Combined effect of linolenic acid and tobramycin on *Pseudomonas aeruginosa* biofilm formation and quorum sensing

WARREN CHANDA[1*], THOMSON PATRICK JOSEPH[1*], ARSHAD AHMED PADHIAR[1], XUEFANG GUO[1], LIU MIN[1], WENDONG WANG[1], SAINYUGU LOLOKOTE[2], ANHONG NING[3], JING CAO[3], MIN HUANG[1] and MINTAO ZHONG[1]

[1]Department of Microbiology, College of Basic Medical Sciences, Dalian Medical University; [2]Department of Epidemiology and Biostatistics, School of Public Health, Dalian Medical University; [3]Laboratory of Pathogen Biology, Experimental Teaching Center for Basic Medical Sciences, Dalian Medical University, Dalian, Liaoning 116044 P.R. China

Received April 6, 2017; Accepted August 22, 2017

DOI: 10.3892/etm.2017.5110

Abstract. *Pseudomonas aeruginosa* is a ubiquitous Gram negative opportunistic pathogen capable of causing severe nosocomial infections in humans, and tobramycin is currently used to treat *P. aeruginosa* associated lung infections. Quorum sensing regulates biofilm formation which allows the bacterium to result in fatal infections forcing clinicians to extensively use antibiotics to manage its infections leading to emerging multiple drug resistant strains. As a result, tobramycin is also becoming resistant. Despite extensive studies on drug discovery to alleviate microbial drug resistance, the continued microbial evolution has forced researchers to focus on screening various phytochemicals and dietary compounds for antimicrobial potential. Linolenic acid (LNA) is an essential fatty acid that possesses antimicrobial actions on various microorganisms. It was hypothesized that LNA may affect the formation of biofilm on *P. aeruginosa* and improve the potency of tobramycin. The present study demonstrated that LNA interfered with cell-to-cell communication and reduced virulence factor production. It further enhanced the potency of tobramycin and synergistically inhibited biofilm formation through *P. aeruginosa* quorum sensing systems. Therefore, LNA may be considered as a potential agent for adjunctive therapy and its utilization may decrease tobramycin concentration in combined treatment thereby reducing aminoglycoside adverse effects.

Correspondence to: Dr Mintao Zhong or Professor Min Huang, Department of Microbiology, College of Basic Medical Sciences, Dalian Medical University, 9 Western Section, Lvshun South Road, Lvshunkou, Dalian, Liaoning 116044, P.R. China
E-mail: dyzhongmt@163.com
E-mail: huangminchao@163.com

*Contributed equally

Key words: Pseudomonas aeruginosa, linolenic acid, biofilm, quorum sensing, tobramycin

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a ubiquitous Gram negative bacterium capable of surviving in several environmental niches such as mammals (including humans), insects, nematodes, soil, water and plants (1,2). In humans, *P. aeruginosa* rarely causes community acquired pneumonia (3,4), but has an increased incidence of causing hospital infections like hospital-acquired pneumonia, sepsis, urinary tract infections, and is prevalent among wound and burn patients (1,5). The bacterium causes severe pulmonary damage and is a leading cause of mortality in cystic fibrosis patients (6-8). This opportunistic pathogen poses a huge challenge in clinical settings as compared to other pathogens because of its highly intrinsic resistance to most antibiotics including β-lactams, fluoroquinolones and aminoglycosides (9-11). Each year, the bacterium is estimated to cause 51,000 healthcare-associated infections out of which 6,000 cases are multidrug resistant with roughly 400 deaths occurring per year in the United States (9). In addition, *P. aeruginosa* has the ability to switch from free-living (planktonic) to biofilm phenotypic mode of living. This ability contributes to antibiotic resistance and is governed by quorum sensing systems that regulate virulence factor production as well (1,12-14). Unlike other pathogens, *P. aeruginosa* has a unique virulence potential, its natural resistance to several antibiotics and other resistance mechanisms like antibiotic modification and energy-dependant drug efflux make infections very problematic to control (15,16). Therefore, there is need to search for more compounds with antimicrobial potential to curb *P. aeruginosa*-related infections.

In the recent past, fatty acids (FA) have received attention because of their antimicrobial and anti-inflammatory properties (17,18). They possess beneficial effects against cancerous cells, body fats, and in conditions such as depression, heart problems, neurodegenerative diseases, joint and bone conditions (19,20). One of the widely discussed fatty acid family based on nutrition and human health benefits is the omega-3 family in which linolenic acid (C18:3n-3, LNA) is a parent fatty acid compound (21). Evidence has shown that LNA and its derivatives, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) possess antimicrobial properties against many

microorganisms (22-26). Thus far, bacterial resistance towards free fatty acids is yet to be reported due to their broader spectrum activities (27). However, the last option of antimicrobial agents for multidrug resistant *P. aeruginosa* related infections include aminoglycosides and polymyxins, which besides losing their efficacy are also accompanied with side effects such as neurotoxicity, nephrotoxicity and ototoxicity (28-30). Nonetheless, these toxic side effects can be managed with reduced concentration and duration of tobramycin therapy. Besides, some studies have shown that phytochemical compounds can produce synergistic and additive effects with aminoglycosides in various bacterial infections thereby improving their (aminoglycosides) efficacious potential (31).

Despite several reports on the antibacterial activity of LNA and its omega-3 derivatives (EPA and DHA), the anti-biofilm mechanism and their interaction with aminoglycosides on *P. aeruginosa* pathogens have not been elucidated. Therefore, the present study aimed at assessing the anti-biofilm activity and mechanism of linolenic acid alone and in combination with tobramycin on *P. aeruginosa*, with the view to evaluating the potency of the compound alone or in combination for treating infections associated with this pathogen.

Materials and methods

Materials. Linolenic acid (Sigma-Aldrich), Azocasein (Sigma-Aldrich), AlarmaBlue cell viability assay kit (KeyGen BioTECH, China), Tobramycin (Solarbio, China), Crystal violet (Solarbio, China), LIVE/DEAD BacLight Bacterial viability kit (Molecular Probes, USA), Biozol Total RNA extraction reagent (BioFlux, China), TransScript Green Two-Step qRT-PCR SuperMix kit (Transgen Biotech, China), Trans2 K DNA ladder (Transgen Biotech, China), 2X Taq PCR MasterMix (TianGen, China), Ethidium bromide (Sigma, China), Cetrimide agar base (Xiya, China), Dimethyl sulfoxide (AMRESCO, Ohio-USA), and any other reagents were of analytical grade.

Bacterial strains. *Pseudomonas aeruginosa* ATCC 27853 strain, and *Pseudomonas aeruginosa* clinical strains were collected from the Laboratory of Pathogen Biology, Experimental Teaching Center for Basic Medical Sciences, Dalian Medical University while environmental strains were isolated from beach soil, water pond soil and garden soil in different locations on Dalian Medical University campus.

Primer design. The quantitative real time polymerase chain reaction (qRT-PCR) primers used in this study were designed with an online Primer-Blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/; accessed March 11, 2016) whereas identification primers for *P. aeruginosa* were those published by Jaffe *et al* (32) (Table I) and all were synthesized by Invitrogen Biotechnology Co. Ltd, China.

Culture and identification of bacteria strains. Clinical isolates of *P. aeruginosa* and the ATCC strain were inoculated on cetrimide agar and incubated at 37˚C for 24 h. For environmental strains, 1 g of soil was dissolved in 1 ml PBS, pH 7.4 with vigorous shaking and centrifuged at 5,000 g for one minute then 0.1 ml was inoculated on cetrimide agar and incubated at 37˚C for 24 h. A bacterial suspension was prepared by dissolving a single colony from an overnight culture plate into 50 μl PBS pH 7.4 from which, a) 30 μl was added to a 15 ml-tube containing 3 ml fresh LB media for overnight culture at 37˚C with 180 rpm shaking, then stored in 8% (v/v) glycerol at -80˚C for use in subsequent assays; b) 1 μl was used for identification with duplex colony polymerase chain reaction (PCR).

Duplex colony PCR. The PCR conditions were performed according to Jaffe *et al* (32) with modifications. Briefly, a 10 μl reaction mixture was prepared to consist of 1 μl bacteria suspension, 0.5 μM forward and reverse primers apiece, 5 μl of 2X Taq PCR MasterMix, 5% Dimethyl sulfoxide (DMSO) and 1.5 μl distilled water to bring the total volume to 10 μl. DMSO was included to improve primer binding specificity during amplification since *Pseudomonas* species have a high GC content. The thermal cycling conditions were set as follows: Initial denaturation at 94˚C for 3 min, a 30 cycle involving denaturation at 94˚C for 30 sec, annealing at 58˚C for 30 sec and extension at 72˚C for 30 sec, and a final extension at 72˚C for 45 sec. After thermocycling, 10 μl was then loaded into a 1% (w/v) agarose gel and the electrophoresis was run for 40 min at 70 volts in 1X Tris-acetate-EDTA (TAE) Buffer mixed with 0.2 mM ethidium bromide, then the images were taken by ChemiDoc XRS$^+$ machine (Bio-ad).

Minimum inhibition concentration determination. The minimum inhibition concentration (MIC) of linolenic acid (LNA) and tobramycin (TOB) were performed following CLSI recommendation using the macrodilution (Tube) broth method and broth microdilution method, respectively (33). The stock solution of LNA (100 mg/ml) was prepared in DMSO and a surfactant Tween 20 (2% final concentration) was added for the uniform distribution of LNA and two-fold serial dilutions were made. Firstly, an overnight *P. aeruginosa* culture was diluted to an optical density (OD) of 0.1 at 600 nm (Multiskan Ascent Microplate Reader, Thermo Scientific) with fresh LB broth (~10^8 CFU/ml) and 10 μl of bacteria suspension (to achieve a final bacterial concentration of ~10^6 CFU/ml) was added to the test tubes containing 1 ml media with serial dilutions of LNA, incubated at 37˚C for 24 h with gentle shaking at 110 rpm. Similarly, a two-fold dilutions of TOB from 20 mg/ml to 0.0391 mg/ml were prepared in a 96-well plate and incubated at 37˚C for 24 h. The highest dilution of LNA or TOB showing visible inhibition of bacterial growth after 24 h of incubation was taken as MIC of the drug. Three independent assays were performed.

Growth curve analysis. To understand the activity of LNA alone or in combination with TOB on the *P. aeruginosa*, sub-MIC concentrations of both LNA and TOB were selected for this study and were assessed through the growth curve analysis as described by Kalia *et al* (34). Briefly, overnight bacteria culture was inoculated into 10 ml of LB broth supplemented with five different sub-MIC concentrations of LNA (*i.e*. 3/4, 5/8, 1/2, 3/8 and 1/4 of MIC) and TOB (1/4 of MIC). The flasks were incubated at 37˚C and OD_{600} was monitored at time intervals 0, 3, 6, 9, 12, 18 and 24 h.

Biofilm control assay. As described elsewhere (34,35), *P. aeruginosa* biofilm inhibition and biofilm metabolic

Table I. Primer list for PCR and RT-qPCR.

Target gene	Primer name	Sequence (5'-3')	Amplicon (bp)	(Refs.)
oprL (PA0973)	oprL-Forward	ATGGAA ATGCTGAAATTCGGC	504	(32)
	oprL-Reverse	CTTCTTCAGCTCGACGCGACG		
16SrRNA	16SrRNA-Forward	GAGGAAGGTGGGGATGACGT	233	(32)
	16SrRNA-Reverse	AGGCCCGGGAACGTATTCAC		
lasI (PA1432)	LasI-Forward	TGAAGCCCAGGTTTTCGGTT	131	This study
	LasI-Reverse	AACGGCTGAGTTCCCAGATG		
lasR (PA1430)	LasR-Forward	TCGAACATCCGGTCAGCAAA	128	This study
	LasR-Reverse	GTTCACATTGGCTTCCGAGC		
rhlI (PA3476)	RhlI-Forward	CATCCGCAAACCCGCTACAT	124	This study
	RhlI-Reverse	GGGTTTCGCTGCACAGGTA		
rhlR (PA3477)	RhlR-Forward	GAAATGGTGGTCTGGAGCGA	132	This study
	RhlR-Reverse	GGAAAGCACGCTGAGCAAAT		
pilJ (PA0411)	pilJ-Forward	GACAAGCAGTACATCGGCCA	137	This study
	pilJ-Reverse	CGTTTCTCGAAGTCGTTGCG		
algU (PA0762)	algU-Forward	CCATCAACACCGCGAAGAAC	148	This study
	algU-Reverse	ATCTCATCCCGCAACATCGC		
pqsH (PA2587)	pqsH-Forward	AGACGCTGATCCTGTTCCAG	131	This study
	pqsH-Reverse	GCGAACGAGGGTATTCCTCA		
mvfR (PA1003)	mvfR-Forward	TCGTTCTGCGATACGGTGAG	136	This study
	mvfR-Reverse	CGTCGATGGTGATGGCGATA		
oprF (PA1777)	oprF-Forward	CAGTACCCGTCCACTTCCAC	146	This study
	oprF-Reverse	TTCACGCGACCACCTTCTAC		
oprI (PA2853)	oprI-Forward	AGAAACCGAAGCTCGTCTGA	137	This study
	oprI-Reverse	CGTTAGCCTCGTCAGCAGT		
phzR/qscR (PA1898)	phzR-Forward	GCTGACCGCGCCTAAATATC	134	This study
	phzR-Reverse	TCCAGATCAGCGGGGTGTAT		
lasB (PA3724)	lasB-Forward	TGTCCAAACTCCCCAGCAAG	149	This study
	lasB-Reverse	GAATTGCTCGTAGCGGGTGA		

activity quantification were assessed in the presence/absence of LNA, TOB or in combination (LNA+TOB). Briefly, one microliter of overnight bacteria suspension adjusted to OD_{600} of 0.1 (~10^8 CFU/ml) was added to a well in a microtiter plate containing 100 μl of fresh LB media mixed with appropriate doses of drugs, and this plate was incubated at 37˚C for 24 h from which subsequent assays were performed as follows:

Biofilm mass quantification. After incubation, the unattached cells were gently aspirated and discarded, and the wells were washed with 0.85% sodium chloride (normal saline) twice and stained with 200 μl of 0.1% crystal violet for 15 min. The stain was discarded, and the wells were washed with distilled water, air dried and the dye was re-solubilized with 160 μl of 33% (v/v) glacial acetic acid (36). The OD was measured at 570 nm using Multiskan Ascent Microplate Reader (Thermo Scientific). Biofilm inhibition was given by:

$$\%BI = \{(OD_C - OD_T) / OD_C\} \times 100.$$

where %BI is the percentage of biofilm inhibition, OD_C is the 570 nm absorbance value of untreated sample and OD_T is the 570 nm absorbance value of treated sample (35).

Biofilm metabolic activity quantification. In order to assess the bacterial activity of biofilm cells, the alamarblue (7-hydroxy-3H-phenoxazin-3-one-10-oxide) cell viability assay was applied as a commended, reliable and reproducible assay for assessing biofilm susceptibility (35,37). After incubation as mentioned above, the supernatant was removed and the wells were gently washed twice with LB media. Then, 200 μl fresh LB media containing 10% (v/v) alarmablue staining reagent was added and incubated at 37˚C for 5 h, as recommended by the manufacturer (KeyGen Biotech, China). The absorbance was then measured at 570 nm and Biofilm inactivation was given by:

$$\%BI = \{(A_C - A_T) / A_C\} \times 100.$$

where %BI is the percentage of biofilm inactivation, A_C is the 570 nm absorbance value of the untreated sample and A_T is the 570 nm absorbance value of treated sample (35).

Microscopic analysis. To visualize the effect of biofilm formation in the presence/absence of sub-MIC doses of LNA, TOB or LNA+TOB, microscopic analysis of *P. aeruginosa* clinical strain C2 biofilms was performed as described previously (11,38,39) with modifications. Briefly, 10 μl of an

overnight culture as described above, was added to 1 ml LB media supplemented with appropriate sub-MIC doses in a 24-well plate. Positive control wells contained media supplemented with the greatest concentration of DMSO used (0.75%) while the negative control wells contained media only (for sterility check), and the plate was then incubated at 37˚C for 24 h. Afterwards, the wells were gently washed with sterile normal saline and 200 μl of staining solution in LB media containing 2.09 μM of Syto 9 and 12.5 μM of Propidium iodide (PI) using LIVE/DEAD BacLight Bacterial Viability Kit L7012 was added to each well, incubated at room temperature for 15 min in the dark and images were captured with an inverted fluorescence microscope (Olympus IX71).

Synergy analysis. The interaction of LNA with TOB was analyzed by a checkerboard method in an 8x8 arrangement using a 96-well plate as previously described (24). Briefly, biofilms were allowed to build up for 24 h and then wells were gently washed with sterile LB medium and later supplemented with LB medium consisting of different combinations of LNA and TOB. The combinations were done in such a way that a fixed dose of one agent and increasing doses of the second agent was put in each column (or row). Then incubated at 37˚C for 24 h, washed gently and the biofilm metabolic activity was assessed with alamarblue staining reagent for assessing biofilm susceptibility (35,37). For all the wells with combined drug concentrations, the sum of the fractional inhibitory concentrations (FIC) index was calculated according to the equation below:

$$\text{FIC index} = FIC_A + FIC_B = A / MIC_A + B / MIC_B.$$

where A and B are the MICs of LNA (A) and TOB (B) in combination, MIC_A and MIC_B are the MICs of LNA and TOB alone, FIC_A and FIC_B are the FICs of LNA and TOB, respectively. The FIC index results were interpreted as synergistic effect (FIC index ≤0.5), additive or indifferent effect (FIC index >0.5 and ≤1) and antagonistic effect (FIC index >1) (40).

Inhibition of virulence factors mediated by QS

Swarming assay. The swarming ability of *P. aeruginosa* C2 strain was investigated in small 35x10 mm plates containing swarming motility media 0.5% (w/v) Bacto agar, 0.5% (w/v) peptone, 0.2% (w/v) yeast extract and 1.0% (w/v) glucose (41). A 2.5 μl aliquot from an overnight culture in the presence/absence of drug was spotted at the center of the agar surface and the plates were incubated at 37˚C for 24 h, and later 2 h at room temperature (42) and the diameters were measured. Three independent assays were performed.

Pyocyanin production. As described by Das *et al* (11) and Kalia *et al* (34), the pyocyanin production was assayed by collecting supernatants from overnight cultures of *P. aeruginosa* grown in the presence/absence of sub-MIC doses of LNA, TOB and LNA+TOB at 37˚C for 24 h. A 5 ml culture supernatant was extracted with 3 ml of chloroform and then with 1 ml of 0.2 N hydrochloric acid to produce an orange-yellow to a pink colored solution which was measured at 520 nm.

LasA staphylolytic assay. In the presence/absence of LNA, TOB and LNA+TOB drugs, the ability of *P. aeruginosa* culture supernatants to lyse boiled *Staphylococcus aureus* cells were determined by LasA protease activity (11). An overnight culture of *S. aureus* cells (OD_{595} of 1.0) was centrifuged at 7,000 rpm for 3 min and the pellet was suspended in 0.02 M Tris-HCl (pH 8.5) then boiled for 10 min and diluted with the same buffer to an OD_{595nm} of 0.8. Diluted *S. aureus* suspension was added to each cell-free culture supernatant of *P. aeruginosa* in the ratio 9:1 and the absorbance was measured at 595 nm after 0, 15, 30, 45 and 60 min.

Azocasein protease assay. The assay was performed to study the effect of protease production by C2 strain in the presence/absence of LNA, TOB and LNA+TOB. This was performed following methods published elsewhere with minor modifications (11,34). Briefly, cell-free supernatant was collected from centrifuged overnight cultures in the presence/absence of drugs at 10,000 rpm for 5 min. 150 μl of supernatant (in absence/presence of the drug) was added to 1 ml of 0.3% azocasein in 0.05 M Tris-HCl (pH 7.5) and incubated at 37˚C for 15 min. The reaction was then stopped with 0.5 ml 10% trichloroacetic acid and the mixture was later centrifuged at 10,000 rpm for 5 min. The clear supernatant was collected and absorbance was measured at 420 nm.

Quantitative real-time polymerase chain reaction (qRT-PCR). The real-time PCR reactions for quantification of *Pseudomonas aeruginosa* quorum sensing target genes (*lasI, lasR, rhlI, rhlR*,), virulence factor-related genes, and the reference gene (*16S rRNA*) were performed on ABI StepOne analyser using TransScript Green Two-Step qRT-PCR SuperMix kit following the manufacturer's instructions (Transgen Biotech, China) with the primers listed in Table I. Firstly, *P. aeruginosa* was cultured in the absence/presence of sub-MIC concentrations of LNA, TOB and LNA+TOB for 24 h at 37˚C. Total RNA was then extracted using biozol total RNA extraction reagent following the manufacturer's instructions (BioFlux, China) and the concentration was measured with NanoDrop 2000C (Thermo Scientific). One microgram of the respective extracted RNA was used for cDNA synthesis using TransScript Green Two-Step qRT-PCR SuperMix kit as per manufacturer's instructions. Secondly, the synthesized cDNA was diluted ten-fold (1:10 ratio) and one microliter of diluted cDNA was mixed with 0.2 μM forward and reverse primers apiece, 10 μl of 2X TransStart Tip Green qPCR SuperMix, 1X passive reference dye I and water to a total volume of 20 μl as per instructions. The dissociation stage consisted of 94˚C for 30 sec, followed by 40 cycles involving 94˚C for 5 sec, 58˚C for 15 sec, and 72˚C for 10 sec. Target gene primers were designed to give products between 120 and 150 bp, and the *16SrRNA* gene was used as an internal control. Each qRT-PCR run was performed in triplicate and three independent experiments were performed. The calculated threshold cycle (Ct) was normalized to the Ct of *16SrRNA* amplified from the corresponding sample. Finally, the relative quantification of target transcripts were calculated by the comparative $2^{-\Delta\Delta Ct}$ method (43).

Statistical analysis. To gain statistical significance, each experiment was performed at least in triplicate. Data values of experimental results were recorded as mean ± SEM or median with interquartile range. Significance was determined by using analysis of variance (one- and two-way), and cited as P<0.05 (*), P<0.01 (**) and P<0.001 (***). Statistical analyses were performed using GraphPad Prism 5.0 statistical software.

Results

Antimicrobial and anti-biofilm effects of linolenic acid and tobramycin on Pseudomonas aeruginosa isolates. The present study examined five clinical strains, five environmental strains, and one standard (ATCC 27853) strain. The clinical and environmental strains were identified by duplex colony PCR using intragenic primer sets for bacterial *16S rRNA* and the peptidoglycan-associated lipoprotein (*oprL*: PA0973) gene sequences for specific detection of *P. aeruginosa* (data not shown).

Several reports suggest that FA of the omega-3 family are effective on various pathogenic microorganisms (22,23,25,26,44). We therefore attempted to assess the antimicrobial activity of LNA on *P. aeruginosa* ATCC 27853, clinical and environmental strains. The median MIC values of LNA and TOB were 1.56 and 0.3125 mg/ml, respectively. Due to this anti-pseudomonas activity, we hypothesized that LNA can affect biofilm formation when used alone or in combination with TOB. Therefore, five sub-MIC doses of LNA (1.17, 0.975, 0.78, 0.585 & 0.39 mg/ml), and 1/4th MIC (0.078 mg/ml) of TOB were selected for this study and their activity on planktonic cells were assessed through the growth curve analysis. The growth curve results showed nonsignificant results on planktonic cells (Fig. 1), but we went on to evaluate the anti-biofilm activities.

Interestingly, the ATCC strain was highly sensitive to TOB and LNA+TOB treatment in comparison with clinical and environmental stains (Fig. 2). An identical dose dependant pattern was exhibited in LNA+TOB and LNA groups with their respective highest median inhibitory percentage being 48 and 38% (Fig. 2A). However, clinical strain C2 was noticed with extreme resistance to TOB dosage used while the rest of the strains were significantly affected (P<0.001, Fig. 2B). The moderate inhibitory effect of LNA alone or in combination (Fig. 2A) provoked further analysis of biofilm cells. We attempted to assess the metabolic effect with alarmablue staining reagent to quantify the viability of cells (37). The assay revealed a significant inactivation of biofilm cells (P<0.001, Fig. 2C); 60% inactivation with LNA+TOB involving 1.17 mg/ml dosage, 50% for LNA (1.17 mg/ml) alone and 42% for TOB alone (Fig. 2C). The slight difference between the two assays (inhibition and inactivated) was probably due to their different staining principles. Unlike alarmablue reagent, crystal violet staining does not differentiate live cells from dead cells but stains every attached biofilm cell whether live, about to die or dead ones, thus giving a slight difference between the two assays. After analyzing the inhibition and inactivation effects, *P. aeruginosa* clinical strain C2 was seen with more resistance especially with TOB (Fig. 2B and D). Therefore, we hypothesized that understanding the anti-biofilm action of LNA alone or in combination treatment could be better in a strain where TOB is exerting an insignificant effect. Moreover, the ATCC strain very sensitive to the selected sub-MIC doses that further dilution was required to re-establish its MIC doses (data not shown). So, clinical strain C2 was selected for microscopic evaluation, virulence factor production and gene expression with qRT-PCR.

Table II. Interaction study between LNA and TOB.

P. aeruginosa strain	Combined doses (LNA+TOB) (mg/ml)	FIC index	Interpretation
C2	1.17+0.078	1.00	Additive
C2	0.78+0.078	0.75	Additive
C2	0.39+0.078	0.50	Synergy

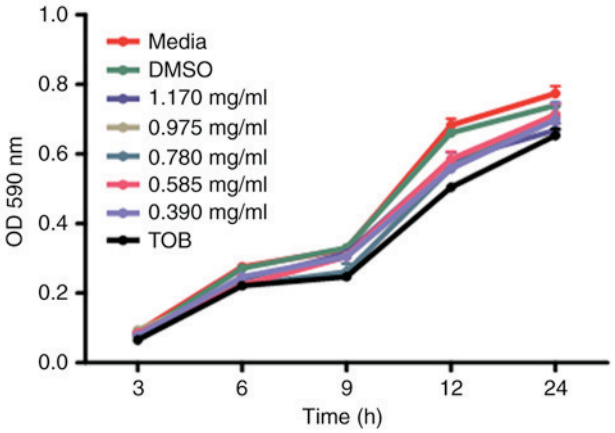

Figure 1. Growth curve evaluation with sub-MIC concentrations on *Pseudomonas aeruginosa* C2 strain. Media (positive control), DMSO (solvent control), sub-MIC doses of LNA (1.17-0.39 mg/ml), and TOB (0.078 mg/ml). Each value is an average of triplicate experiments where data are presented as mean ± SEM and three independent assays were performed. TOB, tobramycin; LNA, linolenic acid.

Microscopic visualization was performed with a fluorescence microscope (Olympus IX71). Images of the untreated control showed robust biofilm biomass but slightly reduced in TOB treated cells. LNA treated cells managed to reduce the biofilm formation by disintegrating the biomass while combination treatment exhibited strong inhibitory activity (Fig. 3). This effect reduced significantly in a dose-dependent manner in combined treatment groups. The findings here were in agreement with the biofilm inhibition (Fig. 2A) and inactivation (Fig. 2C) assays which also showed similar effects with reference to drug concentration used. Moreover, the interaction effect between LNA and TOB determined by the FIC index showed additive and synergistic effects on C2 strain (Table II), which could explain the strong attenuation effects of biofilm in combined treatment with reference to untreated control cells.

Effect of linolenic acid and tobramycin on virulence factor production. *P. aeruginosa* is known to produce virulence factors that include pyocyanin and protease production that tends to counteract host defenses and can directly damage

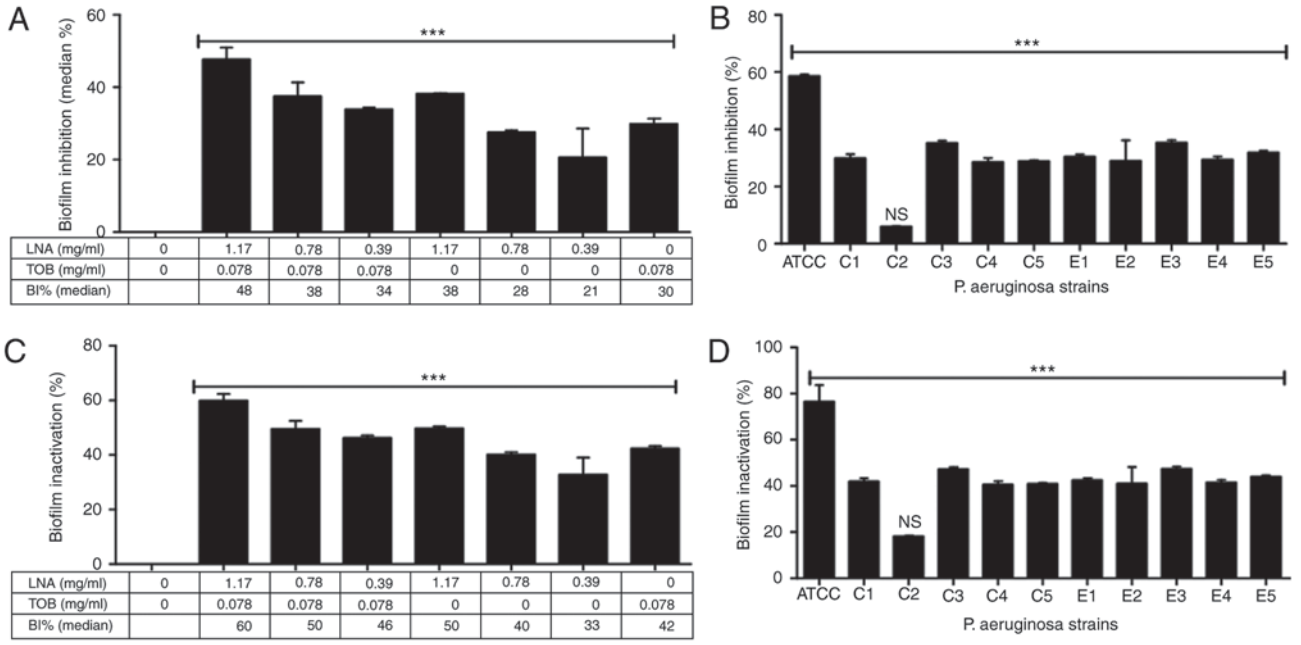

Figure 2. Biofilm inhibition and inactivation effects of linolenic acid, tobramycin, and LNA+TOB on *P. aeruginosa* strains. (A) Median inhibition percentages of all strains with respect to sub-MIC doses, (B) the inhibitory effect of TOB alone, (C) the median inactivation percentages of all strains with respect to sub-MIC doses, and (D) the inactivation effect of TOB alone are presented. The data are presented as median with interquartile ranges. LNA, linolenic acid; TOB, tobramycin; ATCC, ATCC 27853 strain; C, clinical strains; E, environmental strain; and ***P<0.001. Three independent assays were performed.

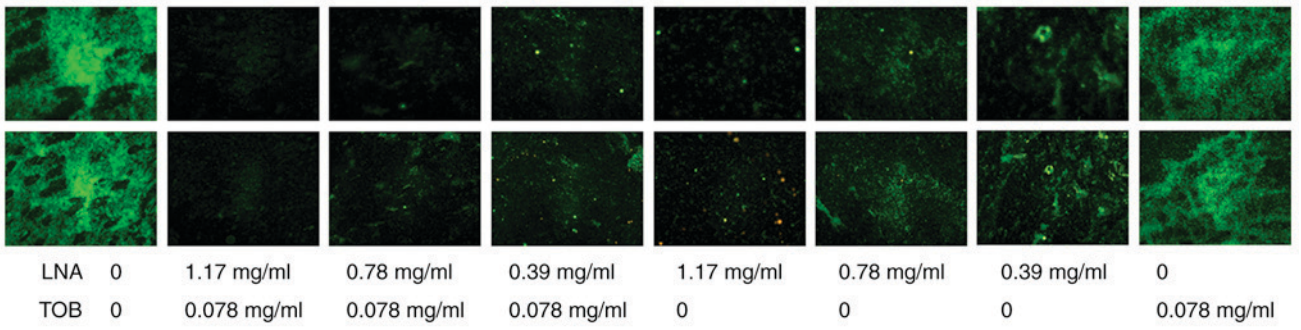

Figure 3. Effects of linolenic acid, tobramycin, and LNA+TOB on biofilm formation in a 24-well plate. Biofilm cells were stained with SYTO9/PI staining solution and images were captured with a fluorescence microscope (Olympus IX71). LNA, linolenic acid; TOB, tobramycin.

host tissues (45). Since virulence factor production and biofilm formation are controlled by the QS systems, we hypothesized that LNA alone or in combination with TOB can attenuate virulent factor production. So we assessed swarming motility, pyocyanin production, lasA and azocasein activities on *P. aeruginosa* strain C2.

Swarming motility. Swarming motility is the ability of bacteria to move across a semisolid surface and *P. aeruginosa* uses a flagellar motility (42) and participates in increasing production of virulence factors and antibiotic resistance (46). We found that the treated group in comparison with the untreated control inhibited the swarming movement of *P. aeruginosa* C2 strain (Fig. 4). Interestingly, all the sub-MIC doses used in combination and single treatment showed significant inhibition effect on swarming motility with reference to the untreated control cells of *P. aeruginosa* C2 strain (P<0.001, Fig. 4).

Virulence factor production. *P. aeruginosa* virulence and pathogenesis include secretion of virulence factors such as pyocyanin and pyoverdine. Pyocyanin disrupts the redox system and electron transport pathways of a host cell whereas pyoverdine is a siderophore that captures iron usually from iron-binding proteins such as ferritin, lactoferrin, and transferrin (45,47), and are important for virulence and biofilm formation (48). On the other hand, LasA protease possesses a high staphylolytic activity on cleaving the peptide bonds of pentaglycine bridges within the peptidoglycan of *S. aureus* cells and enhances the activity of LasB elastase in degrading the Gly-Gly peptide bonds in elastin, a component of connective tissue, blood vessels, and lung tissue (49). The effect of these virulence factors were examined in the presence/absence of sub-MIC doses of the drugs. The activity of LNA+TOB was more effective than single dose treatments (Fig. 5A-C). Therefore, combining the phenotypic findings and genotypic expression pattern, we presented the schematic diagram

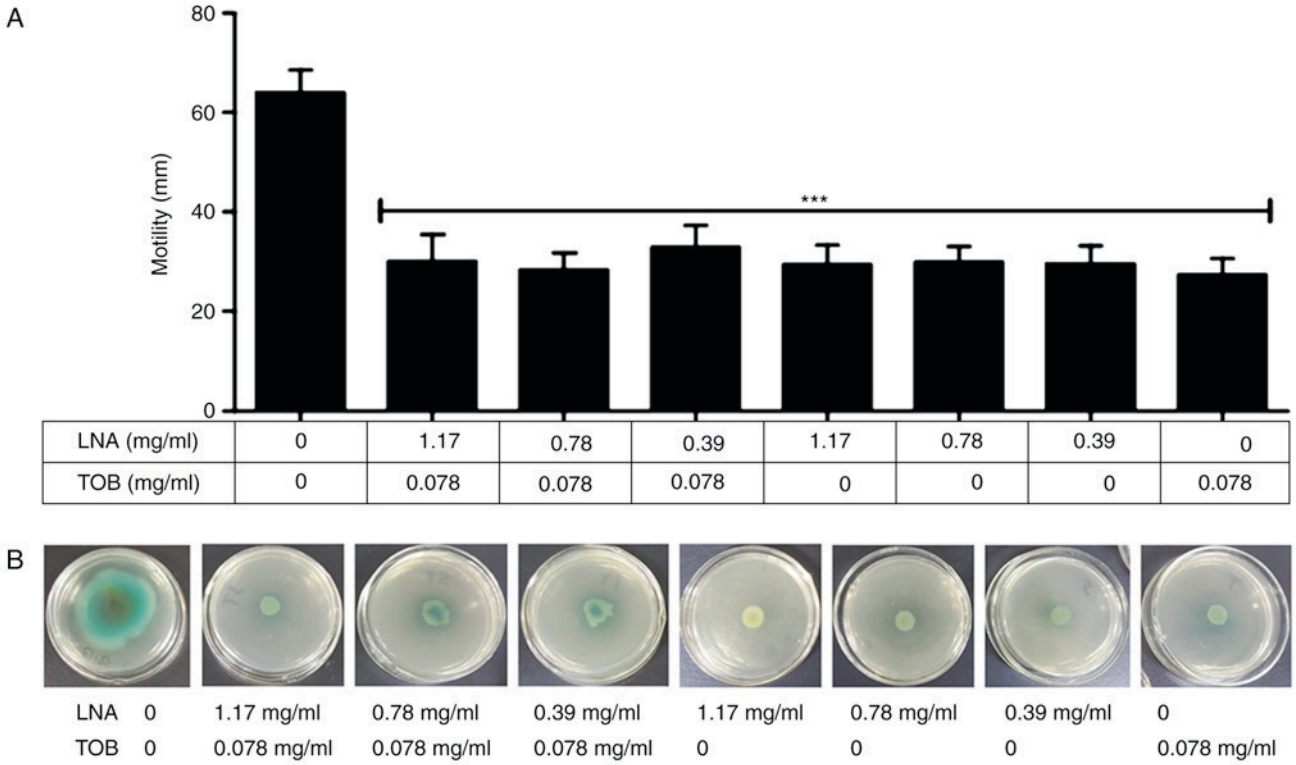

Figure 4. Effect of linolenic acid, tobramycin, and LNA+TOB on the swarming motility of *Pseudomonas aeruginosa* C2 strain. (A) Each value is an average of triplicate experiments where data are presented as mean ± SEM. (B) One representative data set for three independent experiments is shown. Control, untreated; TOB, tobramycin; LNA, linolenic acid; ***P<0.001.

(Fig. 5D) illustrating the suggested targeted sites of LNA+TOB in the QS system.

The anti-biofilm mechanism of linolenic acid, tobramycin, and LNA+TOB on P. aeruginosa C2 strain. To analyze the effects of QS genes and virulence factor-related gene expressions by *P. aeruginosa* C2 strain in the presence/absence of sub-MIC doses of the drugs, quantitative real-time PCR (qRT-PCR) was performed to assess the relative expression of our target genes (*lasI, lasR, rhlI, rhlR, pqsH, mvfR, oprF, oprI, pilJ, algU, phzR*/*qscR*, and *lasB*). For this analysis, 0.39 mg/ml (LNA) and 0.078 mg/ml (TOB) were used in single and combined treatment on C2 strain because these concentrations exhibited synergism in the interaction study as shown by the FIC index (0.5; Table II).

Quantitative RT-PCR results showed nonsignificant effects of both LNA and TOB on QS genes but their expressions reduced by -91fold (*lasI*; PA1432), -20 fold (*lasR*; PA1430), -29 fold (*rhlI*; PA3476) and -25 fold (*rhlR*; PA3477) due to LNA+TOB treatment (P<0.001; Fig. 6A). These genes are the major regulators of Las system (lasI/lasR) and Rhl system (rhlI/rhlR). In spite of these two systems, the bacterium uses another system, the Pseudomonas quinolone signal system. For this system, the activated lasR expresses *mvfR* that interacts with pqsABCDE loci to control the production of *pqsH* and 2-heptyl-4-quinolone (HHQ), and the latter is converted into 2-heptyl-3-hydroxy-4-quinolone (PQS) by *pqsH* (50), as depicted in Fig. 5D. The product, PQS regulates various virulence factor related genes including elastase and pyocyanin coding genes (50). Also, evidence suggests that alterations in *mvfR* expression disrupt PQS synthesis and pyocyanin production (51,52). In addition, *mvfR* mutant was shown to reduce virulence in an animal model (53). In the present study, we observed that the expression of *pqsH* (PA2587; P<0.001) and *mvfR* (PA1003; P<0.001) were downregulated by LNA+TOB whereas the cells treated with LNA or TOB showed no statistical significance (Fig. 6B). This suggested that LNA had no influence on the expression of our selected genes belonging to the three interrelated QS systems. However, due to the anti-biofilm effects that were exhibited in treatment groups (Fig. 2), we thought to assess the expression levels of *oprI* (PA2853), *oprF* (PA1777) and *algU* (PA0762) genes. OprF is an outer membrane protein that plays a role in maintaining the cell shape and contribute to *P. aeruginosa* virulence (54,55). The counterpart outer membrane protein oprI also play a part in membrane integrity and normal cell shape (56). A study by Fito-Boncompte *et al* (55) reported that the absence of OprF disturbs bacterial cell adhesion to animal cells, release of ExoT and ExoS toxins through the type III secretion system (T3SS), and production of the QS-associated virulence factors such as lectin PA-1 L, elastase, pyocyanin, and exotoxin A. In addition, there was reduction and retardation in production of the signalling molecules 3oxoC12HSL and C4HSL respectively in the oprF mutant. In our study, LNA+TOB reduced the expression of *oprF* (P<0.01) but not the outer membrane lipoprotein precursor (*oprI*) as compared to the untreated control (Fig. 6C). Moreover, *algU* is involved in regulating alginate production and inactivation of this gene increased the susceptibility of *P. aeruginosa* PAO1 killing by chemically generated reactive oxygen species, J774 murine macrophages and human

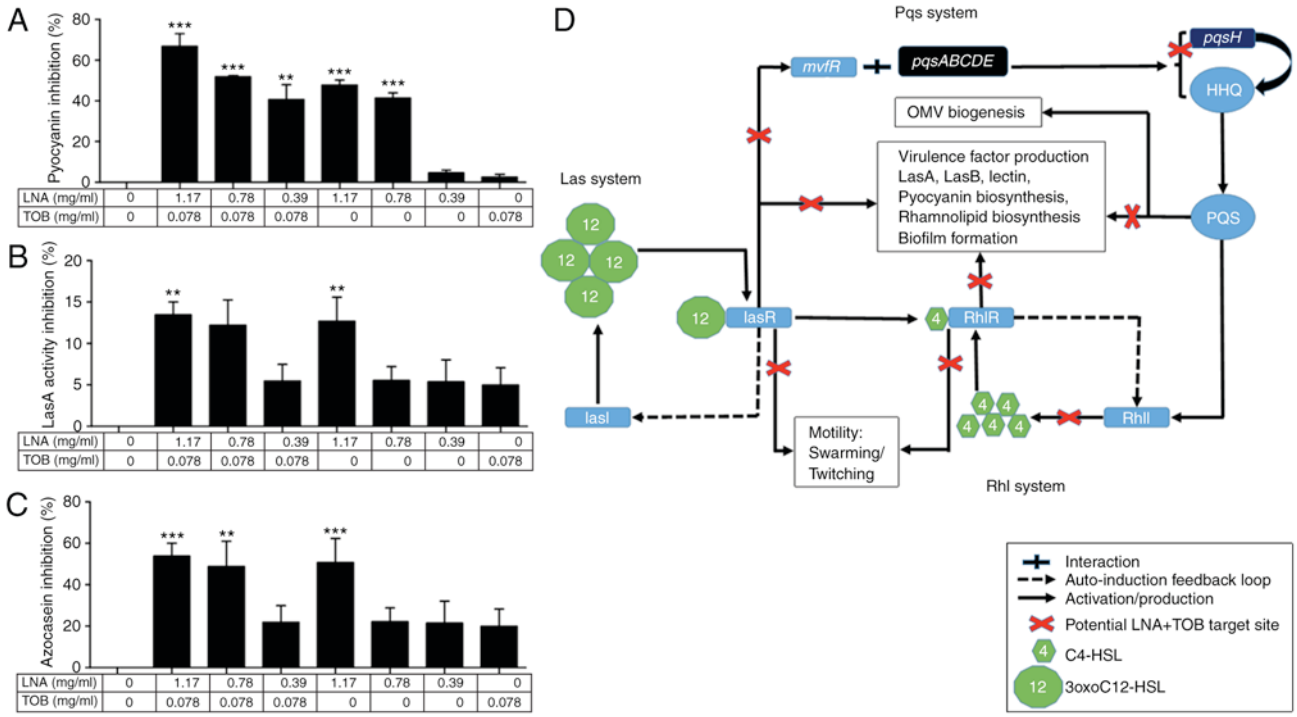

Figure 5. Effect of linolenic acid, tobramycin, and LNA+TOB on QS-regulated virulence factor production by *Pseudomonas aeruginosa* C2 strain. Percentage inhibition of (A) pyocyanin production, (B) LasA staphylolytic activity and (C) Azocasein protease production with respect to untreated control. (D) Schematic presentation of the activity of LNA+TOB on QS-regulated virulence factor production. Control, untreated cells; TOB, tobramycin; LNA, linolenic acid; **P<0.01 and ***P<0.001 vs. untreated control. Three independent assays were performed.

neutrophils (57). Moreover, algU regulates *rsmA* expression, a posttranscriptional regulator involved in virulence factor production and biofilm formation (58). Interestingly, *algU* was downregulated by LNA+TOB (P<0.01) but upregulated in LNA or TOB treated groups with similar statistical magnitude (Fig. 6B). Furthermore, we selectively decided to assess the expression levels of *pilJ* (PA0411), *phzR/qscR* (PA1898) and *lasB* (PA3724) that contribute to bacterial virulence. We observed the downregulation of *pilJ* (PA0411; P<0.001) and *phzR/qscR* (PA1898; P<0.001) genes in LNA+TOB treated cells while LNA alone upregulated the *pilJ* (P<0.001) expression (Fig. 6C). Surprisingly, no statistically significant effect was noticed on *lasB* gene expression in all treatment groups as compared to the untreated control.

Discussion

Pseudomonas aeruginosa is a medically important pathogen that causes nosocomial and serious life threating infections due to its ability to produce biofilm that has contributed to the emergence of drug resistance towards currently utilized antibiotics. Considering the antimicrobial and anti-inflammatory properties of fatty acids (17,18), assessing their biofilm prevention properties on *P. aeruginosa* may supplement in finding alternative agents for antimicrobial resistance problem. Moreover, evidence suggest that LNA and its derivatives, EPA and DHA possess antimicrobial properties against various pathogenic microorganisms (22-26).

Biofilm formation is an active process that is dependent highly on environmental signals which sensitize a cell to undergo stages of cycle growth. This process involves production of a matrix comprising of polysaccharides, proteins, lipids and extracellular DNA (eDNA) that provide a physiological barrier to antimicrobial diffusion and concentrate secreted extracellular enzymes such as β-lactamases, whereas the limitation of oxygen and nutrients support the anaerobic growth of *P. aeruginosa* which decelerates cell division (2,10,59). During this stage, the bacterial community develops resistance to various antimicrobial agents, like β-lactam and aminoglycoside antibiotics that target actively and aerobically growing bacterial cells, respectively (59). Our study has shown that LNA, indeed possesses antibacterial and anti-biofilm effect against *P. aeruginosa* as evidenced by the MIC and biofilm inhibition/inactivation assays. We had observed that LNA alone and in combination with TOB had the ability to disrupt biofilm formation and decrease the metabolic activities of biofilm cells by causing cell death in a dose-dependent manner (Figs. 2 and 3). Besides, the stronger effects exhibited by the combined treatment suggested that LNA did not antagonize the activity of TOB but synergistically and additively exerted their combined effects on biofilm as supported by their FIC index results (Table II).

Furthermore, quorum sensing is a major player in *P. aeruginosa* virulence factor production and biofilm formation, making it an interesting target for an antipseudomonal compound search. Evidence has shown that inhibiting *P. aeruginosa* QS system disrupts biofilm formation and virulence factor production (34,60). Our study showed that LNA+TOB had a great impact in downregulating the genes associated with the three interrelated QS systems (Las, Rhl and Pqs systems) (Fig. 6A and B). Since the bacterium uses QS systems to control biofilm formation and virulence factor secretion, attempts

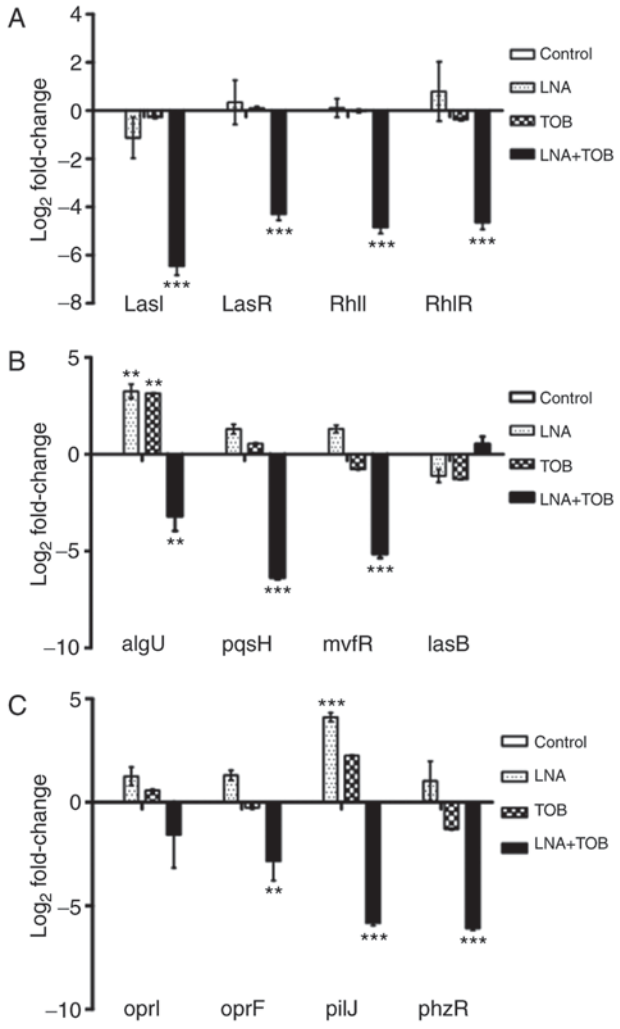

Figure 6. Log2 fold change normalized quantification of QS- and virulence factor-related genes of *Pseudomonas aeruginosa* C2 strain of untreated and treated cells. (A) Key genes for Las and Rhl systems, (B) key genes for pqs system and virulence factor genes (algU and lasB genes) and (C) selected virulence factor genes. Each value is an average of triplicate experiment where data are presented as the mean ± SEM. Target gene quantification was normalized to *16SrRNA* reference gene. **P<0.01, ***P<0.001 vs. untreated control. TOB, tobramycin; LNA, linolenic acid.

were made to analyze some virulence factor associated genes. *P. aeruginosa* produces outer membrane vesicles (OMV) as a tool for genetic information and toxins dissemination (61), and they require the signaling molecule PQS for their biogenesis which accumulates in the LPS-rich outer leaflet of the outer membrane and causes bleb formation (62). Previous evidence suggests that outer membrane proteins oprI and oprF participate in OMV formation but the absence of these proteins and presence of PQS still increased OMV production by *P. aeruginosa* (63). This implies that PQS play a major role in OMV biogenesis making it an attractive target for anti-biofilm exploration. We have shown that LNA+TOB is capable of interfering with OMV biogenesis at gene transcription level (Fig. 6B and C). On the other hand, alginate is associated with chronic lung infection though only secreted by a subsection of *P. aeruginosa* species, as most strains can either secrete Psl (polysaccharide synthesis locus) or Pel (pectate lyase) polysaccharides (64,65). Our study has shown that LNA+TOB is effective in disrupting alginate production (Fig. 6B). This suggests that LNA+TOB can be effective in reducing chronicity resulting from alginate production in lung infections. Moreover, we have shown that LNA+TOB treatment can prevent swarming and twitching motility thereby reducing the spread of infection. Therefore, we can deduce that LNA+TOB is more effective in preventing biofilm formation and virulence factor production than single LNA or TOB in targeting the QS system of *P. aeruginosa*. This could be due to the additive and synergistic effects that may occur once the two compounds are combined.

Although tobramycin is currently used in cystic fibrosis lung infections, it fails to clear chronic infection associated with *P. aeruginosa* completely (66). However, various compounds combined with tobramycin had shown synergism in attenuating biofilm formation by this pathogen (67,68). Our study revealed that LNA can improved the efficacy of TOB and could be considered in *P. aeruginosa* related infections. In spite of the differences in the genotypic and phenotypic assays' outcomes, it is no doubt that LNA is capable of reducing biofilm formation and virulence factor production as shown from our phenotypic analyses such as biofilm, swarming, phenazine, and proteases for being affected by LNA alone. However, the mechanism is definitely not associated with QS system but perhaps utilizing a different route to cause biofilm inhibition. For that, we hypothesized that LNA could be interacting with the cell membrane, increasing the membrane fluidity thereby disrupting its permeability causing the cell to leak out as well as transporting TOB in biofilm cells (17,69), but substantialized analysis is needed. On the other hand, diet supplementation of omega-3 PUFA have been shown to improve the cardiovascular and respiratory conditions (19,70), and was reported to be safe for human use (71), suggesting that LNA supplementation may serve as an immunomodulatory and antibiotic agent.

In conclusion, the present study demonstrates that linolenic acid has the capacity to interfere with *P. aeruginosa* biofilm formation and virulence factor production though experimented on the C2 strain. This strain exhibited stronger resistance to the selected doses than any other strain used in this study. It is therefore not acceptable to generalize the findings of one strain with the rest. However, it is imperative to note that these findings have shown the effectiveness of a combination therapy (LNA+TOB), which should stimulate critical analysis for antimicrobial consideration. Moreover, the combination therapy has shown strong synergism in disrupting the *P. aeruginosa* quorum sensing systems and decreasing its virulence factor production. Therefore, it can be deduced that linolenic acid used alone can attenuate the formation of biofilm and can improve the action of tobramycin in targeting the quorum sensing systems. This may decrease tobramycin concentration when in fact treated in combination with linolenic acid (in the form of dietary supplements) thereby reducing the adverse effects of aminoglycosides. However, this study is only based on *in vitro* investigations that may not mimic the clinical settings. Thus, the findings here may not be conclusive but stimulate interest in considering such compounds for adjunctive therapy. Hence more studies are required, especially *in vivo* studies with model systems that can simulate the clinical settings to substantiate our findings.

Acknowledgements

We would like to convey our appreciation to Mr. Richardson Joseph (Ph.D. Fellow), Mr. Emeka Okoye (Ph.D. Fellow) and Dr. Brian Ayuka (MD) for reviewing this manuscript. We would like to also thank the Chinese Scholarship Council for their financial support.

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