate inside. Once the solvent reached the top of the plate, it was removed from the chamber and allowed to dry. A solution of 10% sulfuric acid in 100% ethanol was used to coat the plate, and it was subsequently developed on a hot plate at 150°C just until lipids appeared. A Canon CanoScan LiDE 210 was used to scan the plate immediately after developing.

2.4 UPLC-MS

2.4.1 Preparing lipids for UPLC-MS

Empty Waters LCMS Certified Amber Screw-Top vials and lids were weighed in triplicate on a Mettler Toledo XS205 DualRange analytical balance. Pasteur pipettes were used to resuspend lipid extracts in 2:1 chloroform: methanol and to transfer lipids in solution from test tubes to amber vials. This transfer process was repeated three times to ensure all lipids were transferred. A total of approximately 1-mL of the 2:1 solution was used for each
transfer. Lipid solutions in amber vials were dried to constant mass under a stream of N₂ gas to remove all solvent. Vials now containing dried lipids were weighed in triplicate, and masses of dried lipids were determined by subtracting average mass of empty amber vials from the average mass of amber vials containing dried lipids. A table of the triplicate weighings and final mass values is provided in the supplemental material. Parent solutions of dried lipid extracts were made by adding 1-mL 4:1 chloroform: methanol and vortexing to ensure all lipids were in solution. Working solutions of 400-ppm total lipid extract were then prepared from the parent solutions. Appropriate aliquots of parent solutions were transferred to empty Waters LCMS Certified Max Recovery vials and allowed to dry under N₂ gas on an N-EVAP 111 Nitrogen Evaporator. The dried lipids were then suspended in 100-μL MeOH (B1) with vortexing. To this solution, 100-μL 30:70 25-mM ammonium acetate: MeOH (A1) was added, such that the final solution was a suspension of extracted lipids in 200-μL of 50:50 A1:B1.

2.4.2 UPLC-MS Full Scan Parameters

Ultra performance liquid chromatography and tandem mass spectrometry measurements were performed on a Waters ACQUITY-UPLC System interfaced to a Quattro Micro API triple quadrupole mass spectrometer with electrospray ionization (ESI) source. Full scans of the 400-ppm lipid extractions were performed on a BEH C-8 reversed phase column with gradient elution. The solvents used as the mobile phase were defined as A1 (30:70 25mM ammonium acetate: MeOH) and B1 (MeOH). All solvents used were Optima grade (Fisher Scientific), and water was 18.2-MΩ cm from a Direct Q3 Milli-Q system. A flow rate of 0.300-mL/min was used and 5-μL of the 400-ppm working solution was injected onto the column for analysis. Gradient elution consisted of 50:50 A1:B1 held constant for 2
minutes, followed by a constant increase in the amount of B1 present until the mobile phase consisted of 100% B1 at 10 minutes. Immediately after the establishment of 100% B1, B1 was rapidly decreased back to initial conditions (50:50 A1: B1) by 10.3 minutes and held constant until the end of run time at 11 minutes.

Following separation via LC, the analytes entered the Quattro Micro API triple quadrupole mass spectrometer where they were ionized via ESI in negative mode with a capillary voltage of 1.5-kV. Inert nitrogen gas at a flow rate of 600-L/h and a temperature of 350°C was used in desolvation of the sample. After ionization, negatively charged molecules passed through a 50-V cone into the quadrupole mass spectrometer, where scans were conducted from 200 m/z to 1500 m/z. Scan time was 0.5 seconds with an interscan delay of 0.01 seconds. The photomultiplier tube detector was operated at 650 V. All chromatograms and mass spectra were analyzed with MassLynx V4.1 software.

2.4.3 SIR Scans

In addition to full scans, which are defined above as the constant scanning from 200 m/z to 1500 m/z, selected ion recording (SIR) scans were also conducted on all lipid samples of *A. salmonicida* grown in the presence of a PUFA. All UPLC and MS parameters remain the same as those used for full scans, but instead of scanning across a range of m/z values, a select few m/z values were scanned. Specifically, the m/z values corresponding to the [M-H]- values of the most likely modified PLs were scanned. Table 2.1 shows the m/z values that were selected for scanning for each different PUFA. This scanning method allows for increased sensitivity by decreasing noise that can be found in full scans. The selectivity of SIR scans can also be a downfall in analysis of modified PLs, as discussed further in results.
Table 2.1 m/z values corresponding to the [M-H]- values of common possible modified lipids where x corresponds to the identity of the modifying PUFA

<table>
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<th>PG 16:0/x</th>
<th>PG 16:1/x</th>
<th>PG 18:0/x</th>
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</table>

2.4.4 Fatty acid calibration curves

The fatty acids linoleic acid (18:2) and docosahexaenoic acid (22:6) were used to create calibration curves by plotting chromatographic peak area vs. concentration. Concentrations of 0.5, 1, 2, and 5 ppm of each of the fatty acids were made in 50:50 A1:B1, and these were analyzed by the UPLC-MS parameters described above with the exception that chromatography on the same gradient was only run for four minutes and m/z values were scanned from 100 to 400. QuanLynx software was used to determine peak area of chromatographic peaks and develop linear regressions.

2.5 Growth Curves

Cultures were prepared from *A. salmonicida* cultures grown overnight in LB. Prepared cultures were grown with shaking in CM9 (0.4% glucose, 0.4% Casamino Acids) at a starting OD_{600} of 0.05 either in the presence or absence of 300-μM of each of the PUFAs (note: slightly higher concentration of Casamino Acids was supplemented into media used in assays as opposed to that used in growth of cultures for TLC and UPLC-MS analysis). One
set of cultures was grown at 23°C in a Thermo Scientific MaxQ 4000 incubator with shaking and another set of cultures was grown at 28°C in a Thermo Scientific Precision incubator with shaking. OD$_{600}$ readings of all cultures were taken with a Thermo Scientific Genesys 10S UV-Vis Spectrophotometer every hour for eleven hours.

2.6 Biofilm Formation Assay

Formation of biofilm was tested by a procedure designed by O'Toole. Inoculum at an OD$_{600}$ of 0.12 in CM9 was prepared from A. salmonicida cultures grown overnight in LB. Additionally, dilutions of the individual PUFAs were prepared in CM9. 170-μL of inoculum was pipetted via multichannel pipette into the wells of a 96-well microtiter plate. 30-μL of either a PUFA dilution or CM9 with no PUFA was added, such that the biofilm formation of A. salmonicida in the presence of each of the seven PUFAs and no PUFA was performed in octuplet. The final starting OD$_{600}$ was 0.1, and the PUFA dilutions were designed such that the final concentration was 300μM. The microtiter plate was statically incubated at 28°C for 24 hours.

After 24 hours of incubation, cultures were removed from the microtiter plate by shaking them out. The plate was gently rinsed by submerging in deionized water and shaking again. Excess liquid was removed by tapping on paper towels, and the plate was allowed to dry before proceeding. After dry, 225-μL of a 0.1% solution of crystal violet (CV) was added via multichannel pipette to each well. This was allowed to sit at room temperature for 15 minutes. CV solution was removed by gentle rinsing with deionized water, as described above. The plate was then allowed to dry completely.

Colorimetric analysis of biofilm was performed by adding 225-μL of 30% acetic acid solution to each well via multichannel pipette and allowing them to sit for 15 minutes, such
that CV would go into solution. Acetic acid-CV solutions were then transferred to a clean microtiter plate, which was read on a BioTek Synergy microplate reader at 590-nm. Significance (P < 0.01) was established with Student’s t-test (two tailed, paired).

2.7 Permeability Assay

Permeability was tested by a CV uptake assay. Eight 6-mL cultures of *A. salmonicida* either in the presence or absence of 300-μM of one of the PUFAs were allowed to grow with shaking at 28°C in CM9 minimal media to OD$_{600}$ of approximately 0.9. These cells were captured by centrifuging and washed with Phosphate Buffered Saline (PBS). Cultures were normalized to OD$_{600}$ of 0.80 in 5-mL PBS and CV was added to the cultures such that each contained 5-mg/mL CV. Readings were taken by capturing 1-mL of each culture. These cultures were pelleted via centrifugation for one minute at 1000 rpm in a Fisher Scientific AccuSpin Micro 17 Centrifuge. Following centrifugation, the supernatant was collected and analyzed by a Thermo Scientific Genesys 10S UV-Vis Spectrophotometer at 590-nm. Readings were taken every five minutes for 25 minutes, and cultures were allowed to gently shake at room temperature between readings. Because the reading of the supernatant corresponded to the amount of crystal violet not taken up by the cells, readings were converted to percentage of uptake by comparison to the reading of a sample of PBS containing 5-mg/mL CV but no bacteria. Significance (P < 0.001) was established with Student’s t-test (two tailed, paired).

2.8 Polymyxin B Susceptibility Assay

Cultures were grown with shaking at 28°C in CM9 minimal media in either the presence or absence of 300-μM of one of the PUFAs to OD$_{600}$ of approximately 0.9. These cultures were centrifuged, washed with CM9, and used to create cultures of OD$_{600}$ 0.12 that were
supplemented with 353-μM of one of the PUFAs, where appropriate. Additionally, dilutions of Polymyxin B (PMB) were made in CM9, with concentrations increasing two-fold. Microtiter plates were prepared by use of multichannel pipette to add 170-μL of the prepared cultures to wells followed by 30-μL of one of the PMB dilutions, such that the wells contained 200-μL altogether at a starting OD\textsubscript{600} of 0.10 and each trial was run in triplicate. Microtiter plates were incubated with shaking in a Thermo Scientific MaxQ 4000 incubator at 28°C in for 24 hours. Plates were lightly covered with aluminum foil to minimize evaporation.

A recent study has found that significant amounts of cationic antimicrobial peptides (cAMPs) can adsorb to glassware and certain plasticware, causing inadvertent lowering of concentrations in studies using cAMPs.\textsuperscript{29} In order to minimize the effect of this adsorption, PMB dilutions used for this study were made in Protein LoBind tubes, and assays were run in polypropylene microtiter plates to ensure accurate concentrations PMB. In addition, polypropylene plates interfere with UV-Vis readings at 600 nm, so cultures were transferred to polystyrene microtiter plates immediately before reading on a BioTek Synergy microplate reader at 600 nm. Significance (P < 0.01) was established with Student’s t-test (two tailed, paired).

2.9 Bioinformatics

The National Center of Biotechnology Information’s Basic Local Alignment Search Tool (BLAST) was utilized to search for homologs of \textit{E. coli} MG1655 FadL, FadD, PlsB, PlsC, PlsX, and PlsY. Only homologs with a maximum score greater than 200 were reported. Homologs are reported for \textit{A. salmonicida subsp. salmonicida} (A449).
Chapter 3: Results

3.1 Introduction to Results

The purpose of this project was to determine the ability of *A. salmonicida* to incorporate exogenous PUFAs into its lipid profile and any biological effects thereof. The first part of this project consisted of analysis of extracted lipids by separation via UPLC and characterization via MS. After confirmation of incorporation a series of biological assays were run to test various biological functions, including growth, biofilm formation, permeability, and antimicrobial peptide susceptibility.

3.2 Thin-layer chromatography of *Aeromonas salmonicida* samples

Figure 3.1 shows the TLC conducted on extracted lipids of *A. salmonicida* samples. The PG and PE regions are labeled, and brackets show examples of the presence of new phospholipid species as a result of PUFA incorporation.
Figure 3.1 Results of TLC conducted on lipids extracted from A. salmonicia samples grown in either the presence or absence of one of the seven PUFAs studied. Splitting indicates the presence of PL species not present in the control sample, likely due to incorporation of PUFAs into lipids.

3.3 UPLC-MS Analysis of Aeromonas salmonicida Samples

The UPLC separation method described in Section 2.4.2 allows for the separation of free fatty acids (FFAs) from glycerophospholipids, and even allows for somewhat, though not entirely, distinct regions of phosphatidylglycerols (PGs) and phosphatidylethanolamines (PEs). This separation is based on the differences in polarity between the species of interest. Figure 3.2 shows the chromatogram of the A. salmonicida control sample, with the FFA and PL regions labeled, and Figure 3.3 shows a closer look at the PL region, with the PG and PE regions labeled to demonstrate this chromatographic separation.

It should be noted that characterization of lipids was conducted in ESI (-) mode, under which conditions glycerophospholipids appear as singly deprotonated [M-H]⁻ ions. Because
the lipids are singly charged, m/z values directly relate to the mass of the lipids less one hydrogen. Several methods of confirmation were used in the characterization of lipids. The LIPID Metabolites and Pathways Strategy (LIPID MAPS) database was utilized to confirm potential identities of parent masses. Additionally, the cone voltage utilized in the mass spectrometry analysis (50 V) was high enough to result in mild fragmentation of the lipids, referred to as cone fragments. Often the most abundant cone fragments correspond to the loss of fatty acyl chains as carboxylate ions from the sn-1 or sn-2 position of the glycerophospholipids. These cone fragments in conjunction with the parent masses and chromatographic separation allow for the positive identification of phospholipids.

The lipids extracted from the sample of *A. salmonicida* grown in the absence of any of the seven PUFAs of interest were used as a reference to compare the native lipid profile of *A. salmonicida* to the modified cultures, confirming that the PUFAs of interest were non-native.

Figure 3.3 also shows the identities of the major chromatographic peaks. These identities were determined according to the mass spectra of the peaks. Figure 3.4 shows the average mass spectrum of the peak at 8.06 minutes in the control sample. The 688.5 m/z value was searched in the LIPID MAPS database, showing that the value corresponds to the [M-H]⁻ m/z value of PE 16:0/16:1. Cone fragments further confirmed the identity of the lipid, as the 253.2 m/z value corresponds to a 16:1 acyl chain and the 255.2 m/z value corresponds to a 16:0 acyl chain. A similar process was followed in determining the identities of the other peaks labeled in Figure 3.3, but the mass spectra have been omitted for the sake of brevity.
Figure 3.2. Chromatogram of A. Salmonicida control sample, distinguishing between the free fatty acid (FFA) and phospholipid (PL) regions.
Figure 3.3. Phospholipid region of A. Salmonicida control sample. Separation of PGs and PEs is indicated by region, and major peaks are identified according to the corresponding mass spectra data.
Figure 3.4. The average mass spectrum of the peak at 8.06 minutes in the A. salmonicida control sample
Figure 3.5 shows the chromatogram, limited to the phospholipid region, of the *A. salmonicida* sample grown in the presence of linoleic acid (18:2). Two peaks of interest are labeled A and B, and the average mass spectra of these peaks are shown in Figures 3.6 and 3.7, respectively. Analysis of the average mass spectrum of peak A (Figure 3.5) shows it to be PG 16:0/18:2. The 745.5 m/z value corresponds to the [M-H]- of PG 16:0/18:2, which was confirmed by use of the LIPID MAPS database. Additionally, the cone fragments of 255.2 m/z and 279.2 m/z correspond to acyl chains 16:0 and 18:2, respectively. Similarly, the identity of B was elucidated from the 714.5 m/z value corresponding to the [M-H]- of PE 16:0/18:2, which was confirmed in the LIPID MAPS database. Because B has the same fatty acyl chains as A, the same cone fragments of 255.2 m/z and 279.2 m/z can be found, also corresponding to 16:0 and 18:2. This also demonstrates the chromatographic separation of PGs and PEs. Despite having the same identifying acyl chains, A and B chromatograph over a minute apart, as shown in Figure 3.5, with the more polar PG species eluting earlier than the analogous PE species in this reversed-phase chromatography.
Figure 3.5. Chromatogram of the A. Salmonicida sample grown in the presence of linoleic acid (18:2); only the phospholipid region is shown.
Figure 3.7. Average mass spectrum of the peak labeled A (6.92 minutes) of the 18:2 sample
Figure 3.8 Average mass spectrum of the peak labeled B (8.32 minutes) of the 18:2 sample
The phospholipid region of the chromatogram of the *A. salmonicida* sample grown in the presence of α-linolenic acid (18:3α) is shown in Figure 3.8. The average mass spectrum of the peak at 7.00 minutes, labeled A, is shown in Figure 3.9. The 684.5 m/z value is the [M-H]- value of PE 14:0/18:3α, which was confirmed via the LIPID MAPS database. Additionally, cone fragments of 227.2 m/z and 277.2 m/z were found, which correspond to 14:0 and 18:3α, respectively. Additionally, the peak at 424.2 m/z indicates the neutral loss of the sn-2 acyl chain (18:3α) as a ketene (R2-CH=O). Specifically, the 424.2 m/z corresponds to the mass remaining after this loss, which can be indicated as [M-H-R2-CH=O]-. This is another cone fragment, caused by the high cone voltage used in the MS parameters.
Figure 3.8. Chromatogram of A. salmonicida sample grown in the presence of α-linolenic acid (18:3α), only the phospholipid region is shown.
As 18:3 alpha; Fullscan

Figure 3.9. Average mass spectrum of the chromatographic peak labeled A (7.00 minutes) in Figure 3.7
Figure 3.10 shows the phospholipid region of the chromatogram of the *A. salmonicida* sample grown in the presence of 18:3γ. The average mass spectrum of the chromatographic peak at 7.89 minutes, labeled A, is shown in Figure 3.11. The mass spectrum peaks at 255.2 m/z and 277.2 m/z indicate cone fragments of 16:0 and 18:3γ, respectively. Because the peak at 712.5 m/z is an even value, this would indicate that the phospholipid that chromatographed at 7.89 minutes is PE 16:0/18:3γ. The LIPID MAPS database confirmed at 712.4 m/z corresponds to the [M-H]⁻ of PE 16:0/18:3γ.
Figure 3.10. Chromatogram of the A. salmonicida sample grown in the presence of γ-linolenic acid (18:3γ), only the phospholipid region is shown.
Figure 3.11. Average mass spectrum of the peak labeled A (7.89 minutes) in the chromatogram of the 18:3γ sample, above
In addition to fullscan parameters, selected ion recording (SIR) scans were also utilized to identify modified phospholipids. SIR scans only scan for a few select m/z values rather than the entire range of 200-1400 m/z that is scanned under fullscan parameters. This allows for increased sensitivity, which can be very useful in the detection of low abundance molecules.

Figure 3.12 shows two chromatograms of the *A. salmonicida* sample grown in the presence of dihomo-γ-linolenic acid (20:3). The chromatogram labeled 1 is from the SIR scan of the sample, which selectively scanned for masses corresponding to possible modified PLs. The chromatogram labeled 2 is from the fullscan of the sample. A chromatographic peak appears in the SIR scan at 8.55 minutes, labeled A. While there is no corresponding peak in the fullscan chromatogram, the same region has been labeled A. The average mass spectra of these chromatographic peaks are shown in Figure 3.13. The mass spectrum labeled 1 corresponds to that taken from the peak at 8.55 minutes in the SIR chromatogram. The mass spectrum labeled 2 corresponds to approximately the same time region, but taken from the fullscan chromatogram.

While SIR scans allow for increased sensitivity in regard to certain ions, they do not offer the same comprehensive identification of PLs as full scans, which also include identifying cone fragments. The mass spectrum taken from the SIR scan shows one major peak at 766.5 m/z and a few smaller peaks at the other masses that were scanned for, but includes little other information. By comparison, the mass spectrum taken from the fullscan contains an abundance of fragments. The peak at 766.5 m/z corresponds to the [M-H]- of PE 18:1/20:3, which was confirmed by searching the LIPID MAPS database. Additionally,
cone fragments can be found at 281.2 m/z and 305.2 m/z, which correspond to 18:1 and 20:3.

The chromatogram also includes many fragments that are unrelated to PE 18:1/20:3. For instance, the peak at 740.5 m/z corresponds to the [M-H]- of PE 16:0/20:3, confirmed by the LIPID MAPS database. This also explains the presence of a peak at 255.2 m/z, which corresponds to 16:0. Additionally, the peak at 434.2 m/z corresponds to the neutral loss of the sn-2 group (20:3) from PE 16:0/20:3.

The peak at 8.42 minutes in the fullscan chromatogram was identified as PE 16:0/20:3, and the presence of related m/z values in the mass spectrum at 8.55 minutes can be explained by slight sampling off the right hand tail of the peak at 8.42 minutes.
Figure 3.12. The chromatogram labeled 1 shows the SIR scan of the *A. salmonicida* sample grown in the presence of dihomo-γ-linolenic acid (20:3), which selectively scanned for masses corresponding to potentially modified PLs. The chromatogram labeled 2 is the phospholipid region of the fullscan chromatogram of the same sample. The peak chosen for analysis is labeled A on both chromatograms.
Figure 3.13. The mass spectrum labeled 1 is the average mass spectrum of the peak labeled A scanned under SIR conditions. The mass spectrum labeled 2 is the average mass spectrum of the peak labeled A scanned under fullscan parameters.
Figure 3.14 shows two chromatograms of the sample of *A. salmonicida* grown in the presence of arachidonic acid (20:4). The chromatogram labeled 1 is a SIR scan of the sample, and the chromatogram labeled 2 is from a full scan of the same sample. The same chromatographic peak at 7.03 minutes is labeled A in both chromatograms. Figure 3.15 shows the average mass spectra corresponding to the chromatographic peak A for both the SIR scan and full scan. The mass spectrum labeled 1 was taken from the SIR scan and the mass spectrum labeled 2 was taken from the full scan. Further analysis will only refer to mass spectrum 2. The peak found at 795.5 m/z corresponds to the [M-H]- value of PG 18:1/20:4, as confirmed by the LIPID MAPS database. Additionally, a cone fragment corresponding to 20:4 can be found at 303.2 m/z. While not visible in the mass spectrum in Figure 3.14, a small peak at 281.2 m/z was found by use of the MassLynx software, corresponding to a cone fragment of 18:1.
Figure 3.14. Two chromatograms of the A. salmonicida sample grown in the presence of arachidonic acid (20:4). The chromatogram labeled 1 comes from an SIR scan conducted that exclusively scanned m/z values that correspond to possible modified PLs. The chromatogram labeled 2 is the PL region examined under fullscan parameters. The mass spectra of the peaks labeled A are shown in Figure 3.14.
Figure 3.15. The average mass spectra of the peaks at approximately 7.03 minutes from the 20:3 sample. The mass spectrum labeled 1 corresponds to that taken from the SIR scan, and the mass spectrum labeled 2 corresponds to that taken from the scan conducted with fullscan parameters.
Figure 3.16 shows the phospholipid region of the chromatogram of the *A. salmonicida* sample grown in the presence of eicosapentaenoic acid (20:5). The average mass spectrum of the chromatographic peak at 7.00 minutes, labeled A, is shown in Figure 3.17. The mass spectrum peak at 708.5 m/z corresponds to the [M-H]⁻ of PE 14:0/20:5, which was confirmed by referencing the LIPID MAPS database. Further confirmation of this identity can be established by the cone fragments at 227.2 m/z and 301.2 m/z, which correspond to 14:0 and 20:5, respectively.

The mass spectrum in Figure 3.17 shows several additional peaks that do not correspond to any fragmentation of PE 14:0/20:5. Specifically, the peak at 733.5 m/z could correspond to the [M-H]⁻ of PG 16:0/cyclo17:0 (cy17:0), as confirmed by the LIPID MAPS database. This would also explain the presence of a cone fragment at 255.2 m/z, which corresponds to 16:0. Additionally, the peak at 465.2 m/z corresponds to the neutral loss of of the sn-2 acyl chain (cy17:0) as a ketene, specifically corresponding to the m/z value of [M-H-R₂-CH=CH=O]⁻. Although not labeled in the mass spectrum in Figure 3.17, a cone fragment of 267.2 m/z was found, corresponding to cy17:0. This demonstrates the slight overlap in elution of PGs and PEs that occurs at around seven minutes.
Figure 3.16. Chromatogram of the A. salmonicida sample grown in the presence of eicosapentaenoic acid (20:5), only the phospholipid region is shown. The peak labeled A is at 7.00 minutes.
Figure 3.17. Average mass spectrum of the chromatographic peak labeled A in Figure 3.15 (7.00 minutes)
Figure 3.18 shows the phospholipid region of the chromatogram from the full scan run on the *A. salmonicida* sample grown in the presence of 300-μM of docosahexaenoic acid (22:6). The chromatographic peak of interest at 8.12 minutes is labeled A, and the average mass spectrum of this peak is shown in Figure 3.19. The peak in the mass spectrum at 762.5 m/z corresponds to the [M-H]- ion of PE 16:0/22:6, confirmed by consulting the LIPID MAPS database. Further confirmation of this identity can be established by the presence of cone fragments at 255.2 m/z and 327.2 m/z, which correspond to 16:0 and 22:6, respectively. Additionally, the peak at 714.5 m/z corresponds to the [M-H]- ion of PE 16:1/18:1, confirmed via the LIPID MAPS database. This explains the presence of a peak at 253.2 m/z, which corresponds to 16:1. Additionally, a small peak at 281.2 m/z was observed in the MassLynx software, which corresponds to 18:1, further confirming the identity of 714.5 m/z as PE 16:1/18:1.
Figure 3.18 Chromatogram of the A. salmonicida sample grown in the presence of docosahexaenoic acid (22:6), only the phospholipid region is shown. The average mass spectrum of the chromatographic peak at 8.12 minutes (labeled A) is shown in Figure 3.18.
Figure 3.19 The average mass spectrum corresponding to the chromatographic peak labeled A from the A. salmonicida sample grown in the presence of 22:6
3.4 UPLC-MS Analysis of Fatty Acids

The linoleic acid (18:2) and docosahexaenoic acid (22:6) utilized to supplement samples of *A. salmonicida* were analyzed by UPLC-MS according to the procedure detailed in Section 2.4.4 to create calibration curves correlating chromatographic peak area to concentration of free fatty acid. QuanLynx software was utilized to determine peak areas and create a linear regression of the data. Figure 3.20 shows the calibration curve of 18:2.

![Figure 3.20 Calibration curve of 18:2 correlating chromatographic peak area to concentration. The $R^2$ value is 0.998.](image)

To avoid redundancy, the calibration curve of 22:6 is omitted, but the linear regression and correlation value are given in Equation 1.

$$y = 5.04E+06x - 1.06E+06 \quad R^2 = 9.98E-01$$  \hspace{1cm} \text{Equation (1)}

Both calibration curves have $R^2$ values greater than 0.99, indicating a strong linear relationship between concentration and chromatographic peak area for both fatty acids.
3.5 Growth Curves

Growth curves of *A. salmonicida* in either the presence or absence of 300 μM of one of the seven PUFAs were determined at both 28°C and 23°C. The growth procedure is outlined in more detail in Section 2.5. The growth curve conducted at 28°C is shown in Figure 3.21, and the growth curve conducted at 23°C is shown in Figure 3.22. The presence of 300 μM of one of the seven PUFAs did not cause any significant increase or decrease in growth rate compared to the control sample grown without PUFA supplementation. However, temperature did affect growth rates as the samples grown at 28°C were found to grow more quickly than the samples grown at 23°C. For this reason, growth of *A. salmonicida* for subsequent assays was conducted at 28°C.
Figure 3.21 Growth curve of A. salmonicida conducted at 28°C
Figure 3.22 Growth curve of A. salmonicida conducted at 23°C
3.6 Biofilm Results

Biofilm formation of *A. salmonicida* either in the presence or absence of 300-μM of one of the seven PUFAs was tested by a method outlined by O'Toole and described in Section 2.6. Biofilms form when communities of microbes attach to surfaces and are distinctive in their resistance to antimicrobials. Biofilms were stained with CV and analyzed via UV-Vis at 590-nm. Figure 3.23 shows the results of biofilm formation. Student’s t-test (two tailed, paired) was used to determine significant (P < 0.01) differences in biofilm formation from the control sample (grown without PUFA supplementation). It was found that the sample grown in the presence of 18:2 and the sample grown in the presence of 22:6 produced significantly lower amounts of biofilm than the control sample.

![Figure 3.23 Biofilm formation of A. salmonicida, standard deviations are represented by error bars, and asterisks indicate samples that varied significantly (P < 0.01) compared to the control sample](image)

---

*Figure 3.23 Biofilm formation of A. salmonicida, standard deviations are represented by error bars, and asterisks indicate samples that varied significantly (P < 0.01) compared to the control sample*
3.7 Permeability Results
Membrane permeability of *A. salmonicida* was tested by measuring the uptake of the hydrophobic compound CV as described in Section 2.7. Figure 3.24 shows the results of this assay, with samples that varied significantly from the control sample (grown without supplementation of PUFAs) marked by an asterisk (*). Significance (P < 0.001) was established by Student’s t-test (two tailed, paired). It was found that samples grown in the presence of 18:3γ, 20:4, and 22:6 exhibited significantly more uptake of CV than the control sample.

*Figure 3.24 Results of permeability assay via CV uptake, samples that varied significantly (P < 0.001) from the control sample are marked with an asterisk (*)*
3.8 Polymyxin B Susceptibility Results

Susceptibility of *A. salmonicida* samples to an antimicrobial peptide (AMP) was tested by exposure to Polymyxin B (PMB) at two-fold increasing concentrations according to the procedure described in Section 2.8. Figure 3.25 shows the results of PMB susceptibility testing. Samples that varied significantly from the control sample are circled with a dashed line. Significance (*P* < 0.001) was established by Student’s *t*-test (two-tailed, paired). The samples grown in the presence of 20:5 and 22:6 showed twofold protection, and the sample grown in the presence of 18:3γ showed fourfold protection against PMB compared to the control.

![Figure 3.25 Results of Polymyxin B susceptibility assay, samples that varied significantly (*P* < 0.001) from the control are circled with a dashed line](image-url)
3.9 Bioinformatics Results

The results of utilizing the BLAST tool to compare known FadD, FadL, PlsB, PlsC, ad PlsX genes of *E. coli* (MG1655) are shown in table 3.1. All homologs listed had a score of greater than 200. All homologs correspond to *A. salmonicida subsp. salmonicida* (A449).

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Chapter 4: Discussion

4.1 Discussion

*A. salmonicida* is a Gram-negative fish pathogen that can have devastating effects on cultivated fish populations.\textsuperscript{22, 23, 26, 31} Currently, several methods are employed to keep infections of *A. salmonicida* at a minimum, including the use of a vaccine, selective breeding, and the use of antibiotics in case infections do arise.\textsuperscript{22, 32, 33} However, vaccination is a limited method and has been shown to have some adverse effects.\textsuperscript{34} Additionally, the efficacy of vaccination of non-salmonids has yet to be explored, and *A. salmonicida* often displays antibiotic resistance.\textsuperscript{26, 35} Thus, the need for novel treatments of *A. salmonicida* infections still exists. Previous research conducted on Gram-negative bacteria has shown certain species capable of incorporating polyunsaturated fatty acids (PUFAs) into their lipodomes, specifically as acyl chains in membrane phospholipids (PLs). These studies also found subsequent alteration of biological functions, such as motility, permeability, biofilm formation, and antimicrobial susceptibility.\textsuperscript{9-12} The purpose of this project was to explore the ability of *A. salmonicida* to incorporate PUFAs into membrane PLs and determine any biological effects thereof, potentially in the light of future therapeutic efforts against furunculosis infections.

The first part of this project consisted of confirming that *A. salmonicida* was capable of assimilating PUFAs into its PL profile. This was done by analyzing lipids extracted from *A. salmonicida* cultures grown at 23°C either in the presence of one of the seven PUFAs of interest at a concentration of 300-μM or without PUFA supplementation. The PUFAs used in this study were linoleic acid (18:20), α-linolenic acid (18:3α), γ-linolenic acid (18:3γ), dihomo-γ-linolenic acid (20:3), arachidonic acid (20:4), eicosapentaenoic acid (20:5), and
docosahexaenoic acid (22:6). One set of lipid extracts was analyzed by thin-layer chromatography (TLC), the results of which are shown in section 3.2. The presence of splitting can be seen in samples grown in the presence of one of the PUFAs that is not present in the control sample, indicating that the PL profile has been altered, likely by incorporation of PUFAs.

While TLC offered proof of concept, ultra performance liquid chromatography and tandem mass spectrometry (UPLC-MS) was utilized for more thorough analysis of the PL profile of *A. salmonicida* samples, though it was still only used as a qualitative measure rather than quantitative. Gradient elution on a BEH C-8 column, explained in section 2.4.2, allowed for chromatographic separation of lipid species, resulting in distinctive free fatty acid and PL regions. Additionally, chromatography resulted in almost, though not entirely, distinct phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) regions. This is to be expected as certain combinations of headgroup and acyl chain compositions can result in identical (or nearly so) polarities which can be observed as chromatographic co-elution. For example, even though PG has a more polar headgroup than PE, if the acyl chains attached to PG are extremely hydrophobic (e.g. long-chain, fully saturated), the polarities of that species, and therefore the retention time, could be the same as that of a PE species containing less hydrophobic acyl chains (e.g. shorter-chain, unsaturated).

PL identities were elucidated by mass spectrometry analysis conducted in negative electrospray ionization mode (ESI (-)). By scanning in ESI (-), fragmented acyl chains from PLs, which cleaved as a result of the cone voltage (50-V) as carboxylate ions, were able to be detected and assist in identification of PLs. Section 3.3 shows the identification process of PLs by mass spectrometry analysis, which was assisted by the use of the LIPID MAPS
database. *A. salmonicida* was shown to be capable of incorporating all seven PUFAs into its lipid profile.

Phospholipids were identified by their (PX sn-1/sn-2) notation, where PX is the identity of the headgroup, and sn-1 and sn-2 are the identities of the acyl chains (carbons: number of double bonds) at the sn-1 and sn-2 positions. It should be noted that acyl chains on the sn-1 position are usually saturated or monounsaturated, compared to the sn-2 position, which has an established preference for unsaturated chains. Additionally, studies conducted on the analysis of PGs and PEs have found preferential loss of the sn-2 acyl chain in triple quadrupole mass spectrometry analysis also conducted in ESI (−) mode. This results in a higher signal of the cone fragment corresponding to the sn-2 chain than that of the sn-1 chain, though this is not always observed. The fact that the sn-2 position preferentially takes saturated fatty acids coupled with the observed intensities of the cone fragments influenced the order in which the acyl chains were reported in the PLs identified in Section 3.3.

One potential interference with the use of cone fragment intensity in specifying the identity of the sn-1 and sn-2 positions is the sensitivity of the fatty acids. The linoleic acid (18:2) and docosahexaenoic acid (22:6) utilized to supplement samples of *A. salmonicida* were analyzed by UPLC-MS and used to create calibration curves ranging in concentration from 0.5- to 5-ppm. Both fatty acids yielded strong correlation values ($R^2 > 0.99$), but 18:2 showed greater than ten times more sensitivity than 22:6, as seen by the values of the slopes (m) of the calibration curves ($m_{18:2} = 5.04 \times 10^6$, $m_{22:6} = 4.72 \times 10^5$). This is likely due to increased fragmentation of the more highly unsaturated fatty acids. This also explains the lower peaks corresponding to free fatty acids of more highly unsaturated PUFAs as
compared to more saturated PUFAs, as signal of the [M-H]· ion is lost to fragmentation. Chromatograms and mass spectra demonstrating this fragmentation and lower signal can be found in supplemental material S1.

Selected ion recording (SIR) scans were also performed on lipid extractions of all A. salmonicida samples that were grown in the presence of one of the seven PUFAs. This mass spectrometry technique selectively scans for specified m/z values. For each of the seven samples, SIR scans were designed with ten m/z values, which are given in Table 2.1. Eight of those values corresponded to the m/z values of the [M-H]· ions of the most probable substituted PLs, and the other two m/z values corresponded to the [M-H]· ions of a doubly substituted PG and PE. SIR scans allow for increased sensitivity, which can be particularly useful as modified PLs are lower abundance than naturally occurring PLs. However, SIR scans do not allow for the same positive identification of PLs as full scan analysis does, because cone fragments are not scanned for. Additionally, certain m/z values can correspond to other PLs that are not of interest. For example, 714.5 m/z corresponds to the [M-H]· of PE 16:0/18:2, but it also corresponds to the [M-H]· of PE 16:1/18:1, which could occur natively. Another such example is that 743.5 m/z corresponds to PG 16:0/18:3 (for both 18:3α and 18:3γ), but it also corresponds to the [M+1-H]· of PE 18:1/18:1, which could occur natively. Thus, SIR scans cannot be used by themselves to identify phospholipids, but they can be useful in identifying chromatographic peaks of modified phospholipids that may have otherwise been lost to noise under full scan parameters. As shown in the analysis conducted in section 3.3, once chromatographic peaks of interest have been identified with SIR scans, the mass spectrum corresponding to the same
chromatographic region of the equivalent full scan run can be analyzed for fragmentation that confirms the identity of the lipid of interest.

After confirmation of PUFA incorporation via TLC and UPLC-MS analysis was established, a series of assays were conducted to test the effect of PUFAs on growth, biofilm formation, permeability, and susceptibility to polymyxin B (PMB). Growth curves of *A. salmonicida* were conducted at 23°C and 28°C according to the procedure detailed in Section 2.5. Optimal growth temperatures for *A. salmonicida* are reported to be between 18°C and 26°C, with little growth at temperatures as low as 4°C and typically no growth seen at 37°C. The presence of PUFAs did not have any significant effect on growth of *A. salmonicida* at either temperature. It was found that *A. salmonicida* samples grown at 28°C grew at a quicker rate than those grown at 23°C up until the eighth hour of growth, when both sets approach the stationary phase of growth. This was unexpected, considering 28°C is above reported optimal temperatures for *A. salmonicida* growth. Because *A. salmonicida* grew more quickly at 28°C, this temperature was used for growth in all subsequent assays.

The formation of biofilm was tested by a method designed by O'Toole and outlined in Section 2.6. Biofilms are communities of microorganisms that are attached to a surface, such that they cannot be removed by gentle rinsing. Biofilms are often notable for their antimicrobial resistance as compared to planktonic microbes. The biofilm assay conducted on *A. salmonicida* in either the presence of one of the seven PUFAs or without PUFA supplementation showed significantly (*P* < 0.01) lower production of biofilm by the cultures supplemented with 18:2 and 22:6 compared to the control culture. However, some of the standard deviations were quite large, such as 22.6% for the 18:3γ sample and 21.6% for the 20:3 sample. It was shown that biofilm formation can be affected significantly by
PUFAs as with the 18:2 and 22:6 samples, but further testing should be conducted before settling on conclusive results of biofilm testing due to the high deviations seen in some samples.

Membrane permeability was tested by measuring uptake of the hydrophobic compound crystal violet (CV) by the procedure described in Section 2.7. Membrane permeability can have a major impact on bacteria, as it determines the passage of molecules between the cell and its environment. Membrane permeability has been linked to functions such as antibiotic resistance. The results of permeability testing showed that the A. salmonicida samples grown in the presence of 18:3γ, 20:4 and 22:6 showed significantly (P<0.001) greater permeability than the control sample. The increase in double bonds and specifically the cis-configuration of the bonds could contribute to the increased permeability, though this does not explain why only those three PUFAs affected permeability significantly.

Antibiotic resistance is a major concern in regard to A. salmonicida. Antimicrobial peptides (AMPs) are a class of antibiotics that specifically target the bacterial membrane and are promising in that the use of AMPs is less likely to lead to resistance than traditional antimicrobials. Because AMPs target the bacterial membrane, membrane modification caused by the incorporation of exogenous PUFAs could affect the efficacy of AMPs. The susceptibility of A. salmonicida samples grown in the presence of one of the seven PUFAs or without PUFA supplementation to the AMP Polymyxin B (PMB) was tested according to the procedure outlined in Section 2.8. It was found that cultures grown in the presence of 20:5 and 22:6 had two-fold minimum inhibitory concentration (MIC) protection against PMB as compared to the control sample (P<0.001). Additionally,
cultures grown in the presence of 18:3γ showed fourfold MIC protection against PMB compared to the control sample (P<0.001). None of the PUFAs studied increased the susceptibility of *A. salmonicida* to PMB.

The increased permeability caused by 18:3γ, 20:4, and 22:6 would cause one to expect increased susceptibility to an AMP as it specifically targets the bacterial membrane. PMB specifically functions by altering membrane permeability by binding to a negatively charged site in the lipopolysaccharide layer.\(^{40}\) However, because of the structure of Gram-negative membranes, PMB must permeabilize both the outer and inner membranes, and the barrier posed by the inner membrane often takes longer to permeabilize.\(^{39}\) It is possible that the modification allowed for increased permeability of hydrophobic compounds such as CV, while providing some form of protection against AMPs due to mechanistic differences by which the molecules permeate the membrane.

The incorporation of exogenous PUFAs into membrane lipids in the model organism *E. coli* has been connected to an outer membrane transporter of long-chain fatty acids (FadL), an acyl-CoA synthetase (FadD), and acyltransferases (PlsB, PlsC, PlsX, and PlsY).\(^{9,13,14,41}\) Previous research has found that the presence of homologs of these genes in other species can indicate their capacity for membrane modification.\(^{9,10}\) The use of the National Center of Biotechnology Information’s Basic Local Alignment Search Tool (BLAST) revealed that *A. salmonicida* subsp. *salmonicida* (A449) has two FadL homologs, two FadL homologs, and four homologs corresponding to PlsB, PlsC, PlsX, and PlsY. These homologs are given in Table 3.1. The identification of these homologs further confirms that *A. salmonicida* should be capable of membrane modification by exogenous PUFA incorporation, which has been shown by this research.
Interestingly, *A. salmonicida* samples grown in the presence of 22:6 showed significant deviations from the control sample in biofilm formation, permeability, and antimicrobial resistance. Docosahexaenoic acid is an ω-3 fatty acid that is extremely prevalent in fish. Because *A. salmonicida* is a fish pathogen, it is possible that it has developed a specific affinity for 22:6.

4.2 Future work

Future work to be conducted on *A. salmonicida* includes revisiting the effects of PUFAs on biofilm formation to be able to draw stronger conclusions. Additionally, the effect of temperature on growth could continue to be explored to see at what temperature the growth rate of *A. salmonicida* declines. Additionally, the fatty acid calibration curves created could be used in first attempts at quantitative analysis of modified lipids by quantifying the amount of free fatty acid that elutes in a sample and referencing the original 300-μM concentration of the fatty acid during growth to determine how much was used for incorporation by difference. Future projects could also see if similar Gram-negative aquatic pathogens experience as much phenotypic alteration following incorporation of 22:6 as *A. salmonicida*. 
References


## Supplemental Material

### Table S.1 Masses of *A. salmonicida* Dried Lipid Extracts

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mass of empty LC vial (g)</th>
<th>Mass of LC Vial with Dried Lipid Extract (g)</th>
<th>Average Mass of Dried Lipid Extract (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. salmonicida</em> (-)</td>
<td>2.55094</td>
<td>2.55151</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.55094</td>
<td>2.55150</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.55091</td>
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<td><em>A. salmonicida</em> 18:2</td>
<td>2.59898</td>
<td>2.60002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.59893</td>
<td>2.60001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.59892</td>
<td>2.60003</td>
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</tr>
<tr>
<td><em>A. salmonicida</em> 18:3α</td>
<td>2.51958</td>
<td>2.52026</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.51956</td>
<td>2.52022</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.51955</td>
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<tr>
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<td>2.55526</td>
<td>2.55616</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.55524</td>
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<td></td>
</tr>
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<td></td>
<td>2.55525</td>
<td>2.55614</td>
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<td>2.60128</td>
<td>2.60291</td>
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<tr>
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<td>2.60124</td>
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<td></td>
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<td></td>
<td>2.52410</td>
<td>2.52484</td>
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<td><em>A. salmonicida</em> 20:5</td>
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<tr>
<td></td>
<td>2.59007</td>
<td>2.59109</td>
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<tr>
<td></td>
<td>2.59005</td>
<td>2.59112</td>
<td>1.04</td>
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<td><em>A. salmonicida</em> 22:6</td>
<td>2.55992</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>2.55989</td>
<td>2.56044</td>
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<tr>
<td></td>
<td>2.55992</td>
<td>2.56041</td>
<td>0.52</td>
</tr>
</tbody>
</table>
Figure S.1 Chromatogram of 5 ppm 18:2 sample, peak at 1.87 minutes corresponds to elution of the fatty acid.

Figure S.2 Chromatogram of 5 ppm 22:6 sample, peak at 1.81 minutes corresponds to elution of the fatty acid.
Figure S.3 Overlaid chromatograms of 5 ppm 18:2 (red) and 5 ppm 22:6 (green), vertical axis is linked to show the greater intensity of the 18:2 peak, even though both samples were sized to 5 ppm.

Figure S.4 Average mass spectrum of the chromatographic peak at 1.87 minutes of the 5 ppm 18:2 sample. Peak at 279.2 m/z corresponds to [M-H]- carboxylate ion of 18:2 and peak at 280.2 m/z corresponds to the M+1 isotope of 18:2 carboxylate ion.
5 ppm 22:6 in 50:50; Fullscan

062518_5ppm_FA_22_6_NEG_01 174 (1.814) Cm (171:176)

Scan ES-4.32e6

Figure S.5 Average mass spectrum of the chromatographic peak at 1.81 minutes of the 5 ppm sample of 22:6. The peak at 327.2 m/z corresponds to the carboxylate ion of 22:6. The peak at 283.2 m/z corresponds to the [M-H-C=O-O] fragment of 22:6. The overall increase in fragmentation compared to 18:2 could explain the lower intensity of the 22:6 sample.