Polyunsaturated fatty acids cause physiological and behavioral changes in *Vibrio alginolyticus* and *Vibrio fischeri*

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Abstract

Vibrio alginolyticus and *Vibrio (Aliivibrio) fischeri* are Gram-negative bacteria found globally in marine environments. During the past decade, studies have shown that certain Gram-negative bacteria, including *Vibrio* species (*cholerae*, *parahaemolyticus*, and *vulnificus*) are capable of using exogenous polyunsaturated fatty acids (PUFAs) to modify the phospholipids of their membrane. Moreover, exposure to exogenous PUFAs has been shown to affect certain phenotypes that are important factors of virulence. The purpose of this study was to investigate whether *V. alginolyticus* and *V. fischeri* are capable of responding to exogenous PUFAs by remodeling their membrane phospholipids and/or altering behaviors associated with virulence. Thin-layer chromatography (TLC) analyses and ultra-performance liquid chromatography-electrospray ionization mass spectrometry (UPLC/ESI-MS) confirmed incorporation of all PUFAs into membrane phosphatidylglycerol and phosphatidylethanolamine. Several growth phenotypes were identified when individual fatty acids were supplied in minimal media and as sole carbon sources. Interestingly, several PUFAs acids inhibited growth of *V. fischeri*. Significant alterations to membrane permeability were observed depending on fatty acid supplemented. Strikingly, arachidonic acid (20:4) reduced membrane permeability by approximately 35% in both *V. alginolyticus* and *V. fischeri*. Biofilm assays indicated that fatty acid influence was dependent on media composition and temperature. All fatty acids caused decreased swimming motility in *V. alginolyticus*, while only linoleic acid (18:2) significantly increased swimming motility in *V. fischeri*. In summary, exogenous fatty acids cause a variety of changes in *V. alginolyticus* and *V. fischeri*, thus adding these bacteria to a growing list of Gram-negatives that exhibit versatility in fatty acid utilization and

highlighting the potential for environmental PUFAs to influence phenotypes associated with planktonic, beneficial, and pathogenic associations.

Keywords: *Vibrio*, *Aliivibrio fischeri*, *Vibrio alginolyticus*, biofilm, fatty acids, motility, phospholipids

Introduction

Vibrio alginolyticus and *Vibrio fischeri* are Gram-negative aquatic bacteria, each possessing unique physiological characteristics, and environmental associations. *V. alginolyticus* is a medically important bacteria known for causing diseases in marine vertebrates and invertebrates, as well as being responsible for wound and ear infections (Kahla-Nakbi et al., 2009; Chang et al., 2008; Pezzlo et al., 1979). As with other *Vibrio* species, the emergence of antibiotic resistance has complicated the prevention and treatment of *V. alginolyticus* infections. *V. fischeri* is known to have a non-obligatory symbiotic relationship with the bobtail squid *Euprymna scolopes*. This unique relationship is thought to provide safe, nutritious shelter for *V. fischeri,* while the bacteria's bioluminescence deters predation of the squid while causing a multitude of metabolic changes in the invertebrate (Stabb et al., 2007; Ruby & Lee, 1998; Lyell et al., 2008; McFall-Ngai, 2014; Kohl & Carey, 2019; Koch et al., 2020). The dynamics of strain-specific ecological and evolutionary adaptations observed in *V. fischeri* have guided recent research avenues (Bongrand & Ruby, 2019).

Previous research has shown that Gram-negative bacteria such as *Escherichia coli, Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Vibrio*

species are capable of acquiring exogenous PUFAs for both membrane phospholipid incorporation and behavioral modification (Moravec et al., 2017; Baker et al., 2018; Hobby et al., 2019; Eder et al., 2017; Herndon et al., 2020). Membrane remodeling in Gram-negative bacteria, first described in *E. coli*, can originate from the import of fatty acids through the long-chain fatty acid transporter FadL, followed by activation by the long-chain fatty acyl-CoA synthetase FadD, and subsequent assembly into phospholipids by membrane-associated acyltransferases (PlsB/C/X/Y) (Parsons & Rock, 2013). Several Gram-negative bacteria possess multiple homologs of these fatty acid-handling proteins, suggesting a broader impact of fatty acid sensing and utilization capabilities. Other identified fatty acid-mediated effects involve changes in phenotypes associated with virulence, such as biofilm formation and motility. The current study seeks to examine two additional *Vibrio* species to elucidate the extent of their exogenous fatty acid utilization on membrane phospholipid assimilation and phenotypic responses.

The exogenous fatty acid responses of *V. alginolyticus* and *V. fischeri* have not been studied previously, although the observation of squid-associated *V. fischeri* adopting a PUFA-containing fatty acid profile introduced the possibility of PUFA acquisition and assimilation (Wier et al., 2010). As in previous analyses, thin-layer chromatography and UPLC/ESI-MS were used to examine and demonstrate the exogenous fatty acid incorporation into membrane phospholipids. The growth of both species was affected when individual PUFAs were supplemented into media or used as sole carbon sources. Hydrophobic compound uptake assays indicated changes to membrane permeability while swimming motility and biofilm production were differentially affected by several fatty

acids. Importantly, the remodeling of membrane phospholipids with individual fatty acids resulted in dramatic shifts in the MICs to polymyxin B and colistin.

Materials and Methods

Bacterial strains and growth conditions. *Vibrio alginolyticus* Z096 (clinical isolate, East Tennessee State University Clinical Lab (Giles et al., 2011; Pride et al., 2013)) and *Aliivibrio fischeri* ATCC 700601 were used in this study. CM9 (0.4% casamino acids,0.4% glucose, supplemented with 342 mM NaCl) was used for the growth of bacteria in experiments, while sole carbon source experiments were performed in glucose-deficient M9 minimal media. All experiments, except growth curves, were performed with a starting inoculum of OD 0.1. Fatty acids used in this study were purchased from Cayman Chemicals and administered at a concentration of 300 μM for each experiment, except for sole carbon source growth experiments, wherein they were dosed with 1mM.

Growth curves. *V. alginolyticus* and *V. fischeri* were grown in CM9 minimal media at 28°C in the presence and absence of 300μM fatty acid (starting OD = 0.05). *V. alginolyticus* was also grown at 37°C. Absorbances (600nm) were recorded each hour for 12 hours. All growth experiments were performed at least twice.

Bacterial lipid extraction and thin-layer chromatography. Phospholipid extraction, using 14ml of bacterial culture, followed the method of Bligh and Dyer (1957). Lipids were separated on Silica Gel 60 TLC plates in a solvent system of chloroform, methanol, and

acetic acid (65:25:10 v/v/v). Plates were visualized using an aerosol solution of sulfuric acid and 100% ethanol (1:10 v/v), followed by heating to 150°C for 1-2 min. A Canon CanoScan 9000F scanner was used to acquire the final image.

Ultra performance liquid chromatography/ESI-mass spectrometry. Cultures (20 ml) were grown in the presence or absence of 300 μM of each PUFA prior to extraction of phospholipids. Lipid extracts were dried under nitrogen gas, massed using an analytical balance, and resuspended to yield a 400 ppm (total lipid) sample. Diluent consisted of 3.75 mM ammonium acetate in 15:85 H_2O:CH_3OH. All reagents were Optima LC-MS grade (Fisher Scientific). Chromatographic separation was accomplished using reversed-phase gradient elution, from a 5uL injection, on an ACQUITY UPLC system (Waters, Milford, MA) equipped with a BEH C18 column (2.1 x 100 mm; 1.7 μm particles). Mobile phase A was 10 mM ammonium acetate in 90:10 H_2O:CH_3OH and B consisted of 0.01% formic acid in CH_3OH. The gradient began at 60% B for 0.5 min hold, then ramped to 95% B by 2 minutes which was held for 7 minutes. Initial conditions returned by 9.2 minutes and held for 0.8 min to a re-equilibrate column. The mobile phase flow rate was 0.4 ml/min. Detection was done using quadrupole mass spectrometry (Quattro Micro; Waters, Milford, MA) following electrospray ionization in the negative mode. In this mode, phosphatidylethanolamine (PE) species have even m/z and phosphatidylglycerol (PG) species have odd m/z. The scan range was 200 – 800 m/z and the scan time was 0.85 sec. The mass spectrometer used the following conditions: 1.5 kV capillary; 50 V cone; 130°C source; 375°C desolvation using dry nitrogen flowing at 750 L/hr. These temperatures and voltages were sufficient to generate cone fragments derived by

cleavage of the fatty acyl chains from the sn-1 and sn-2 positions of the phospholipids. This method allows unambiguous determination of phospholipid identity - including fatty acyl composition - as illustrated in Appendix Figure A1.

Crystal violet uptake assay. *Vibrio spp.* were grown in 7 ml of CM9 media in the presence and absence of 300 μM each PUFA to logarithmic phase. Cultures were pelleted (2,500 x g, 10 m), washed, and resuspended in phosphate-buffered saline (PBS) at an OD of 0.7. Crystal violet (5 μg/ml) was added to the cells and cultures were gently agitated (50 rpm). Every 5 min, 1 ml was removed, pelleted, and the supernatant was measured (OD_{590}). For each assay, a control (containing CV but no bacteria) permitted normalization of the data. Microsoft Excel was used to convert the amount of dye excluded into a percentage uptake. Three biological replicates were performed and all standard deviations were less than 5%. The Student's t-test (paired, 2-tailed, $p < 0.02$) was used to determine statistical significance.

MIC assays. *Vibrio spp.* were grown in CM9 minimal media (-/+ individual PUFAs) to OD ~ 0.8. Bacterial inoculum constituted 170 μl and contained fatty acid for a final concentration of 300 μM. Two-fold concentrations of each cyclic peptide and beta-lactam antibiotic (30 μl) were administered for a total of 200 μl per well (bacterial starting OD_{600} = 0.1). Following incubation at 37°C for 24 h, a Biotek Synergy microplate reader was used to record absorbances at 600 nm. Two independent experiments were performed in triplicate and the Students t-Test (2-tailed, paired) calculated *p*-values.

Biofilm assay. The amount of biofilm formation was assessed using the protocol by O'Toole (2011). Briefly, *Vibrio spp.* were grown to exponential phase at 28ºC in CM9, and cultures were prepared at an OD of 0.1 in 96-well microtiter plates containing CM9 minimal media or Marine broth supplemented with or without each fatty acid. Following static incubation (48h for CM9; 24h for Marine broth) at 28ºC, planktonic cells were removed and the plates were gently washed three times with dH_2O. A 3% crystal violet solution was added and plates were incubated for 15 min at room temperature. Plates were triple rinsed with dH_2O and allowed to dry. The addition of 30% acetic acid, incubation for 15 min, and transfer of the solution to a new microtiter plate preceded absorbance reading at 590 nm using a Biotek Synergy microplate reader. At least two independent experiments were performed in octuplet and the Students t-Test (2-tailed, paired) generated *p*-values.

Results

Growth characteristics of *V. alginolyticus and V. fischeri* in the presence of exogenous PUFAs. In their respective environments, *V. alginolyticus* and *V. fischeri* thrive in a variety of niches, ranging from free-living to beneficial and/or pathogenic symbiotic associations. For these diverse interactions, fatty acids represent potential external molecules that could be utilized for survival and persistence. The presence of PUFAs has been established in tissues that serve as infection sites for *V. alginolyticus* (Ansari et al., 1970; Harvey, 1989; Saito, 2014; Rueda et al., 2001), as well as native fatty acids for the known symbiont of *V. fischeri* (Wier et al., 2010). The effects of fatty acids

on growth were assessed by administering a physiologically relevant concentration of fatty acid (300 μM) during growth of the *Vibrio* species at 28°C in M9 minimal media supplemented with casamino acids (CM9). In *V. fischeri,* the same two fatty acids (18:2 and 20:3) supported growth regardless of carbon source supplementation (Figure 1a & b). Docosahexaenoic acid as a sole carbon source also elicited a spike in growth during the first 3 hours (Figure 1b). Interestingly, most PUFAs (18:3α, 18:3γ, 20:4, 20:5, and 22:6) disallowed growth over the 12 hours of monitoring. All fatty acids supported the growth of *V. alginolyticus*, with 20:3 causing higher overall growth during the latter half of the curve (Figure 1c). Only 3 PUFAs (18:2, 20:3, and 22:6) supported growth as sole carbon sources, with 22:6 causing a similar spike initially (Figure 1d). When *V. alginolyticus* was grown at 37°C in CM9 (150mM NaCl) there was a significant delay in growth with most fatty acids, while 18:2 and 20:3 supported similar growth to control (Figure 1e). In the absence of a carbon source, 18:2 and 20:3 allowed the growth of *V. alginolyticus* at an earlier stage than compared to 28°C (Figure 1f).

Growth with PUFAs yields altered phospholipid profiles of *V. alginolyticus* and *V. fischeri*. To evaluate the potential for incorporation of exogenous fatty acids into membrane phospholipids, bacteria were grown with or without fatty acid. Bacterial phospholipids were extracted and separated using thin-layer chromatography (TLC). The major phospholipids produced by *Vibrio* species include phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL) (Figure 2). The chromatogram of *V. alginolyticus* revealed species that migrated higher on the plate, a pattern expected with increasing carbon number and unsaturation of the exogenously acquired fatty acid. This

effect is particularly noticeable as double bonds are introduced (eg, from 18:2 to 18:3). The chromatogram of *V. fischeri* phospholipids conveys a similar pattern, with more pronounced shifts and appearance of distinctly unique species (see PE for 20:4 and 22:6). 18:3α and 18:3γ are not represented because they prevented the growth of *V. fischeri*.

UPLC/ESI-MS analyses indicate assimilation of exogenous PUFAs into *V. alginolyticus* and *V. fischeri* phospholipids. The qualitative changes observed by TLC (Figure 2) were further investigated using ultra-performance liquid chromatography/electrospray ionization mass spectrometry (UPLC/ESI-MS). Bacterial lipids were extracted (Bligh and Dyer, 1959) following growth in the presence and absence of individual PUFAs and fractionated using reversed-phase gradient elution resulting in good separation between phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), the two major phospholipids (PL) synthesized by *V. alginolyticus* and *V. fischeri*. Analyses of 400 ppm (total lipid extract) samples yielded chromatograms depicting structural changes to phospholipids in a manner consistent with the exogenously supplied fatty acid. Extracted ion chromatograms (XIC) from the fatty acid exposed cultures are shown in Figure 3. The observation of new chromatographic peaks in all samples exposed to fatty acids confirms that phospholipid profiles have been modified compared to the control. The extracted phospholipids identified by chromatography in this study corresponded to each PUFA supplemented in those cultures.

Similar to TLC, LC-MS analyses detected abundant PE and PG species. Mass spectrometry confirms that these phospholipids are composed of fatty acyl chains

originating from the exogenous fatty acid (eg, Appendix 1, Figure A1). Analysis of the $[M-H]^-$ ions and their cone fragments shows that the new peaks correspond to phospholipid species consisting of at least one acyl chain matching the supplied fatty acid. Thus, the phospholipids PE and PG were modified with all of the fatty acids used in this study.

Exogenous PUFAs affect hydrophobic compound uptake in *V. alginolyticus* and *V. fischeri.* After confirming the assimilation of exogenous PUFAs, it was hypothesized that membrane permeability would be affected. Using the hydrophobic compound crystal violet, permeability assays indicated that most PUFAs caused a decrease in membrane permeability (Figure 4). For *V. alginolyticus* and *V. fischeri*, significant differences ($p < 0.02$) were observed for six fatty acids (18:2, 18:3α, 18:3γ, 20:3, 20:4, and 20:5). Only 22:6 failed to elicit significant changes in permeability. Strikingly, 20:4 and 20:5 decreased permeability by over 20% in *V. fischeri*.

Exogenous PUFAs alter cyclic peptide antibiotic, but not beta-lactam, resistance in *V. alginolyticus* and *V. fischeri.* The adoption of new phospholipid species-containing PUFAs inspired evaluation of the antimicrobial effect of membrane-active agents. Specifically, the cyclic peptide antibiotics polymyxin B and colistin were administered to PUFA-treated cultures for MIC determination (Figure 5). In *V. alginolyticus*, the MIC of PMB was decreased by 5 of 7 PUFAs and increased only by 20:4 (Figure 5a). A 4-fold decrease in MIC was observed for the 3 fatty acids possessing 3 unsaturations (18:3α, 18:3γ, and 20:3) (Figure 5a). All PUFAs significantly decreased the MIC of colistin, with

18:3α and 18:3γ eliciting a striking 8-fold decrease in MIC (Figure 5b). In *V. fischeri*, the MIC of PMB was lowered 20-fold by 18:2 and 22:6, with 20:3 displaying no effect (Figure 5c). The only PUFA affecting colistin MIC was 18:2, which lowered the MIC 6-fold (Figure 5d). Neither *Vibrio spp*. experienced altered MICs to imipenem (Figure 5e & f), a beta-lactam antibiotic that relies on protein-mediated passage through the outer membrane. The absence of growth for *V. fischeri* exposed to 18:3α and 18:3γ was expected based upon the growth experiments; however, the lack of growth with 20:4 and 20:5 is presumed due to the elevated incubation temperature.

PUFAs variably impact biofilm formation in *V. alginolyticus* and *V. fischeri*. The formation of biofilms allows bacteria to persist and thrive in both environmental and host niches. For *Vibrio spp*., biofilms confer survival advantages in aquatic associations and have been correlated with the availability of specific nutrients and disease recurrence (Karunasagar & Otta, 1996; Nyholm et al., 2000; Sultana et al., 2018; Kierek & Watnick, 2003). During host infection, biofilms play a role in pathogenicity and complicate treatment (Tamayo et al., 2010; Faruque et al., 2006). A crystal violet-based biofilm assay was used to measure biofilm formation of *V. alginolyticus* and *V. fischeri* during exposure to PUFAs. The assays were performed in both M9 minimal media and Marine broth. The presence of PUFAs generally decreased biofilm production in CM9 for both *Vibrio spp*. (Figure 6). However, 18:3γ caused an increase in *V. alginolyticus*, whereas 18:2 and 20:4 significantly increased biofilm for *V. fischeri*. When grown in Marine broth, several PUFAs elicited a higher amount of biofilm production.

Since *V. alginolyticus* is a human pathogen, we also examined biofilm formation in CM9 containing physiological salt concentration (150mM NaCl) and at 37°C (Appendix Figure 2). The results largely mirrored the response to PUFAs in Marine broth, albeit with less than half of the overall biofilm accumulation.

Most PUFAs decrease motility in *V. alginolyticus* while only 18:2 increases motility in *V. fischeri*. A critical bacterial phenotype for successful survival and host interaction is swimming motility. In *Vibrio spp.*, this key behavior is linked to chemotaxis, biofilm formation, colonization, and virulence (Butler & Camilli, 2005; Yilidiz & Visick, 2009; Kumar et al., 1992; Teschler et al., 2015). *V. alginolyticus*, and likely other Vibrio spp., exhibit a speedy three-step motility process characterized by chemoattractant signaling and flagellar transition between run-reverse and run-reverse-flick patterns (Xie et al., 2011; Son et al., 2013). The unique, lophotrichous, sheathed flagella of *V. fischeri* enables motility that is instrumental for establishing symbiosis with *E. scolopes* (McCarter, 2001; Graf et al., 1994). Swimming motility in *V. alginolyticus* was measured for 96 hours, and the supplementation of all PUFAs tested resulted in lower motility (Figure 7a). A motility assay using *V. fischeri* only identified one fatty acid (18:2) that caused heightened swimming motility after 6 hours of incubation (Figure 7b).

Discussion

This study identified similarities and differences with regard to PUFA-mediated responses in *V. alginolyticus* and *V. fischeri*. PUFAs administered during growth were confirmed by UPLC-ESI-MS to be assimilated into bacterial phospholipids, a membrane modification

that resulted in changes to permeability and resistance to cyclic peptide antibiotics. Motility and biofilm formation were differentially affected depending on supplemented PUFA.

As outlined in previous studies, *Vibrio spp*. appear to be genetically equipped for handling a variety of exogenous fatty acids. Moravec *et al.* assembled a table reflecting the myriad of auxiliary proteins involved in fatty acid recognition, uptake, and incorporation into membrane phospholipids (2017). Among Gram-negative bacteria, bioinformatic analyses have indicated that *Vibrio* and *Aeromonas spp*. possess more homologs to fatty acid transporters, acyl-CoA synthetases, and acyltransferases. Further characterization of this machinery will define underlying mechanisms responsible for diverting exogenous fatty acids toward lipid modification versus beta-oxidation or chemotactic response.

Based on TLC data, the separation pattern for *V. fischeri* indicates more distinct incorporation of fatty acids, as visualized by both, duplication of phospholipid species and disappearance of the lower, *de novo*, species (see extracted PLs for 20:4 and 22:6). UPLC-ESI-MS corroborated these findings by identifying the incorporation of all PUFAs into membrane phospholipids. It is important to note that *V. fischeri* was inoculated at a starting OD of 0.1 for extraction of phospholipids after incubation with PUFAs. For comparison, *V. fischeri* required 4-8 hours of growth in the presence of all PUFAs except both linolenic acids (18:3α and 18:3γ) to achieve an OD~1; it required 18 hours of incubation to reach the desired bacterial yield for these PUFAs. Ongoing studies are examining the lengthy delay in growth observed with most fatty acids in *V. fischeri*. The marked preference of *V. alginolyticus* for 18:2 at 37°C is interesting considering the

relative abundance of linoleic acid in human tissue and mucosa (Ciçek et al., 2005; Pezeshkian et al., 2009; Bolt et al. 1965; Lekholm & Svennerholm, 1977).

Both *Vibrio spp*. exhibited similar permeability effects; for example, 22:6 had a negligible impact while 20:4 and 20:5 elicited the largest change. These results are also in agreement with crystal violet assays of other *Vibrio* spp. demonstrating PUFA-mediated decreases in permeability (Moravec et al., 2017). Although more in-depth analyses are warranted for defining PUFA-mediated permeability characteristics, this study demonstrates commonality within *Vibrio* species with regard to exogenous fatty acid assimilation into phospholipids and corresponding, albeit variable, decreases in permeability using crystal violet as the gauge.

Few studies have reported the susceptibilities of *V. alginolyticus* and *V. fischeri* to polymyxins. In *V. alginolyticus*, approximate breakpoints for polymyxin B and colistin have been between 30-50ug/ml, with one study identifying higher resistance to colistin from isolates of farmed and wild fish (Ndip et al., 2002; Zouiten et al., 2017; Beshiru, 2020; Dahanayake, et al., 2019). In *V. fischeri*, polymyxin B has been most effective at 0.7-7 ug/ml, whereas studies involving colistin are conspicuously absent from the literature (Adin et al., 2007). Here, these cyclic peptide antibiotics were used to assess PUFA-adapted bacterial MICs. Although permeability assays revealed decreases in membrane integrity, the MICs to PMB and colistin were largely decreased given PUFA availability. Similarities were observed between MIC results for PMB and colistin, especially with *V. alginolyticus*. For both antibiotics, 18:2, 18:3α, 18:3γ, and 20:3 caused significant decreases in MIC. 18:2 also significantly lowered the MIC of both antibiotics in *V. fischeri*. The evidence for phospholipid composition contributions toward colistin resistance in

Gram-negative bacteria is building. Whereas known mechanisms of polymyxin resistance involve lipid A modification, capsule expression, efflux systems, and enzymatic inactivation (Baron et al., 2016; Moffatt et al., 2019; Li et al., 2019) The remodeling of membrane phospholipids with PUFAs deserves further attention, especially since the effect generally increases, rather than decreases, vulnerability to cyclic peptides. Indeed, a study by Li et al. (Li et al., 2019) used proteomic analysis to identify unsaturated fatty acid biosynthesis as one of the key metabolomic categories differentiating sensitive and resistant strains in *V. alginolyticus*. In a similar study, metabolomics did not implicate unsaturated fatty acid pathways in cephalosporin resistance but demonstrated that the fatty acid biosynthesis inhibitor triclosan caused heightened resistance in *V. alginolyticus* (Shi-Rao et al., 2019). Other known factors influencing antibiotic resistance in *V. alginolyticus* include salinity and capacity for reactive oxygen species (ROS) production (Zhang et al., 2020).

Response to exogenous fatty acids could be important for controlling motility and/or biofilm formation during planktonic or symbiotic lifestyles. Our findings indicate varied effects depending on the media when incubated at 28°C. Although more dramatic differences were observed using CM9 minimal media, the biofilm assays performed in Marine broth are likely to better represent the responses to fatty acids in the aquatic niches of *Vibrio*. These results also emphasize the importance of media composition for observing bacterial behavior, in this case potentially driven by the complex salts mimicking marine environments that may contribute to elevated biofilm production in the presence of PUFAs (Figure 6c&d). In a complex, tightly regulated, relationship, *V. fischeri* initiates biofilm formation on the surface of the squid's light organ prior to dissemination

and entry into the symbiotic organ (Nyholm, Stabb, Ruby, et al 2000; Visick, 2009). In addition to known environmental cues such as calcium and nitric oxide, there are likely more signaling molecules that remain to be discovered (Wang et al., 2010; Tischler et al., 2018). The PUFA-mediated effects on biofilm formation found herein certainly illustrate the sensitivity involved in quorum sensing and exopolysaccharide secretion. The observed trend of increased biofilm formation in both Marine broth (28°C) and CM9 (37°C, 150mM NaCl), in contrast with CM9 (28°C, 342mM NaCl), may indicate temperature dependence and/or important contributions of other mineral/nutrient constituents in Marine broth. Importantly, the potential synergism between colistin and PUFA supplementation may impact treatment outcomes in biofilm-associated infections (Lora-Tamayo et al., 2019). Certainly, the contrasting biofilm responses warrant further investigation with regard to media composition, incubation temperature, and individual PUFA response.

Motility for each *Vibrio* species was examined at 28°C, with *V. alginolyticus* exhibiting weaker swimming motility for all PUFAs and *V. fischeri* responding only to 18:2 (~45% increase) (Figure 7). It would be prudent to assess the motility *of V. alginolyticus* at 37°C to test the effect of the PUFAs expected to be more available at sites of human infection (18:2 and 20:4). The theme of decreased motility in *V. alginolyticus* mostly aligned with similar studies using other pathogenic *Vibrio* species (*cholerae*, *parahaemolyticus*, *vulnificus*) (Moravec et al., 2017). Additionally, since *V. vulnificus* showed increased motility when grown in Marine broth, it may be important to consider the locomotion of *V. alginolyticus* when exposed to media mimicking the marine environment. The observed increase in linoleic acid-mediated motility for *V. fischeri* is

interesting given its bacterial ecology. Perhaps the attraction to 18:2 stems from planktonic survival and the association of linoleic acid with sediment, stemming from high deposits of omega-6 fatty acids from terrestrial plant matter (Whatley et al., 2014; Torres-Ruiz & Wehr, 2010). It would reason that the squid-associated PUFAs (20:4, 20:5, and 22:6) would not increase motility, since symbiotic bacteria downregulate chemotaxis and motility in the crypts of the squid light organ (Bennett et al., 2020).

The control and management of human and aquatic animal infections caused by *V. alginolyticus* have been complicated by the organism's efficient pathogenicity. Access to the human body, normally via contaminated seawater, causes a variety of soft tissue, ear, and wound infections that can be difficult to treat due to continually emerging antibiotic resistance (Horii et al., 2005; Citil et al., 2015; Hernandez-Robles et al., 2016). In aquatic animals, the quick onset and high mortality rate threaten wild and farmed fish populations (Selvin & Lipton, 2003; Lee, 1995; Liu et al., 2004; Ahmed et al., 2015). A potential avenue for controlling pathogens may involve interference with fatty acid sensing, an important virulence pathway in *Vibrio spp*. (Gao et al., 2017; Cronan, 1997; Shi et al., 2015; Kovacikova et al., 2017). The unsaturated fatty acid acquisition also represents a significant facet of the *V. fischeri*-squid symbiosis as evidenced by the adoption of squid fatty acid profiles, identification of fatty acid chemoreceptors, and the necessity of long-chain fatty acids for achieving bioluminescence (Wier et al., 2010; Nikolakakis et al., 2015; Brodl et al., 2018.).

Conclusions

The findings herein further promote exogenous PUFAs as important physiological and behavioral molecules that are likely actively scavenged in the aquatic and host environments of *Vibrio spp*. In addition to assimilation into bacterial phospholipids, exogenous PUFAs differentially influence growth, biofilm formation, motility, permeability, and antimicrobial resistance in *Vibrio fischeri* and *Vibrio alginolyticus*. This metabolic and sensory versatility may guide survival and fitness in aquatic bacteria that have expanded their habitable niches to include symbiotic and pathogenic relationships. Future studies aim to define the underlying mechanisms of PUFA utilization and the potential for PUFA administration in clinical and ecological settings.

Author contributions

David Smith: Conceptualization (supporting); Investigation (lead); Formal Analysis (supporting) Writing—original draft (equal); Writing—review and editing (supporting); **Carina Houck**: Investigation (supporting)**;** Formal Analysis (supporting)**; Allycia Lee:** Investigation (supporting); **Timothy Simmons:** Investigation (supporting); **Olivia Chester:** Investigation (supporting); **Ayanna Esdaile:** Investigation (supporting); **Steven Symes:** Conceptualization (supporting); Formal analysis (supporting); Funding acquisition (equal); Investigation (supporting); Methodology (supporting); Project administration (supporting); Resources (supporting); Validation (supporting); Visualization (supporting); Writing—original draft (equal); Writing—review and editing (supporting); **David Giles:** Conceptualization (lead); Formal analysis (lead); Funding acquisition (equal); Investigation (supporting); Methodology (lead); Project administration

(lead); Supervision (lead); Resources (lead); Validation (lead); Visualization (lead); Writing—original draft (equal); Writing—review and editing (lead).

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Ethics Statement

None Required

Conflict of Interest

None declared

Data Availability Statement

All data generated or analyzed during this study are included in this published article.

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Figure legends

Figure 1. Growth characteristics of *Vibrio alginolyticus* and *Vibrio fischeri* in the presence of exogenous polyunsaturated fatty acids. (a & b) *V. fischeri* was grown at 28°C in CM9 minimal media (342mM NaCl) with PUFAs as supplemental carbon sources [300uM] (a) and as sole carbon sources [1mM] (b). (c & d) *V. alginolyticus* was grown at 28°C in CM9 minimal media (342mM NaCl) with PUFAs as supplemental carbon sources [300uM] (c) and as sole carbon sources [1mM] (d). (e & f) *V. alginolyticus* was grown at 37°C in CM9 minimal media (150mM NaCl) with PUFAs as supplemental carbon sources [300uM] (e) and as sole carbon sources [1mM] (f).

Graphs are representative of at least two biological replicates (standard deviations < 0.05).

Figure 2. Thin-layer chromatography of phospholipids extracted from *Vibrio alginolyticus* and *Vibrio fischeri* grown in the presence of individual polyunsaturated fatty acids. Bacteria were grown to exponential phase (OD ≈ 0.8) in CM9 minimal media (3% NaCl) at 28°C with or without 300 μM of the indicated fatty acids (linoleic acid [18:2], alpha-linolenic acid [18:3α], gamma-linolenic acid [18:3γ], dihomo-gamma-linolenic acid [20:3], arachidonic acid [20:4], eicosapentaenoic acid [20:5] and docosahexaenoic acid [22:6]). Phospholipids were extracted and subjected to separation by TLC (see Materials and Methods). Both *V. alginolyticus* (a) and *V. fischeri* (b) appear to be incorporating PUFAs into their phospholipid membranes. Compared to the 'No FA' culture, the major bacterial phospholipids phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL) display a noticeable migratory shift upward. Linolenic acids (18:3α and 18:3γ) are not represented for *V. fischeri* because the bacteria do not grow to the logarithmic phase.

Figure 3. Ultra performance liquid chromatography/mass spectrometry of extracted lipids from *Vibrio alginolyticus* and *Vibrio fischeri* grown in the presence of a specific fatty acid. The phospholipid region of the chromatograms shows a variety of peaks corresponding to modified phospholipids that are absent in the control. Shown are extracted ion chromatograms for *V. alginolyticus* cultures exposed to (a) 18:2 (b) 18:3α (c) 20:3 and *V. fischeri* cultures exposed to (d) 20:4 (e) 20:5 (f) 22:6. Identifications

of each phospholipid were based on mass spectrometry. See Appendix Figure 1 for assignment rationale.

Figure 4. The effect of exogenous fatty acids on permeability in *Vibrio alginolyticus* and *Vibrio fischeri*. (a) *V. alginolyticus* and (a) *V. fischeri* were grown at 28°C in CM9 (3% NaCl) with and without 300 μM of each PUFA to mid-log phase (OD = 0.8). Cultures were gently pelleted, washed, and prepared in PBS (OD_{600} = 0.7). Measurement of CV remaining in the supernatant at each time interval allowed calculation of the percentage of CV uptake. The graph is representative of three independent experiments. Standard deviations (not graphed for visual clarity) were less than 3% and statistical significance was measured by using all five-time interval values compared to control (Student's t-test, paired, 2-tailed, *$p < 0.002$).

Figure 5. The effect of exogenous fatty acids on polymyxin B, colistin, and imipenem resistance in *Vibrio alginolyticus* and *Vibrio fischeri*. Bacteria were grown at 28°C in CM9 (3% NaCl) with and without 300 μM of the indicated fatty acids to mid-log phase (OD = 0.8). Cultures were pelleted, washed, and resuspended in CM9 to a final desired inoculum of $5x10^5$ cfu ml^{-1}. Fatty acids were added to a final concentration of 300 μM. The bacterial suspension was transferred to microtiter plates containing two-fold concentrations of (a & b) polymyxin B, (c & d) colistin, or (e & f) imipenem. Growth measurements (OD = 600nm) were recorded after 24 h incubation at 28°C. Experiments were performed in triplicate. Circled symbols indicate significant differences ($p < 0.002$) as compared to the control (no fatty acid) at the same antimicrobial concentration.

Figure 6. Incubation with exogenous fatty acids alters biofilm formation in *Vibrio alginolyticus* and *Vibrio fischeri*. Overnight cultures were pelleted, washed, resuspended in the appropriate media, and inoculated onto microtiter plates (starting OD ~0.1) in octuplet. Each culture was grown in the presence of 300μM of the indicated fatty acids. The biofilm assay was performed with *V. alginolyticus* (a & c) and *V. fischeri* (b & d) in CM9 minimal media (a & b) and Marine broth (c & d). Following incubation, the solubilized CV was measured at 590nm. At least two independent biological replicates were performed in octuplets. The Student's t-test was used to determine significant differences in biofilm formation (*,$p < 0.001$).

Figure 7. The effects of PUFAs on swimming motility in *Vibrio alginolyticus* and *Vibrio fischeri*. For *V. alginolyticus*, soft agar motility plates were prepared with 10 g L^{-1} tryptone, 10 g L^{-1} NaCl, 0.35 % agar, and with or without 300 μM of a given PUFA. For *V. fischeri*, soft agar motility plates were prepared using Marine broth containing 0.35% agar, and with or without 300 μM of a given PUFA. Overnight cultures were washed and resuspended to prepare a culture with an OD_{600} of 1.0. Each motility plate was divided into 4 quadrants and 2 μL of the diluted culture was injected into the center of each quadrant. (a) *V. alginolyticus* swimming motility was measured after 48h, 72h, and 96h at 28°C. All fatty acids caused a steady decrease in swimming motility. (b) *V. fischeri* swimming motility was measured after 6h at 28°C. Asterisks indicate significant ($p < 0.01$) deviations from the control sample at that incubation time, as calculated using a Student's t-test (paired, two-tailed distribution).

APPENDIX

Figure A1. Ultra-performance liquid chromatography/mass spectrometry of lipids isolated from *Vibrio alginolyticus* grown in the presence of eicosapentaenoic acid. *Vibrio alginolyticus* was grown to logarithmic phase at 28°C in CM9 (3% NaCl) spiked with 300 μM of eicosapentaenoic acid (20:5). Electrospray ionization-quadrupole mass spectrometry was used to detect $[M-H]^-$ ions produced following gradient elution using a reversed-phase C18 column. The use of elevated cone voltage (50 V) produces cone fragments due to cleavage of the fatty acyl chains from the sn-1 and sn-2 positions. (a) Extracted ion chromatograms (overlain), mass filtered for the parent ions of the indicated phospholipids. These ions are absent in the control culture. (b) The mass spectrum of the chromatographic peak at 5.75 min displays a mass peak of 736.5 m/z which corresponds to a PE 36:5 species (http://www.lipidmaps.org/). Direct observation of the cone fragments at m/z 255.2 and 301.2 confirm the identity as PE 16:0/20:5. All chromatographic peaks were assigned in this way; by analysis of mass spectral parent peaks and their corresponding acyl chain, cone fragments were observable when using high cone voltage in negative mode electrospray ionization.

Figure A2. PUFAs increase biofilm formation of *V. alginolyticus* grown at human physiological temperature (37°C) and salt concentration (150mM NaCl). Overnight cultures were used to prepare fresh inocula in CM9 media (0.4% casamino acids, 0.4% glucose, 150mM NaCl) and transferred onto microtiter plates (starting OD ~0.1) in octuplet. Growth conditions involved administration of 300μM of the indicated fatty acids and incubation at 37°C for 48h. Absorbance values (OD_{590}) were measured and data

were expressed as the mean (± SD) of two independent experiments performed in octuplet. Asterisks indicate significant differences ($p < 0.001$) in biofilm formation compared to control as determined by Student's t-test.

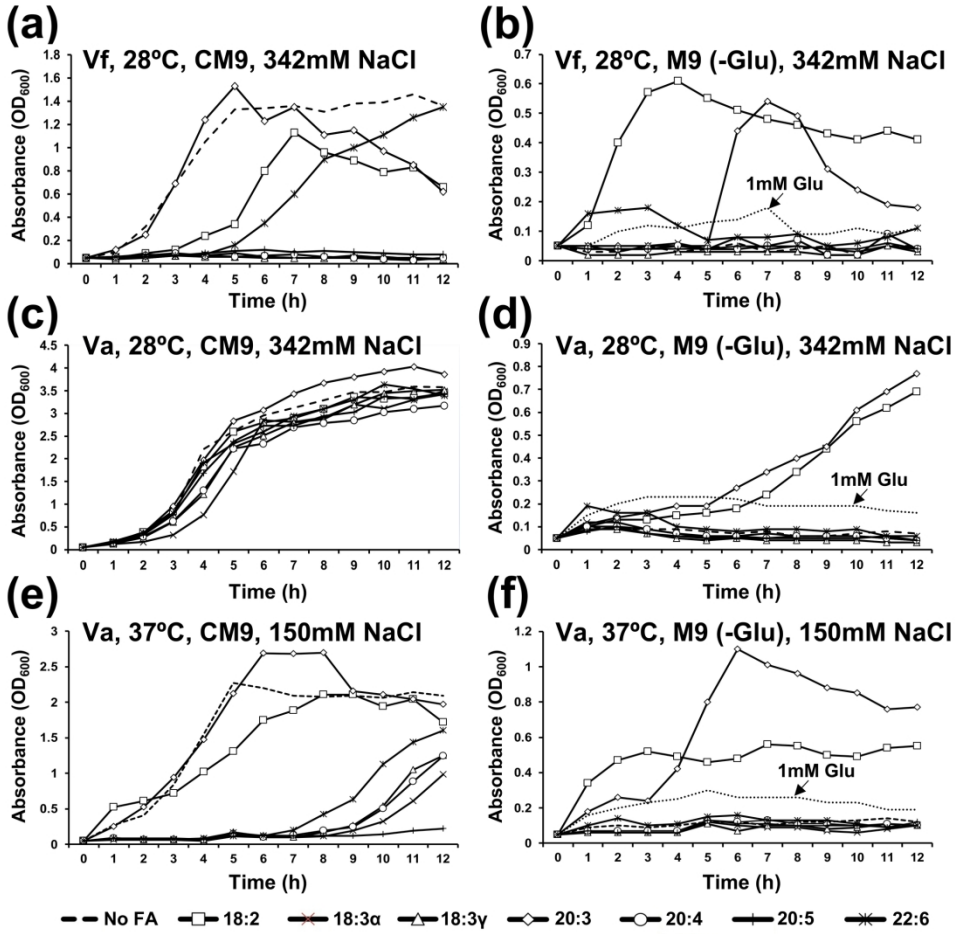
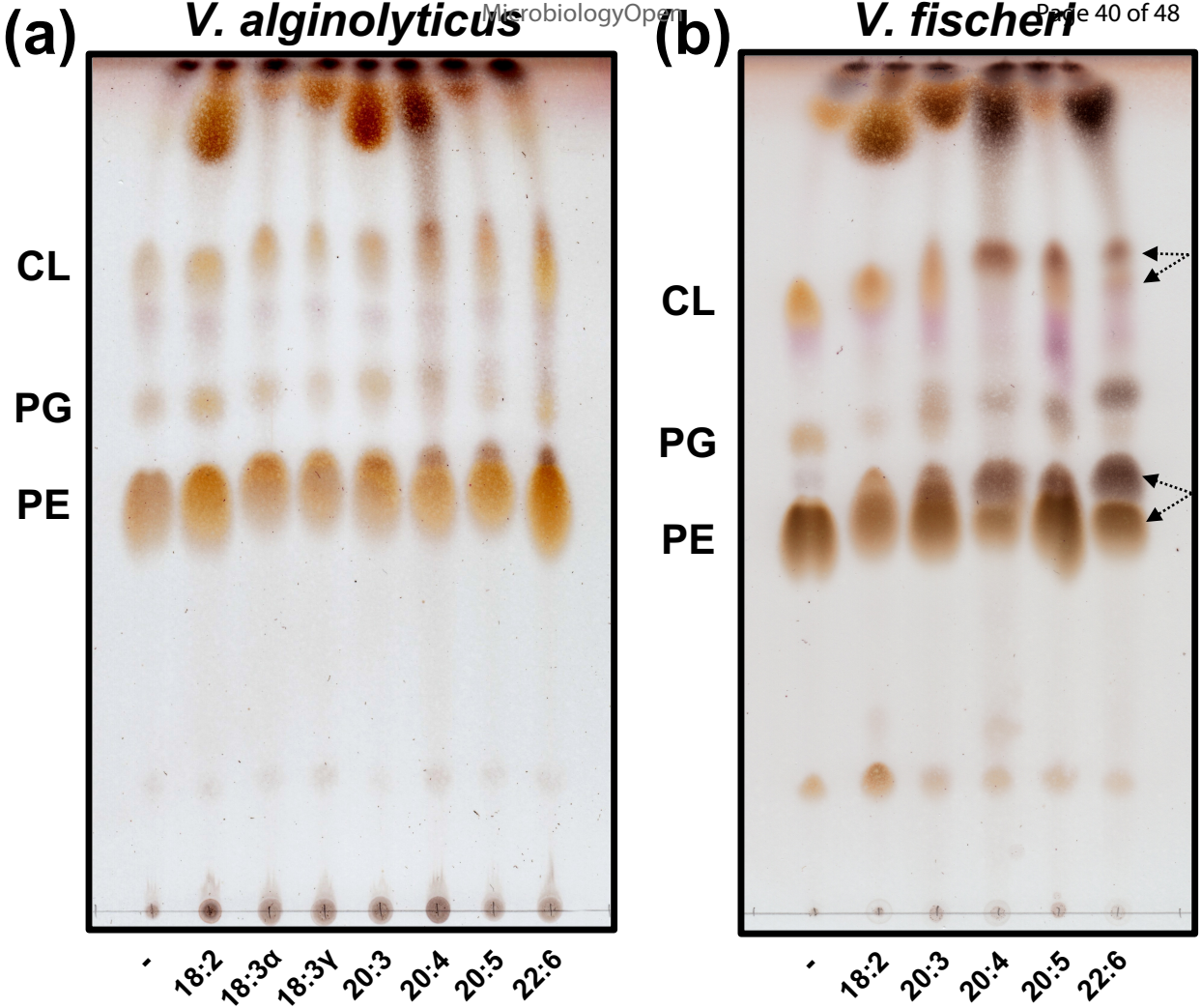
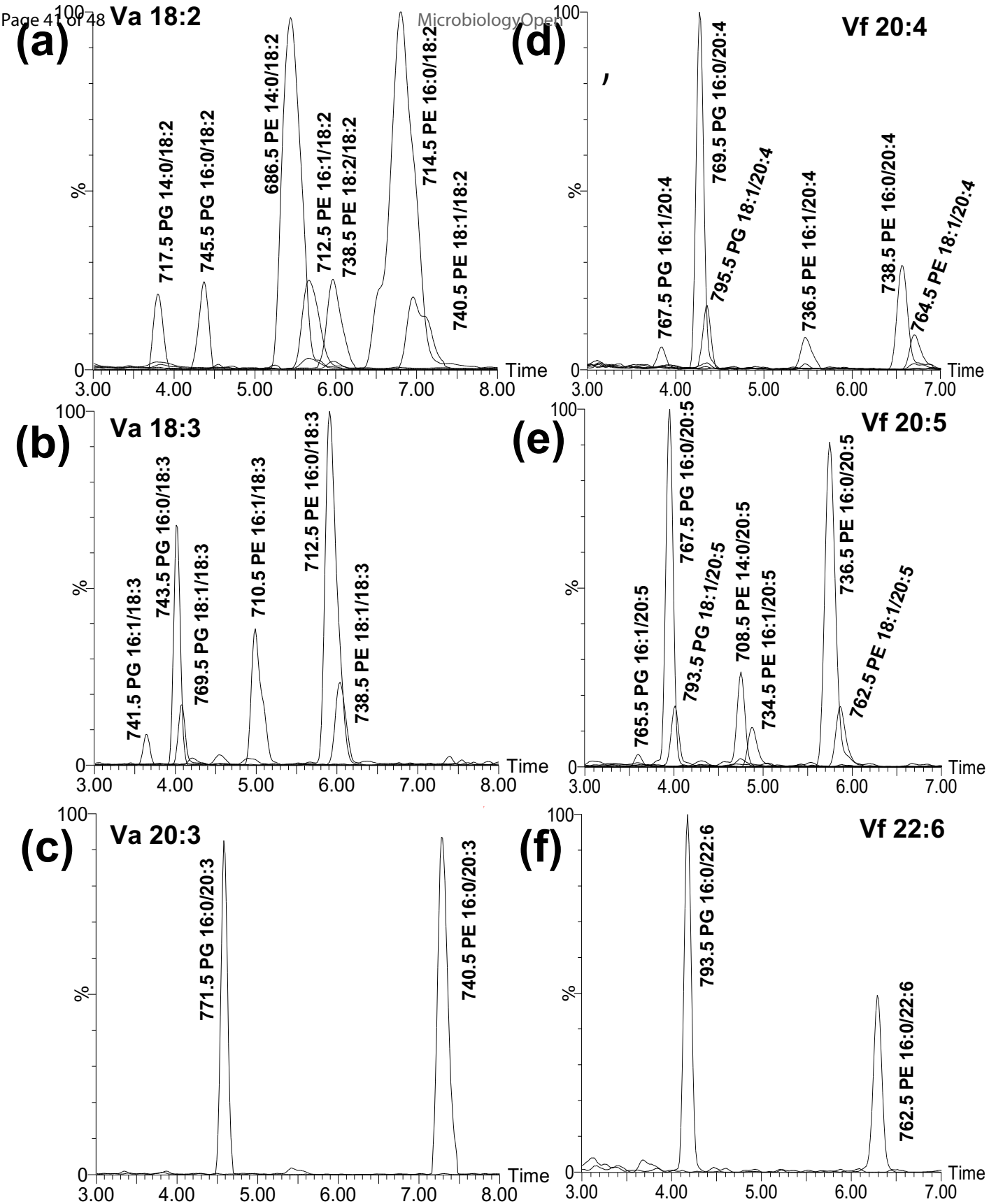
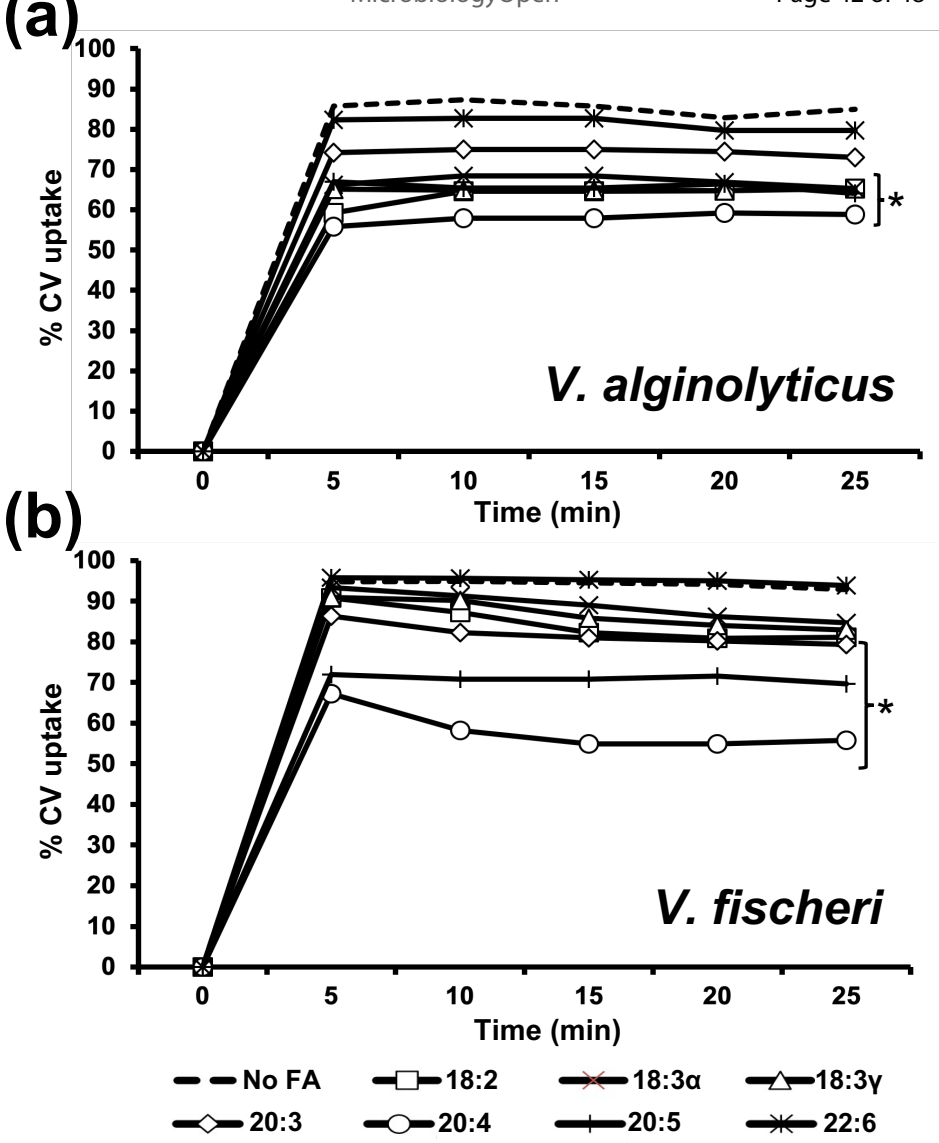


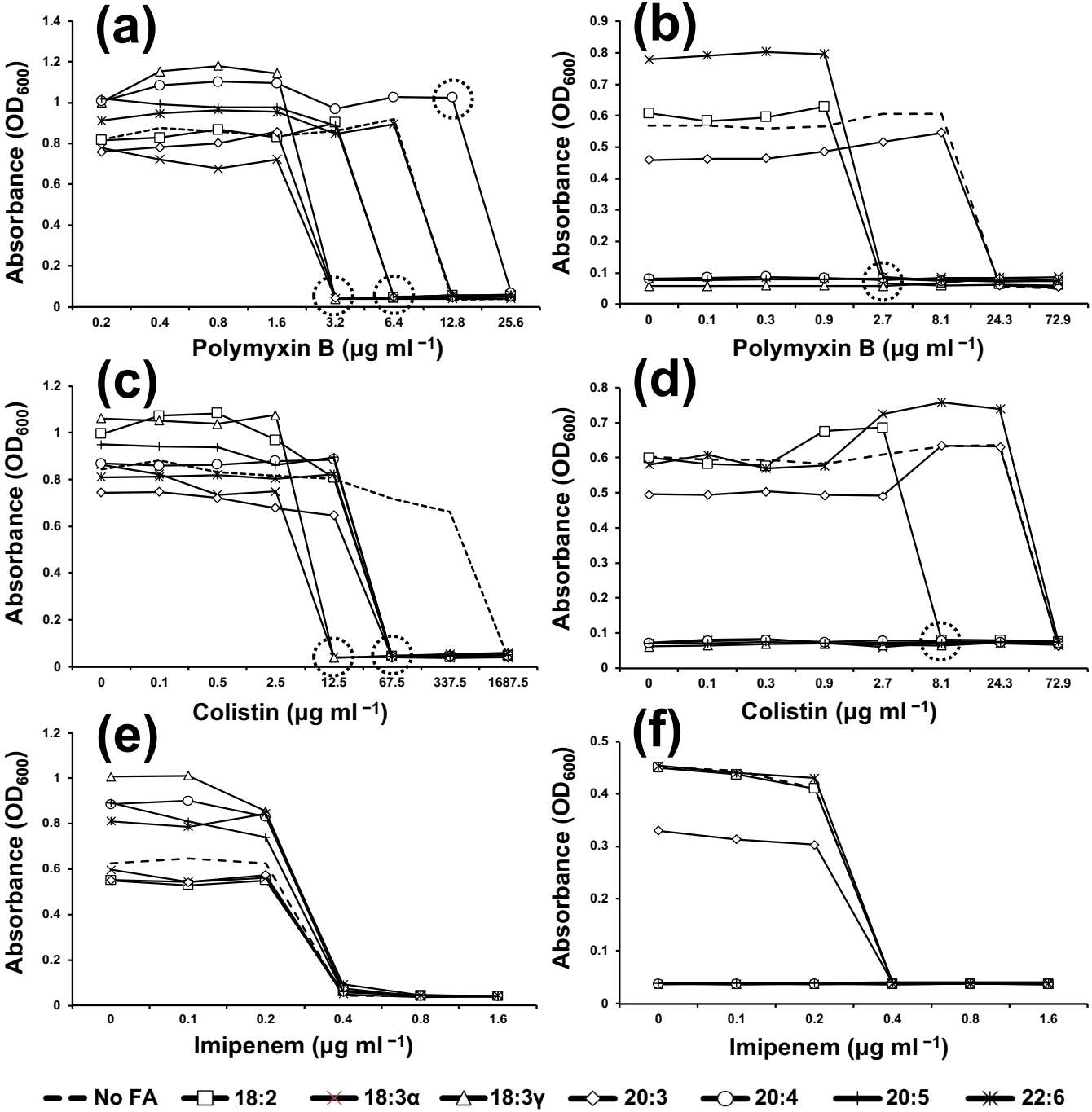
Figure 1. Growth characteristics of *Vibrio alginolyticus* and *Vibrio fischeri* in the presence of exogenous polyunsaturated fatty acids. (a & b) *V. fischeri* was grown at 28°C in CM9 minimal media (342mM NaCl) with PUFAs as supplemental carbon sources [300μM] (a) and as sole carbon sources [1mM] (b). (c & d) *V. alginolyticus* was grown at 28°C in CM9 minimal media (342mM NaCl) with PUFAs as supplemental carbon sources [300μM] (c) and as sole carbon sources [1mM] (d). (e & f) *V. alginolyticus* was grown at 37°C in CM9 minimal media (150mM NaCl) with PUFAs as supplemental carbon sources [300uM] (e) and as sole carbon sources [1mM] (f). Graphs are representative of at least two biological replicates (standard deviations < 0.05).

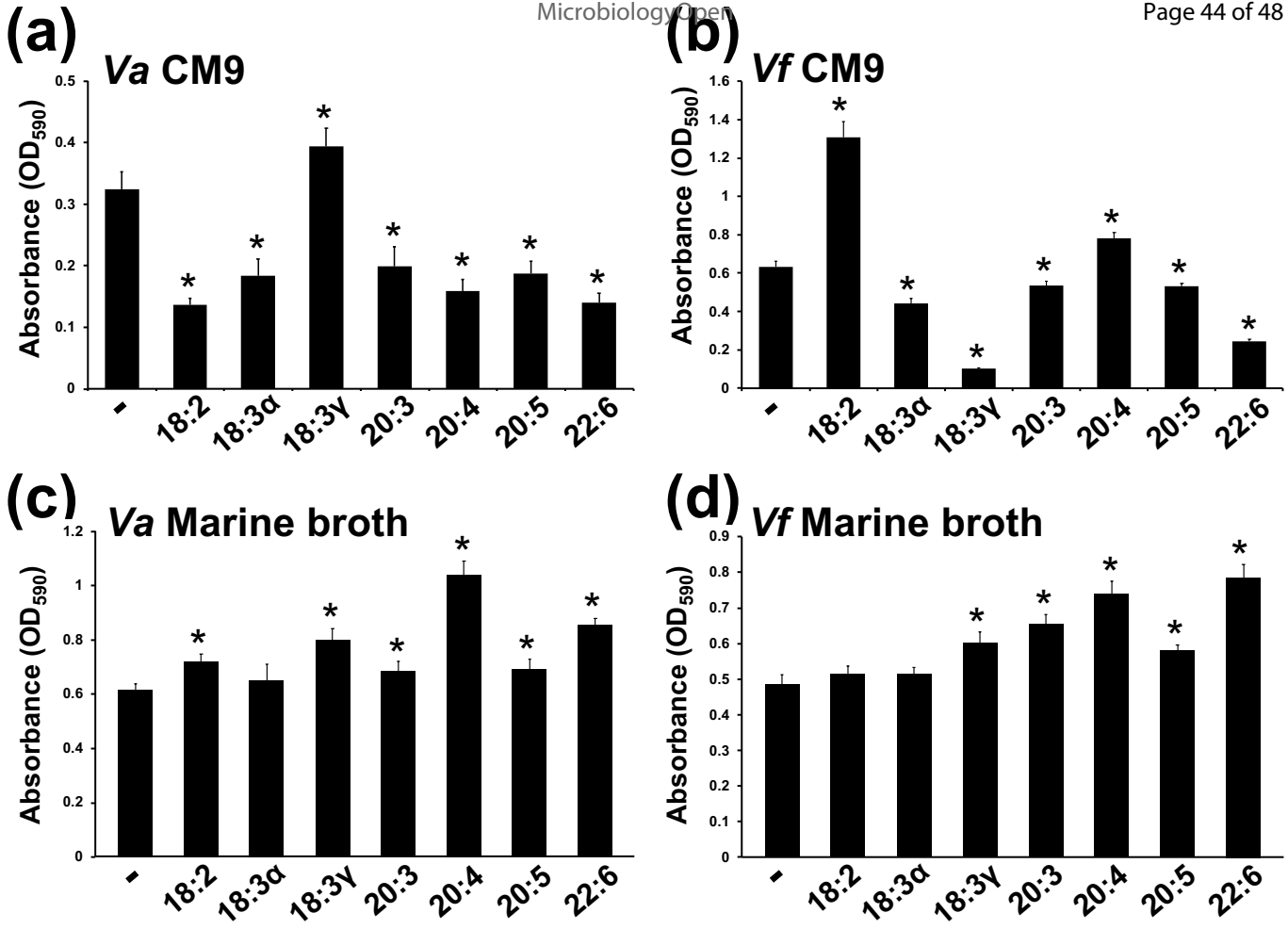
190x183mm (300 x 300 DPI)

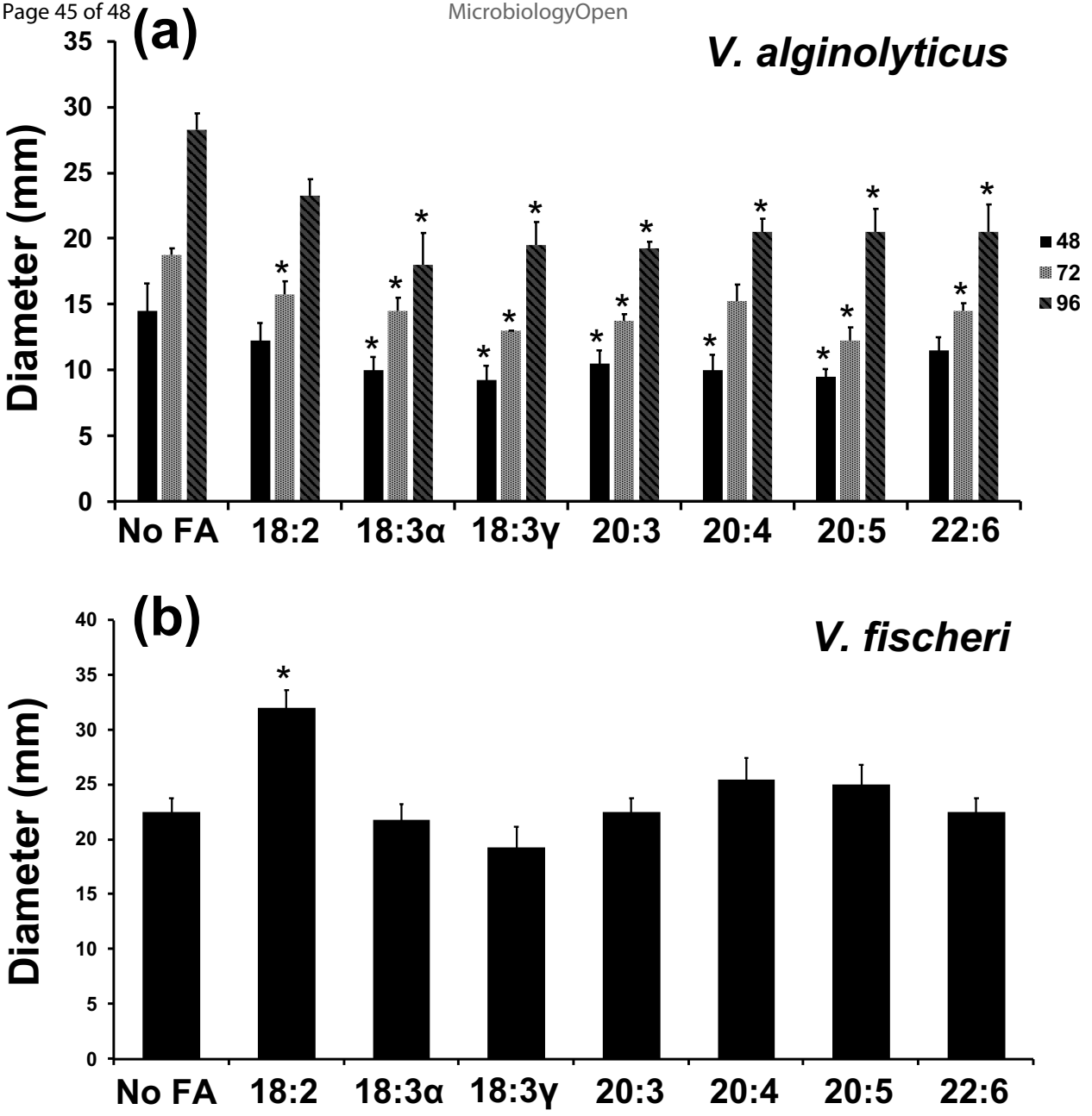

(a)
V. alginolyticus
CL
PG
PE
-
18:2
18:3α
18:3γ
20:3
20:4
20:5
22:6
(b)
V. fischeri
CL
PG
PE
-
18:2
20:3
20:4
20:5
22:6


(a)
Va 18:2
717.5 PG 14:0/18:2
745.5 PG 16:0/18:2
686.5 PE 14:0/18:2
712.5 PE 16:1/18:2
738.5 PE 18:2/18:2
714.5 PE 16:0/18:2
740.5 PE 18:1/18:2
(d)
Vf 20:4
767.5 PG 16:1/20:4
769.5 PG 16:0/20:4
795.5 PG 18:1/20:4
736.5 PE 16:1/20:4
738.5 PE 16:0/20:4
764.5 PE 18:1/20:4
(b)
Va 18:3
741.5 PG 16:1/18:3
743.5 PG 16:0/18:3
769.5 PG 18:1/18:3
710.5 PE 16:1/18:3
712.5 PE 16:0/18:3
738.5 PE 18:1/18:3
(e)
Vf 20:5
765.5 PG 16:1/20:5
767.5 PG 16:0/20:5
793.5 PG 18:1/20:5
708.5 PE 14:0/20:5
734.5 PE 16:1/20:5
736.5 PE 16:0/20:5
762.5 PE 18:1/20:5
(c)
Va 20:3
771.5 PG 16:0/20:3
740.5 PE 16:0/20:3
(f)
Vf 22:6
793.5 PG 16:0/22:6
762.5 PE 16:0/22:6
%
Time
100
0
3.00
4.00
5.00
6.00
7.00
8.00


(a)
% CV uptake
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
Time (min)
*
V. alginolyticus
(b)
% CV uptake
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
Time (min)
*
V. fischeri
No FA
18:2
18:3α
18:3γ
20:3
20:4
20:5
22:6


(a)
Absorbance (OD600)
Polymyxin B (μg ml −1)
(b)
Absorbance (OD600)
Polymyxin B (μg ml −1)
(c)
Absorbance (OD600)
Colistin (μg ml −1)
(d)
Absorbance (OD600)
Colistin (μg ml −1)
(e)
Absorbance (OD600)
Imipenem (μg ml −1)
(f)
Absorbance (OD600)
Imipenem (μg ml −1)
No FA
18:2
18:3α
18:3γ
20:3
20:4
20:5
22:6


(a)
Va CM9
Absorbance (OD590)
0
0.1
0.2
0.3
0.4
0.5
-
18:2
18:3α
18:3γ
20:3
20:4
20:5
22:6
(b)
Vf CM9
Absorbance (OD590)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
-
18:2
18:3α
18:3γ
20:3
20:4
20:5
22:6
(c)
Va Marine broth
Absorbance (OD590)
0
0.2
0.4
0.6
0.8
1
1.2
-
18:2
18:3α
18:3γ
20:3
20:4
20:5
22:6
(d)
Vf Marine broth
Absorbance (OD590)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
-
18:2
18:3α
18:3γ
20:3
20:4
20:5
22:6


(a)
V. alginolyticus
Diameter (mm)
35
30
25
20
15
10
5
0
48
72
96
No FA
18:2
18:3α
18:3γ
20:3
20:4
20:5
22:6
(b)
V. fischeri
Diameter (mm)
40
35
30
25
20
15
10
5
0
No FA
18:2
18:3α
18:3γ
20:3
20:4
20:5
22:6

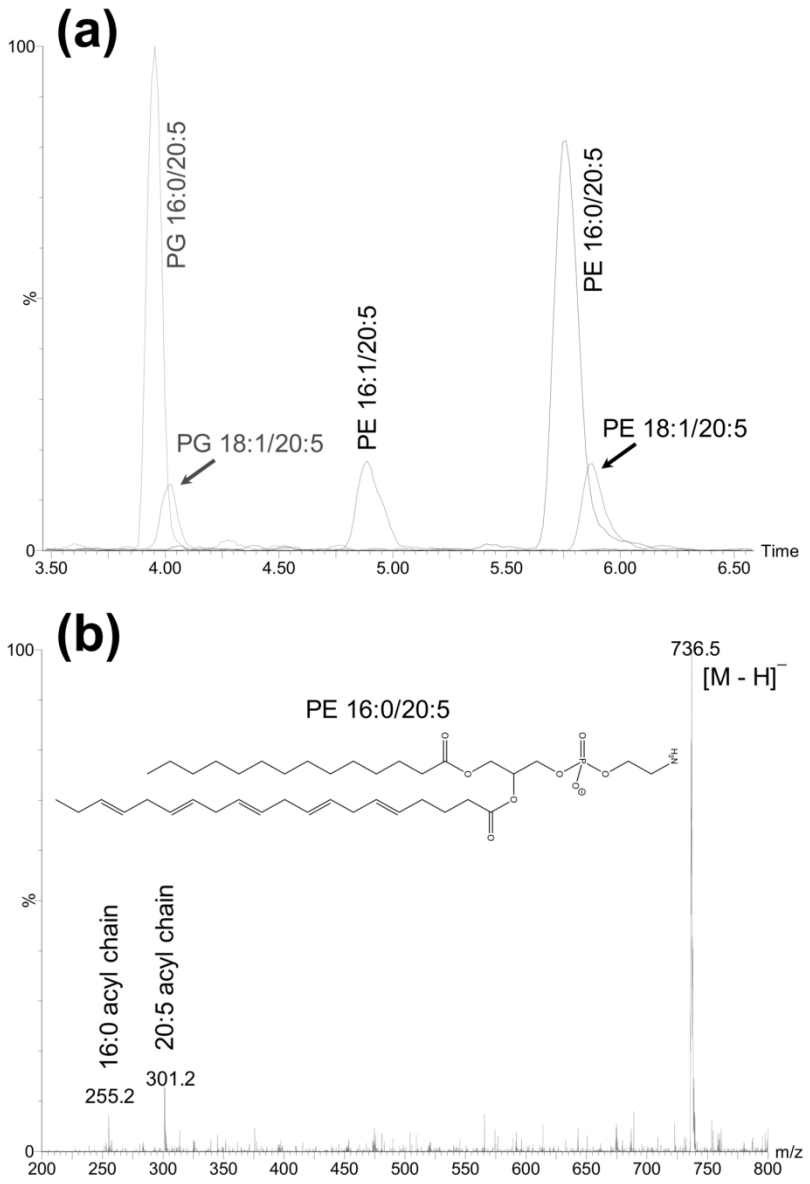
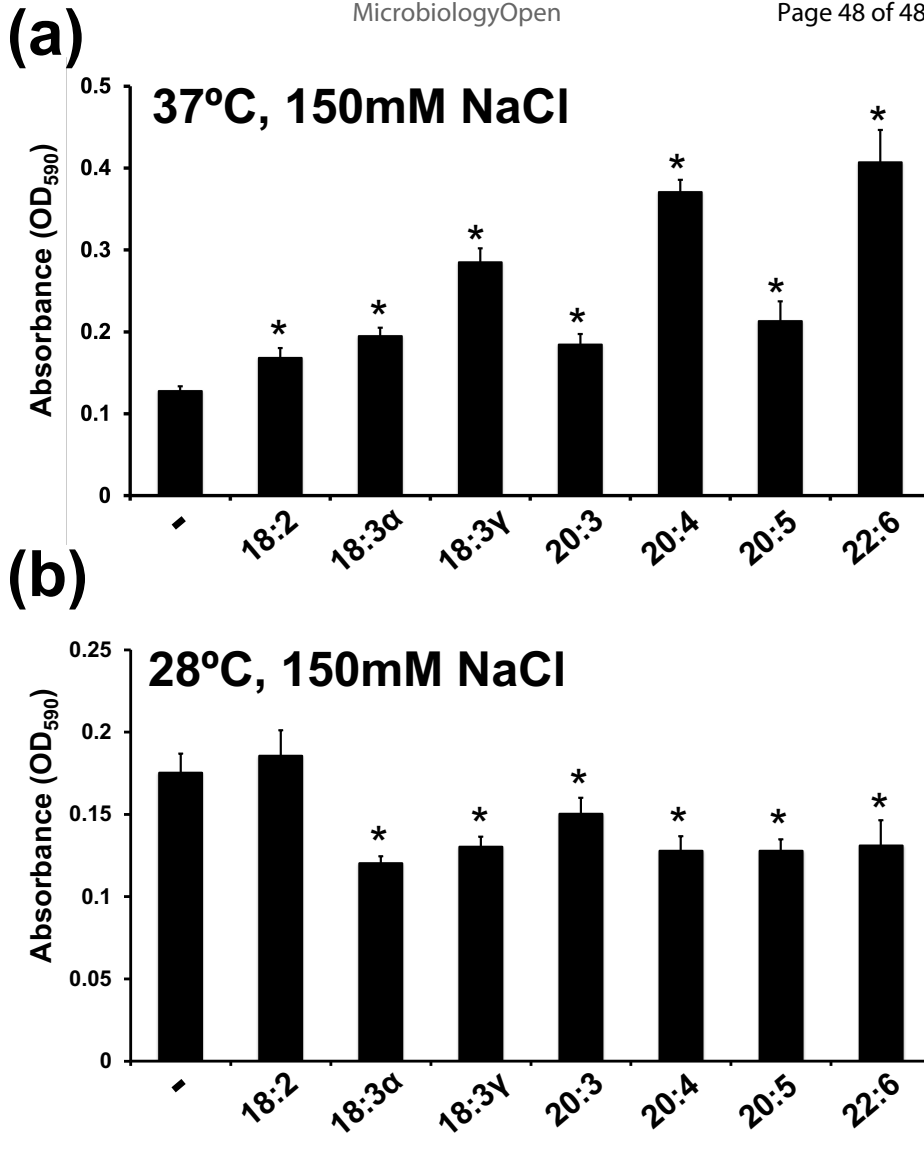


Figure A1. Ultra performance liquid chromatography/mass spectrometry of lipids isolated from *Vibrio alginolyticus* grown in the presence of eicosapentaenoic acid. *Vibrio alginolyticus* was grown to logarithmic phase at 28°C in CM9 (3% NaCl) spiked with 300 µM of eicosapentaenoic acid (20:5). Electrospray ionization-quadrupole mass spectrometry was used to detect [M-H]– ions produced following gradient elution using a reversed phase C18 column. Use of elevated cone voltage (50 V) produces cone fragments due to cleavage of the fatty acyl chains from the sn-1 and sn-2 positions. (a) Extracted ion chromatograms (overlain), mass filtered for the parent ions of the indicated phospholipids. These ions are absent in the control culture. (b) The mass spectrum of the chromatographic peak at 5.75 min displays a mass peak of 736.5 *m/z* corresponding to a PE 36:5 species (http://www.lipidmaps.org/). Direct observation of the cone fragments at *m/z* 255.2 and 301.2 confirm the identity as PE 16:0/20:5. All chromatographic peaks were assigned in this way; by analysis of mass spectral parent peaks and their corresponding acyl chain cone fragments observable when using high cone voltage in negative mode electrospray ionization.

130x195mm (300 x 300 DPI)


(a)
37ºC, 150mM NaCl
Absorbance (OD590)
0.5
0.4
0.3
0.2
0.1
0
-
18:2
18:3α
18:3γ
20:3
20:4
20:5
22:6
(b)
28ºC, 150mM NaCl
Absorbance (OD590)
0.25
0.2
0.15
0.1
0.05
0
-
18:2
18:3α
18:3γ
20:3
20:4
20:5
22:6