ORIGINAL ARTICLE

In-Silico and In-Vitro Assessment of Selected Fatty Acids against Vibrio Spp..

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ABSTRACT

INTRODUCTION: *Vibrio* spp. is a zoonotic pathogen that can cause mild or fatal infection in human and animals. The overuse of antibiotics has led to increasing antimicrobial resistance (AMR). Fatty acids (FAs) offer a potential alternative due to their broad-spectrum antibacterial activity. Hence, this study aimed to investigate the anti-Vibrio effects of oleic acid (OA), lauric acid (LA), palmitic acid (PA), and stearic acid (SA) through in-vitro and in-silico approaches. **MATERIALS AND METHODS:** Molecular docking was performed using PyRx against 21 receptors belonging to *Vibrio* spp. retrieved from the Protein Data Bank (PDB) for in-silico investigation. To validate the findings, LA, OA, PA and SA were experimentally tested. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) assays were conducted in-vitro against *Vibrio alginolyticus* and *Vibrio parahaemolyticus*. **RESULTS:** Molecular docking results revealed that OA exhibited the lowest binding energy (min=-7.2, mean=-4.8). OA also showed the second-highest number of hydrogen bond interactions and ranked third in van der Waals interactions. FAs demonstrated significantly stronger affinity ($p<0.05$) to proteins related to toxin production, nutrient acquisition, and quorum sensing. In-vitro assays aligned with in-silico where OA had the lowest MIC (125 µg/mL) against both *V. alginolyticus* and *V. parahaemolyticus* and exhibited bactericidal activity against *V. alginolyticus* at 500 µg/mL. **CONCLUSION:** Therefore, OA is a promising anti-Vibrio agent that might regulate environmental sensing and interaction with small molecules. The receptors 3MRU, 3WPW, 3A57 and 3X0T, matched in-vitro results and could be used in the design of new treatments for vibriosis.

Keywords
Vibriosis, Vibrio alginolyticus, Vibrio parahaemolyticus, Lauric acid, Oleic acid

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INTRODUCTION

Vibrio spp. are gram-negative, rod-shaped bacteria that inhabit freshwater, estuary, and marine environments.[1] It is a zoonotic pathogen causing severe infection in human and animals.[2] Most *Vibrio* species are known to cause waterborne and foodborne diseases that spread through contaminated water and the consumption of raw and undercooked seafood, causing significant human health hazards.[3] According to WHO, there are up to 4.0 million cases reported annually of *Vibrio cholera* infection, causing mild to acute watery diarrhoea leading to severe dehydration.[4] *Vibrio harveyi* could infect human through an open wound coming in contact with contaminated water, which will cause severe inflammation and necrosis on the wound.[5] Meanwhile, *Vibrio vulnificus* is primarily linked to septicaemia on the skin through direct contact with contaminated seafood or water, which will trigger the immune system.[6] In terms of *Vibrio parahaemolyticus* and *Vibrio alginolyticus*, both species commonly infect farmed animals and lead to mass mortality in aquaculture industry.

The consumption of infected animals could cause serious gastroenteritis, fever, nausea, abdominal cramps, and bloody diarrhea.[7]

The primary medical treatment of these vibriosis through antibiotics has led to multiple antibiotic resistance (MAR) in numerous *Vibrio* species.[8] In a study, 67.2% of *Vibrio* spp. isolated from Malaysian seafood had high antimicrobial resistance (AMR).[9] According to another study on *Vibrio* spp. isolates from water samples, only some were susceptible to clinically relevant antibiotics, including tetracycline-class drugs used in current treatment regimens by the Centres for Disease Control and Prevention (CDC).[10,3] Thus, the adverse effects of the MAR on human and animal risk control have necessitated the development of an improved and more effective option for antimicrobial treatments with the least hazardous risk.

Over the years, fatty acids (FAs) properties have been studied as a potential substitute for conventional antibiotics to combat AMR problems. FAs are crucial for the immune system of organisms to protect against several diseases, including infection with antibiotic resistant bacteria.[11] FAs may function as biofilm inhibitors at low concentrations and exhibit antimicrobial activity at higher concentrations; however, bacterial responses can vary depending on the specific type of FA.[12] Long-chain fatty acids (LCFAs), including oleic, palmitic, and stearic acids, are naturally present in plants and animals via acetyl-CoA, and their carbon chain length varies between 12 to 24 carbons.[13] Meanwhile, lauric acid with a 12-carbon chain is known as a saturated medium chain fatty acid (MCFA) and can be rapidly absorbed and transported.[14] These medium to long-chain fatty acids are known to have antibacterial, antiviral, and antifungal properties. However, several studies reported that FAs are more effective against gram-positive bacteria than gram-negative bacteria.[11] For example, lauric acid demonstrate strong antimicrobial activity against gram-positive bacteria but have limited efficacy against gram-negative bacteria.[15,16]

The amphipathic nature FAs might play an important role in their antibacterial activity, which induces the disruption of the lipid packing leading to increased membrane permeability and leakage of cellular components.[17] Recent studies reported that unsaturated fatty acids are able to inhibit the growth of both gram-negative and gram-positive bacteria.[18] Unsaturated fatty acids, such as oleic and linoleic acid, have shown potential in treating *Helicobacter pylori*, *Staphylococcus aureus*, and *Escherichia coli* by disrupting the cellular membrane, leading to cell lysis.[19] Hence, this study aims to determine the antibacterial activity of selected fatty acids (FAs) against *Vibrio* spp..

MATERIALS AND METHODS

In-silico screening

Molecular docking simulations were done on 21 protein receptors identified from *Vibrio* spp. against 4 fatty acids (FAs), namely, lauric acid (LA) ($C_{12}H_{24}O_2$), oleic acid (OA) ($C_{18}H_{34}O_2$), palmitic acid (PA) ($C_{16}H_{32}O_2$), and stearic acid (SA) ($C_{18}H_{36}O_2$).

Ligand Preparation

The structure of the four different FAs, namely, lauric acid (LA), oleic acid (OA), palmitic acid (PA), and stearic acid (SA) were downloaded from the PubChem database. The ligands were prepared and optimized using Avogadro (v1.2.0), with polar hydrogens added to the 3D conformers. The ligands' geometry was optimized until convergence was reached. The prepared ligands were then saved in PDB format.[20]

Receptor Preparation

A total of 21 protein receptors belonging to *Vibrio* spp. were obtained from the Protein Data Bank (https://www.rcsb.org/) in PDB format.[21] The selected protein receptors were from various functional systems as well as functional classes as showed in Table I. All the water molecules, heteroatoms, and any co-crystallized ligands were removed from the protein structure using the Discovery Studio Version 21.1.0.20298. Finally, polar hydrogen atoms were added, and the protein structures were saved as a PDB file.[20] The active sites for all proteins were determined from previous publications linked to Protein Data Bank database. The grid box for

each receptor was established and the XYZ coordinates are provided in Table I.

Molecular Docking

The docking process was done by loading 1 receptor at a time, together with all 4 FAs into the PyRx software Version 0.8. Universal Forcefield (UFF) was applied due to its flexibility and viability for simulations in various systems which is needed considering the diversity of the 21 receptors used in this study.[22] The active site of the protein receptor was determined based on published research and data provided in the protein data bank database.

Molecular Docking Data Analysis

Molecular docking data was collected in triplicates to facilitate statistical analysis rather than reporting only the lowest free energy of binding (ΔG) or highest number of hydrogen bond. Statistical analyses were then carried out using SPSS software Version 20 to determine significant differences between FAs and protein receptors of *Vibrio* spp.. Univariate Analysis of Variance (ANOVA) and Tukey post hoc test ($p<0.05$) were used to compared FA, receptor's functional system and receptor's functional class in terms of binding energy, while chi-square test was used to compared number of hydrogen bonds and van der Waals interactions at $p<0.05$. Spearman's rho test was also applied to identify correlations between binding energy, number of hydrogen bonds and van der Waals interactions at $p<0.05$.

In-vitro screening

Compound preparation

Lauric acid (LA) ($C_{12}H_{24}O_2$), oleic acid (OA) ($C_{18}H_{34}O_2$), palmitic acid (PA) ($C_{16}H_{32}O_2$), and stearic acid (SA) ($C_{18}H_{36}O_2$) were purchased from Evachem (Malaysia) and were of analytical grade and used without further purification. A stock solution of 10 mg/ml of the 4 different FAs were prepared in 50% Dimethyl sulfoxide (DMSO).

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC and MBC were determined using sterile 96-well microtiter plates according to CLSI (2012) and previously described methods.[23,25] Two pathogenic *Vibrio* spp. maintained in our laboratory collection, namely *V. alginolyticus* (GenBank accession number: PQ044564.1)

Table I. List of selected protein receptor of Vibrio spp. from PDB website

PDB ID	Name	Functional System	Functional Class	*Source species*	Coordinates of binding site
6JV4	VMB-1	Antimicrobial resistance & survival	Hydrolase	*Vibrio alginolyticus*	X:23.12, Y:-10.66, Z:29.14
7CUS	VbrK	Antimicrobial resistance & survival	Signalling protein	*Vibrio parahaemolyticus*	X:3.46, Y:-7.89, Z:-6.27
3VCY	MurA	Antimicrobial resistance & survival	Transferase	*Vibrio fischeri* MJ11	X:-10.91, Y:1.14, Z:22.37
6PXA	Chlo-resist	Antimicrobial resistance & survival	Transferase	*Vibrio fischeri* ES114	X:-4.99, Y:5.37, Z:23.05
3MRU	PepD	Biofilm formation	Hydrolase	*Vibrio alginolyticus*	X:-32.79, Y:13.63, Z:-10.00
4BE5	RbmA	Biofilm formation	Cell adhesion	*Vibrio cholerae* MJ-1236	X:44.31, Y:31.74, Z:13.15
7AGZ	BsrV	Biofilm formation	Peptide-binding protein	*Vibrio cholerae* O1 biovar El Tor str. N 16961	X:8.81, Y:52.59, Z:1.71
2ZF8	MotY	Motility & flagellar assembly	Structural protein	*Vibrio alginolyticus*	X:50.85, Y:9.47, Z:63.36
3W1E	FlgT	Motility & flagellar assembly	Motor protein	*Vibrio alginolyticus*	X:24.1, Y:3.4, Z:32.6
3WPW	PomB	Motility & flagellar assembly	Membrane protein	*Vibrio alginolyticus*	X:16.27, Y:35.28, Z:45.28
6IF6	SflA	Motility & flagellar assembly	Membrane protein	*Vibrio alginolyticus*	X:32.19, Y:23.12, Z:33.09
3CK6	ZntB	Nutrient acquisition	Structural protein	*Vibrio parahaemolyticus* RIMD 2210633	X:15.25, Y:-65.24, Z:53.56
3R5T	ViuP	Nutrient acquisition	Metal transport	*Vibrio cholerae*	X:22.44, Y:14.43, Z:15.09
3LJL	LuxT	Quorum sensing & signalling	Transcription regulator	*Vibrio parahaemolyticus* RIMD 2210633	X:7.03, Y:52.63, Z:36.05
2HJ9	LuxP-LuxQ complex	Quorum sensing & signalling	Signalling protein	*Vibrio harveyi*	LuxP domain (X:13.07, Y:-1.77, Z:59.36)
2WK8	CqsA	Quorum sensing & signalling	Transferase	*Vibrio cholerae*	X:62.44, Y:59.94, Z:-2.65
1ZHH	LuxP	Quorum sensing & signalling	Signalling protein	*Vibrio harveyi*	X:-27.13, Y:82.43, Z:12.23
3A57	TDH	Toxins & toxin-associated regulators	Toxin	*Vibrio parahaemolyticus*	X:26.93, Y:20.79, Z:35.20
3X0T	PirA	Toxins & toxin-associated regulators	Toxin	*Vibrio parahaemolyticus* M0605	X:31.04, Y:41.10, Z:9.08
3X0U	PirB	Toxins & toxin-associated regulators	Toxin	*Vibrio parahaemolyticus* M0605	X:28.35, Y:-11.036, Z:23.77
5KEV	VtrA/VtrC	Toxins & toxin-associated regulators	Signalling protein	*Vibrio parahaemolyticus* RIMD 2210633	X:210.70, Y:50.70, Z:32.58

and *V. parahaemolyticus* (GenBank accession number: PX462024), were used in this study ensuring biological relevance and public health significance. The bacteria were sub-cultured on tryptic soy broth (TSB) supplemented with 1.5% NaCl for 24 hours at 37°C. The inoculum was adjusted to a final concentration of 5×10^5 CFU mL^{-1} based on optical density measured at 600 nm.[24]

Muller Hilton Broth (MHB) with 1% NaCl was used as test media, and two-fold serial dilutions were prepared, yielding a concentration range of 1000-7.8 µg/mL. Tetracycline was used as a positive control, while the negative control was 50% DMSO (5% inside well). The MIC was determined qualitatively by adding 0.01% resazurin dye prepared in sterile distilled water, and the results were then reported in µg/mL.[25]

The wells that showed MIC were then streaked on Muller -Hinton Agar (MHA) with 1% NaCl and incubated at 37° C for 24 hours. Plates yielding no visible growth were taken as the MBC, and the results were reported in µg/ mL.[25] A fatty acid was interpreted as bactericidal if the MBC/MIC ratio was ≤4 and bacteriostatic if otherwise.[27]

RESULTS

In-silico screening of anti-vibrio activity of selected fatty acids

In-silico screening showed that oleic acid (OA) exhibited the lowest binding energy, followed by stearic acid (SA), palmitic acid (PA), and lauric acid (LA), respectively (Figure 1a), indicating potential high binding affinity. In terms of functional system, the FAs showed higher binding affinity to toxin-associated proteins, followed by nutrient acquisition and quorum sensing & signalling. In contrast, lower binding affinities were observed against motility, biofilm formation and antibiotic resistance protein receptors indicated by the higher binding energies. In terms of functional class, the FA had lower binding energy and potentially higher binding affinity towards peptide-binding proteins, metal transporters, toxins, and transferase related receptors.

This preliminary pattern identification was followed by statistical analysis to determine significant differences. Univariate ANOVA was used to determine main factors affecting binding energy showed that FA ($p<0.001$), functional system ($p<0.001$) and functional classes ($p<0.001$) significantly affected binding energy. With regards to FAs, OA and SA showed significantly lower binding energy than LA and PA ($p<0.05$), while OA and SA had similar binding energy ($p>0.05$) (Figure 1a). Functional system also differed significantly where toxin and toxin-associated regulatory proteins were significantly lower that all other system ($p<0.001$) (Figure 1b). This was followed by nutrient acquisition which had similar binding energy to quorum sensing and signalling receptors ($p>0.05$). Motility and flagellar assembly associated receptors showed the highest binding energy, which indicates a significantly lower binding affinity ($p<0.05$).

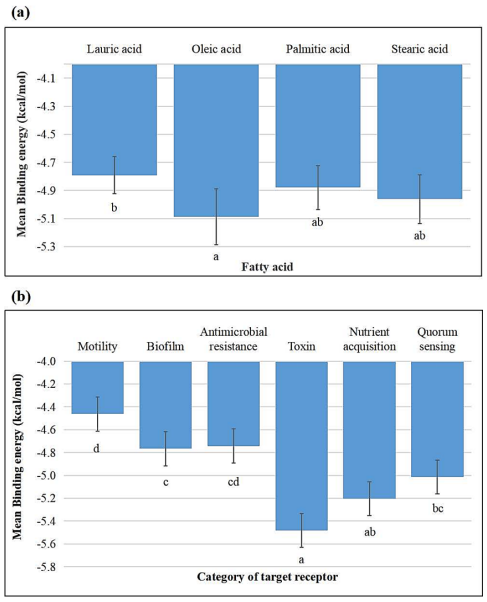

Figure 1 Mean binding energy (kcal/mol) of different fatty acids **(a)** and **(b)** across functional system of target receptors. Significant differences were determined using Univariate ANOVA and Tukey's post hoc test. Different letters (a, b, c, d) indicate statistically significant differences ($p<0.05$).

In terms of protein functional class, significant differences were observed between functional classes and post-hoc analysis showed that peptide-binding proteins, metal transporters, toxins, and transferase related receptors showed lower binding energies ($p<0.05$), which indicated potentially higher affinity to tested FAs (Table II). In contrast, hydrolase related receptors showed significantly higher binding energy compared to all other classes ($p<0.01$), followed by transcription regulators,

membrane proteins, cell adhesion and motor protein, indicating potentially a very poor binding affinity to those classes.

Overall, these results suggest that the selected FAs have potentially high binding affinity to proteins commonly involved in ligand recognition, environmental sensing, and interaction with small molecules. Hence, these FAs might have the potential as modulators of signalling or virulence pathways. In contrast, the FAs showed poor potential in binding catalytic enzyme (e.g. hydrolase) and structural protein.

Table II. Mean binding energy (kcal/mol) of different fatty acids across functional classes of target receptors

Functional Class	Binding Energy (kcal/mol, mean ± SD)
Cell adhesion	-4.68±0.39^{d}
Hydrolase	-4.22±0.21^{e}
Membrane protein	-4.63±0.31^{d}
Metal transport	-5.33±0.36^{ab}
Motor protein	-4.71±0.24^{d}
Peptide-binding protein	-5.39±0.28^{a}
Signalling protein	-5.01±1.03^{bc}
Structural protein	-5.08±0.37^{bc}
Toxin	-5.11±0.47^{abc}
Transcription regulator	-4.54±0.17^{d}
Transferase	-5.24±0.33^{abc}

*Values are presented as mean ± SD. Means sharing different superscript letters are significantly different (Univariate ANOVA followed by Tukey's HSD test, $p<0.05$).

The number of hydrogen bonds and van der Waals interactions exhibited similar patterns across all FAs ($p>0.05$) (Table III). LA exhibited the highest number of hydrogen bonds, followed by OA, whereas stearic acid demonstrated the strongest van der Waals interactions, followed by PA. This indicated that longer chain fatty acids showed more hydrophobic interactions, where shorter chain (LA) and unsaturation (OA) increased hydrophilic interactions. Spearman's rho test showed a weak but significant negative correlation between binding energy and van der Waals interaction energy (ρ=-0.297, $p<0.001$), while number of hydrogen bonds did not correlate with binding energy (ρ=-0.018, $p>0.05$). This indicated that van der Waals interactions were the main factor influencing binding affinity.

Table III. Hydrogen bond and van der Waals interactions of different fatty acids across all tested receptors

Fatty Acid	Hydrogen Bonds			van der Waals interactions		
	Median (IQR)	χ^2	*p*-value	Median (IQR)	χ^2	*p*-value
Lauric Acid (LA)	2 (1)	13.993	0.301	7 (4)	43.410	0.411
Oleic Acid (OA)	2 (1)			8 (4)		
Palmitic Acid (PA)	1 (1)			8 (4)		
Stearic Acid (SA)	1 (1)			9 (4)		

BIOVIA Discovery Studio Visualizer was used to visualize binding pocket of the FAs against selected receptor. The fatty acids produced the lowest binding energy when docked against PDB 5KVE. Superimposition analysis showed that the optimal docking poses of all four FAs occupied the same binding pocket in 5KVE (Figure 2), indicating a shared binding site preference.

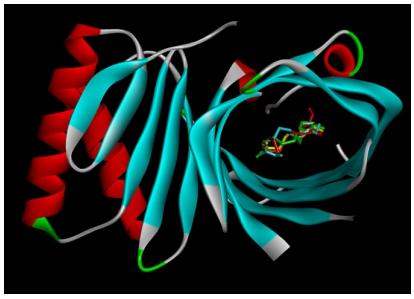

Figure 2 Superimposed structures of lauric acid (yellow), oleic acids (red), palmitic acid (cyan) and stearic acid (green) within the binding site of 5KVE showing similar binding pocket

Despite occupying the same binding site, the molecular interactions between the four FAs and 5KVE differed (Figure 3). LA was limited to alkyl and π–alkyl interactions, whereas PA and SA were limited to van der Waals interactions. In contrast, OAs demonstrated all three interaction types, indicating a comparatively broader interaction profile and supporting its intermediate binding behaviour among the tested fatty acids.

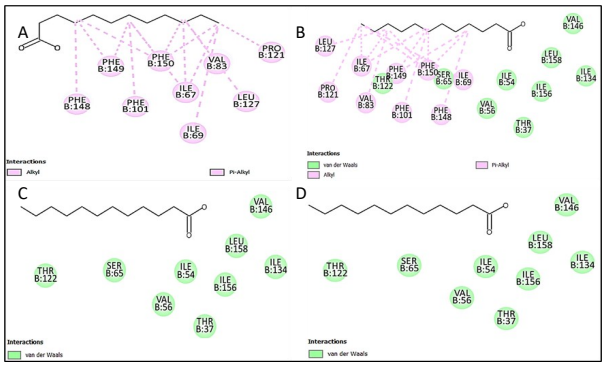

Figure 3 Interactions of lauric acid (a), oleic acids (b), palmitic acid (c) and stearic acid (d) within the binding site of 5KVE showing different interactions within the same binding pocket

In-vitro screening of anti-vibrio activity of selected fatty acids:

In-vitro screening was used to validate *in-silico* predictions based on MIC and MBC. OA and LA were the most effective in inhibiting the tested *Vibrio* spp.., with MIC values of 125 µg/ml and 500 µg/ml, respectively (Table IV). Meanwhile, the lowest MIC for PA and SA were

1000 µg/ml (Figure 4a). The MBC analysis indicates that OA was the most effective compound in killing *V. alginolyticus*, with the lowest observed MBC at 500 µg/mL. Meanwhile, no MBC was obtained against *V. parahaemolyticus* at the highest tested concentration of 1000 µg/mL (Figure 4b). This highlights OA's strong bactericidal activity, making it the most potent among the tested FAs. LA and PA also demonstrated bactericidal properties, effectively killing the *V. alginolyticus* strain, though at a higher concentration than OA.

Table IV. MIC and MBC assay, screened on different concentrations of selected fatty acids against *Vibrio* spp.

Species	Fatty acid	MIC (µg/mL)	MBC (µg/mL)	Category
Vibrio alginolyticus	Lauric acid	500	1000	Bactericidal
	Oleic acid	125	500	Bactericidal
	Palmitic acid	1000	1000	Bactericidal
	Stearic acid	1000	-	Bacteriostatic
	Tetracycline (control +)	<.7.8	<7.8	Bactericidal
Vibrio parahaemolyticus	Lauric acid	500	-	Bacteriostatic
	Oleic acid	125	-	Bacteriostatic
	Palmitic acid	1000	-	Bacteriostatic
	Stearic acid	1000	-	Bacteriostatic
	Tetracycline (control +)	<7.8	<7.8	Bactericidal

(-): No inhibition.

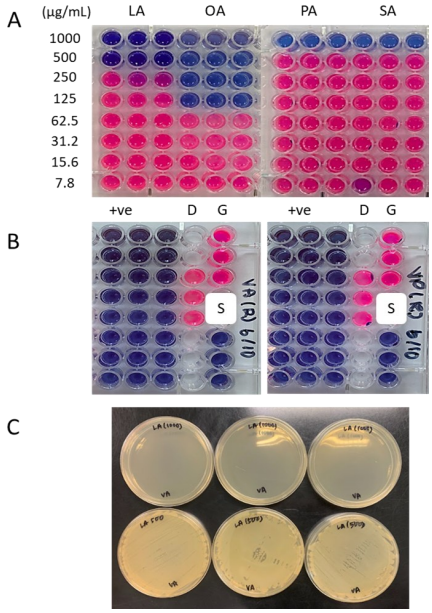

Figure 4, (A) MIC determination of Lauric acid, Oleic acid, Palmitic acid, and Stearic acid against *Vibrio alginolyticus* and *Vibrio parahaemolyticus* using a 96-well plate. (B) MIC determination of control samples where +ve: Positive control (Tetracycline), D: Negative control 50%DMSO, G: Growth control, S: Sterility control. Colour change from blue to pink indicates bacterial growth, while wells that remain blue indicate inhibition. Representative agar plates showing MBC results of Lauric acid against *V. alginolyticus* where 1000 µg/mL showed no growth, indicating bactericidal, while 500 µg/mL shows bacteria regrowth

DISCUSSION

Fatty acids (FAs) are well known for their antimicrobial properties against viruses, fungi, parasitic hosts, and bacterial infection.[11,26] However, research suggests that their antibacterial efficacy is limited to gram-positive bacteria, while they are less effective against gram-negative bacteria due to the presence of the outer membrane.[28] Few studies showed that FAs can be effective against gram-negative bacteria, including *Pseudomonas aeruginosa*, *Helicobacter pylori*, *Escherichia coli,* and *Vibrio* spp..[11]

The efficacy of FAs against gram-negative bacteria was previously linked to several factors, including carbon chain length, degree of saturation, and solubility.[29] In this study, the effect of carbon chain length and saturation on the efficacy of selected FAs against *Vibrio* spp. was investigated. The selected FAs chain length (12 to 18 carbons) was generally classified as long-chain FAs (LCFA) with carbon atoms between 12-26.[30] *In-silico* screening showed that OA had the lowest binding energy, followed by PA and LA (Figure 1). There was also a lack of conventional hydrogen bonds that usually promote stabilization. This was not according to the expected as the presence of several hydrophobic interactions in molecular docking might interfere with the hydrogen bond that improves the binding affinity and reduces the binding energy.[31] LCFA might have produced lower binding energy compared to LA due to the length of their carbon chain that favours hydrophobic interactions which are abundant in tested receptors. OA exhibited the highest affinity among molecules with the same hydrocarbon chain length. This can be linked to the presence of double bonds, which affect the orientation (cis- or trans-), which leads to different conformations and formation of new interactions with receptors.[17] Vaidyanathan et al. (2023) reported similar results where phenolic acid derivatives were tested against human serum albumin and reported that stability of molecular complexes was affected the both hydrogen bonding and hydrophobic interactions.[32] However, hydrophobicity is also critical factor that limits FAs' ability to access bacterial cells in an aqueous environment.[33] Hence, LCFA were expected to perform poorly *in-vitro* by

yielding weak antibacterial activity compared to LA.

The use of Universal Force Field (UFF) in this study was important due to the large number of receptors and their diversity. Despite Merck Molecular Force Field (MMFF94) being more suitable for organic compounds, UFF is computationally less intensive. A key disadvantage of UFF its ability to accurately predict hydrogen bonds[34], however hydrogen bonds were not expected to play major role in the docked fatty acids. UFF are also commonly applied when using PyRx,[35] which were used in this study.

In-vitro screening showed that among saturated FAs, OA (C18:1) exhibited the lowest MIC of 125 µg/mL, followed by LA (C12:0) with MIC of 500 µg/mL. PA (C16:0) and SA (C18:0) had an MIC of 1000 µg/mL (Table IV). Generally, MIC values below 100 µg/mL are considered "good" antibacterial agents, however, none of the fatty acids tested reached this effectivity. An MIC ranging from 100-500 µg/mL they are considered "moderate". MIC values between 500 and 1000 µg/mL are generally regarded as weak, while above 1000 µg/mL are considered inactive.[36] In terms of MBC, OA also had the best MBC 500 µg/mL, LA and PA gave best MBC 1000 µg/mL, while SA did not yield an MBC against either *Vibrio* spp.. LA, PA, and SA behaved as expected *in-vitro* (MIC and MBC) where the increase in carbon chain length has drastically reduced the antibacterial activity of the FAs, with the exception of OA. This could be due to the decreased solubility and therefore fewer interactions with the bacterial cell wall.[29] The primary antibacterial mechanism of FAs is known to involve the integration of the hydrophobic fatty acid alkyl chain into the bacterial lipid membrane causing cell lysis and death of bacteria.[35]

OA showed the strongest antimicrobial properties both *in -silico* and *in-vitro* studies against *Vibrio* spp., which may be attributed to its amphiphilic nature and optimal balance between hydrophobic and hydrophilic characteristics. Although OA contains a long hydrophobic C18 chain, the presence of a polar carboxyl group and a cis double bond enhances molecular flexibility and slightly increase hydrophilicity which improves access to the bacterial cell membrane.[29,36] This might have contributed to OA being more effective than the other FAs through the distinct combination of hydrophilic and hydrophobic properties that facilitate its interaction with the membranes of the microbial cell.[30] The presence of double bonds in the structure might indirectly affect bacterial cell membrane by introducing a kink and disrupting lipid packing. Such physicochemical changes ultimately to increased membrane permeability, leakage of intracellular components, and eventually leads to cell death.[24]

Long-chain saturated fatty acids like PA and SA, demonstrated strong performance in *in-silico* studies but exhibited limited efficacy in *in-vitro* experiments. This discrepancy highlights the necessity of validating theoretical drug candidates through experimental *in-vitro* tests because of various external factors, including solubility.[37] OA and LA were able to form hydrogen bonds *in-silico,* which correlated with enhanced performance in *in-vitro* assays. This finding supports previous research that highlights the critical role of hydrophilic interactions in modulating the solubility, membrane affinity, and overall antimicrobial effectiveness.[17] While OA has a cis double bond that can disrupt tight hydrophobic packing, reduce aggregation and improve dispersion in aqueous environments, LA has significantly shorter carbon chain which reduces its the hydrophobicity. A higher number of hydrogen bonds can also facilitate stronger interactions with microbial cell membranes, potentially leading to membrane destabilization and increased permeability. Additionally, improved solubility of the FAs can increase the interaction with bacterial cells, which will enhance the penetration of FAs and accordingly disrupting the essential cellular processes such as enzyme function and nutrient transport.[15]

In terms of species, the *in-vitro* test on the FAs demonstrated greater effectiveness against *V. alginolyticus* than *V. parahaemolyticus*. LA, OA, and PA all showed bactericidal activity against *V. alginolyticus*, while none of the 4 FAs showed bactericidal activity against *V. parahaemolyticus*. This might be a limitation of following

CLSI guideline on using 1% MHA, which might not be suitable for highly halophilic bacteria like *V. alginolyticus*.[38] It is hypothesized that *V. alginolyticus* might have encountered osmotic stress besides the FAs effect which could have led to it being more susceptible towards the FAs.

In terms of possible applications of selected FAs, *in-silico* analysis showed that FAs have preferential binding to proteins commonly involved in ligand or small-molecule binding, such as signalling proteins, transferase enzymes, peptide-binding proteins and metal transport. Li et al. (2022) summarized that disrupting signalling pathways such as N-acyl-homoserine lactone (AHL) results in increased susceptibility to antibiotics, inhibit biofilm formation and deactivation of efflux pumps.[39] The four FAs showed the highest binding affinity to PDB 5KEV, a key signalling protein involved in toxin production *V. parahaemolyticus*. This is particularly important because toxin production is a key in the pathogenicity of *V. parahaemolyticus*. They are used to regulate type III secretion system (T3SS) proteins, which are responsible for diseases such as Acute Hepatopancreatic Necrosis Disease (AHPND) and Early Mortality Syndrome (EMS) in shrimp aquaculture. These characteristics could also be transferred to human through ingestion of the infected shrimp which could led to gastroenteritis and sepsis.[40,41] The infected human could experience bloody diarrhoea, stomach cramps, nausea, and vomiting.[41,42] Besides that, the FAs also showed high affinity to PDB 7AGZ, a key peptide-binding protein involved in biofilm formation of *V. cholerae*. High inhibition potential was also observed against 6PXA, 2HJ9 and 3R5T. PDB 2HJ9 is quorum sensing & signalling protein in LuxP-LuxQ complex from *V. harveyi*.[43] Quorum sensing is a primary mechanism of intercellular communication in bacteria that enables a population of pathogenic bacteria to synchronize their gene expression.[44] It helps facilitate the collective behaviour of the bacteria to evade host immune responses while producing harmful virulence factors, as well as establish antibiotic-resistant biofilms.[45] The PDB 6PXA from *V. fischeri* is a transferase that aid in chloramphenicol resistance,[46,47] while 3R5T is a metal transporter involved in nutrient acquisition of ferric ion in *V. cholerae*.[48] Interfering with iron (Fe) acquisition via siderophores by *Vibrio* spp. is also a promising finding. Metal ions regulate the expression of various genes in *V. parahaemolyticus,* such as swarming and toxin secretion systems (e.g., T3SS).[49] Hence, the tested fatty acids might have various mechanism to potentially inhibit *Vibrio* spp., that could be developed into targeted drugs for prevention and treatment of Vibrio infections.

CONCLUSION

This study investigates the antibacterial activity of lauric acid (LA), oleic acid (OA), palmitic acid (PA), and stearic acid (SA) against *Vibrio* spp. using both *in-silico* and *in-vitro* approaches. Molecular docking predicted that OA exhibited the lowest binding energy, indicating the highest binding affinity with selected *Vibrio* protein receptors. This is despite OA being second-highest in the number of hydrogen bond interactions and third in terms of van der Waals interactions. These results suggest that its binding affinity is linked to the amphipathic nature, which is attributed to its long hydrophobic carbon chain and a hydrophilic carboxyl head. This is further coupled a single degree of unsaturation which give the structure conformational flexibility, making more chemical interactions and enhancing its binding affinity. *In-vitro* validation through MIC and MBC assays supported the *in-silico* predictions. OA demonstrated the lowest MIC of 125 μg/mL against both *Vibrio alginolyticus* and *Vibrio parahaemolyticus*, as well as bactericidal activity against *V. alginolyticus* at 500 μg/mL. Hence, molecular docking using selected receptors, particularly 3MRU, 3WPW, 3A57 and 3X0T, matched *in-vitro* results can be used to screen lead compounds in the future. Further research is recommended to explore its mechanism of action *in-vitro* and its effectiveness *in-vivo*. A positive outcome in future studies could establish an effective and safe alternative to antibiotics for the treatment of vibriosis.

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