ISSN: 0067-2904

Gentamicin Variably Affects *amrZ* and *rhl* gene Expression in Swarmer Cells of *Pseudomonas aeruginosa*

Khaldoon Hasan Fadhil, Harith Jabbar Fahad Al-Mathkhury
Department of Biology, College of Science, University of Baghdad

Received: 11/1/2022 Accepted: 5/3/2022 Published: 30/7/2022

Abstract

Swarming is one of the most important virulence factors used by bacteria to invade new sites. This study aimed to test the effects of gentamicin on swarming motility of *Pseudomonas aeruginosa*, both phenotypically and molecularly. The present results revealed that 11/25 isolates had gentamicin MIC of 1024 µg/ml. However, gentamicin at sub-minimal inhibitory concentration significantly ($P< 0.05$) reduced the diameter of swarming in all *P. aeruginosa* isolates. Noticeably the mean and median swarming diameter before treatment with gentamicin 5.557 and 5.816 cm respectively had significantly ($P < 0.001$) reduced to 0.871 and 0.766 cm respectively. At the molecular level, *amrZ* (a global regulator of multiple genes) and *rhl* (responsible for rhamnolipid production) were variably affected by gentamicin. More likely it can be concluded that *amrZ* and *rhl* are not fully responsible for swarming in *P. aeruginosa* isolates.

Keywords: Gentamicin, *amrZ*, *rhl*, Swarming, *Pseudomonas aeruginosa*

يؤثر الجنتامايسين بشكل متغاير في التعبير الجيني لـ *amrZ* و *rhl* في خلايا الزوائف الزنجارية العاجة

خلدون حسن فاضل ، حارث جبار فهد المذخوري

قسم علوم الحياة، كلية العلوم، جامعة بغداد

الخلاصه

العج واحد من اهم عوامل الفوعة التي تستعملها البكتريا لغزو اماكن جديدة. اظهرت النتائج الحالية ان 11 من 25 عزلة كانت ذات ادنى تركيز مثبط من الجنتامايسين يساوي 1024 مايكروغرام/مل. على اية حال، قلل الجنتامايسين عند التركيز المثبط تحت الادنى وبشكل معنوي ($P<0.05$) من قطر العج في عزلات الزوائف الزنجارية جميعها بشكل ملحوظ، انخفض معنويا ($P<0.01$) كل من المعدل و الوسيط من5.557 و 5.816 سم، على التتابع قبل المعاملة بالجنتامايسين الى 0.766 و 0.871، على التتابع بعد المعاملة بالجنتامايسين. على المستوى الجزيئي، تأثر كل من *amrZ* (منظم شامل لعدة جينات) و*rhl* (مسؤول عن انتاج الرامنولبد) وبشكل متغاير بالجنتامايسين؛ اغلب الظن، يمكن الخلوص الى كلا من الجينين المذكورين انفا غير مسؤولين بشكل كامل عن العج في الزوائف الزنجارية.

Introduction

Infections caused by *P. aeruginosa* are still difficult to cure. Due to its resistance to antibiotics, it has been termed a "superbug" because of its ability to infect a wide range of

*Email: khfkhf98@gmail.com

organisms [1]. Burn wound is an opportunistic colonization site for organisms of both endogenous and exogenous origin, such as *P. aeruginosa* [2]. Swarming in *P. aeruginosa* is related to its enhanced resistance to most applied antibiotics and its involvement in bacterial spread from localized infection sites to distal organs [3].

Pseudomonads contain a conserved transcription factor known as *amrZ*. It is considered as a global regulator of several genes that are involved in virulence and environmental fitness. According to Hou *et al.* [4] AmrZ has a function in modulating swarming motility which makes it an ideal target for the development of anti-*P. aeruginosa* therapies [5]. Rhamnolipids are the biosurfactants involved in swarming motility and its production is regulated via Rhl, a quorum sensing-based system [6]**.**

The overuse of antibiotics to treat illnesses has resulted in the emergence of antibiotic resistance in bacteria such as *P. aeruginosa*. As a result, numerous antibiotics are being investigated for their effectiveness in impeding pathogen virulence. The antibiotics at sub-minimum inhibitory concentrations (sub-MICs) widely disturb the transcription process in bacteria and may function as signalling mediators instead of targeting microbe growth [7]. Locally, gentamicin has shown to modulate the gene expression of virulence determinants such as *hla* [8], *fnbA, fnbB* [9], *pslA,* and *pelA* [10]. Based on that, the aim of this work was to determine the impact of gentamicin on swarming ability of *P. aeruginosa*.

Materials and Methods

Pseudomonas aeruginosa isolates

A total of 25 gentamicin-resistant swarming *P. aeruginosa* isolates were obtained from the microbiology lab at the Department of Biology, College of Science, University of Baghdad. These isolates were originally isolated from burn patients.

Estimation of Minimum Inhibitory Concentration

Resazurin-based turbidometric assay was used to estimate the minimal inhibitory concentration (MIC) of gentamicin using double serial concentrations (2 – 1024 µg/ml). The test was done for all the 25 *P. aeruginosa* isolates using the broth micro-dilution in microtitre plates. After 24 hrs of incubation at 37°C, 5 µl of resazurin at a concentration of 6.75 mg/ml was placed in wells and eventually incubated at 37°C for an extra 4 hours. Changes in colour, from blue to pink, were recorded [11]. The lowest concentration before the colour change was considered as the MIC.

Swarming Assay

Swarming motility was tested for all the 25 *P. aeruginosa* isolates by the below steps:

BM2 Swarming Agar

It was prepared by dissolving 62 mM potassium phosphate buffer (pH 7), 2 mM $MgSO_4$, 10 µM $FeSO_4$, 0.4% wt/vol glucose and 0.5% wt/vol agar agar [12].

Swarming Protocol

Swarming protocol was performed according to the procedure described by Hou *et al.* [4]. In brief, after solidification, BM2 swarming agar plates were left to dry at room temperature. Thereafter, 10 µl of overnight brain heart infusion broth cultures (compatible to McFarland standard no. 0.5) at 37°C for one week, were spotted on BM2 plate. Experiments were repeated in triplicate. The swarming diameter was measured again using a metric ruler. Similar protocol was followed to test the effects of gentamicin on swarming, except for the BM2 plate containing gentamicin at sub-MIC that corresponded to each isolate.

Gene Expression

A total of five isolates were chosen for testing the impact of gentamicin on the gene expression of *amrZ* and *rhl*. These isolates demonstrated the highest significant differences due to gentamicin effect on swarming. Moreover, the bacterial cells were taken from the peripheral swarming wave (tendril tip) in BM2 plates, with and without gentamicin at sub-MIC.

Following the manufacturer instructions, RNA was extracted from *P. aeruginosa* swarming cells at the tendrils using TRIzol™ Reagent. Nanodrop was employed for estimating the concentration and purity of the extracted RNA. Later on, cDNA was synthesized with the Protoscript cDNA synthesis kit using the supplied protocols.

To evaluate the *amrZ* and *rhl* gene expression, the results were normalized to the expression of *fbp* gene. Table 1 highlights the primers used in this study. The provided primers were dissolved in sterile nuclease-free water to obtain 100 pmol /µl as a final concentration. Subsequently, all prepared primers were kept in a deep freezer. The reaction mixture has been summarized in Table 2. Moreover, after several trials, thermocycler protocol was optimized and the resultant protocol is listed down in Table 3.

Table 1-Primers used in qPCR

Primer		Sequence (5´-3´)	Reference
amrZ	F	TGACAAATTCGTCGTTCGTCTGCC	[13]
	R	AACACCGAGATTGTCTTGCAGCG	
rhl	F	GGCCGAACATTTCAACGTGG	[14]
	R	AGGATTTCCACCTCGTCGTC	
Fbp	F	CCTACCTGTTGGTCTTCGACCCG	[15]
	R	GCTGATGTTGTCGTGGGTGAGG	

Table 2-qRT-PCR Reactants

Reactants	Volume (µl)
qPCR Master Mix	**10**
Forward primer	**1**
Reverse primer	**1**
Nuclease Free Water	**3**
Template cDNA	**5**

Table 3- qRT-PCR protocol

Step	Temperature (°C)	Duration	Cycles
Initial Denaturation	95	60 seconds	1
Denaturation	95	15 seconds	45
Extension	60	30 seconds	

Gene Expression Calculation

Expression levels were quantified using relative quantitation with Livak equation ($2^{-\Delta\Delta Ct}$) [16]. A melting curve was achieved with temperatures extending from 60°C to 95°C, with a 1°C increase per second.

Statistical Analysis

All experiments were performed in triplicate and data was expressed as mean and standard deviations. T test was used to determine the effect of study variables on swarming diameter. These statistical analyses were done using SPSS version software. The differences were considered significant where $P<0.05$. Fold change less than 2 was considered insignificant [17].

Results and Discussion

Minimal Inhibitory Concentration

In the present study, the gentamicin MIC was determined by the resazurin-based assay (Figure 1) which revealed that 11/25 isolates had MIC of 1024 µg/ml. Such finding reflected on the magnitude of gentamicin resistance problematic issue. However, gentamicin kills the bacterial cell via suppressing bacterial protein synthesis by binding to ribosomal 30S subunits [1].

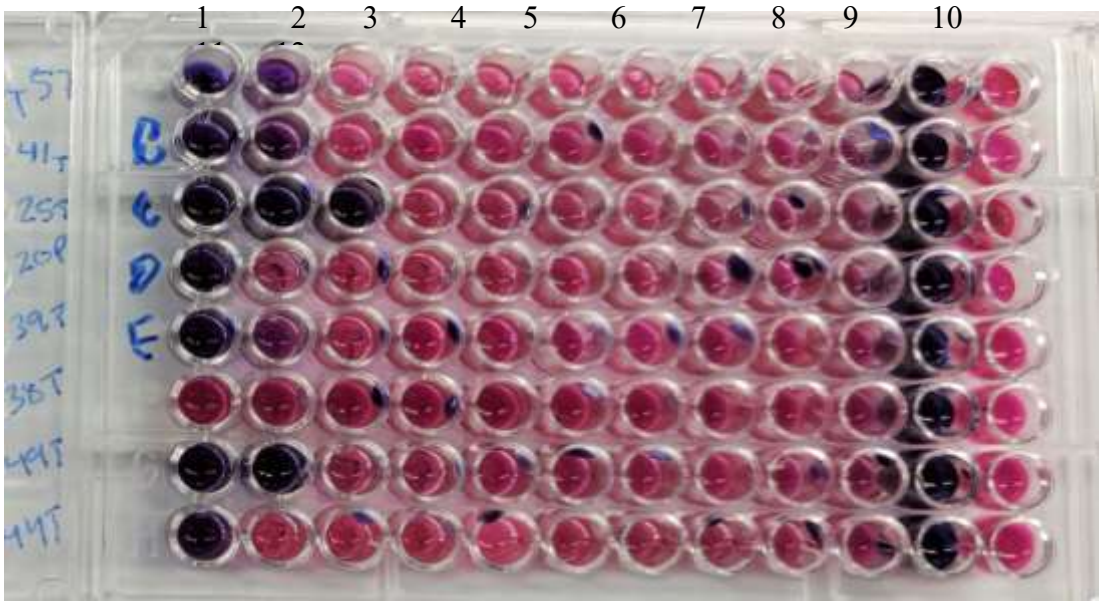

Figure 1- Gentamicin minimum inhibitory concentration of *Pseudomonas aeruginosa* by Resazurin based method. Column 11 represents the negative control showing the natural colour of resazurin (blue/purple). Column 12, a positive control, changed to reduced form (red to pink). Wells 1 – 10 contained gentamicin at concentrations of 2-1024 µg/ml respectively.

MIC is regarded as the "gold standard" for evaluating organism susceptibility to antimicrobials and is hence used to evaluate the efficacy of all other susceptibility testing procedures. MIC is employed in diagnostic laboratories to confirm extraordinary resistance, to provide a conclusive answer when other methods of testing yield a borderline result or when disk diffusion procedures are ineffective. MIC data aids the physicians in monitoring resistance development and clinical pharmacists in determining the optimal pharmacodynamics dosage [18]. The current study's gentamicin MIC result was reasonably consistent with Paduszynska *et al.* [19].

Effect of Gentamicin on Swarming

As depicted in Figure 2, the mean and median swarming diameter before treatment with gentamicin (5.557 and 5.816 cm respectively) significantly ($P < 0.001$) reduced to (0.871 and 0.766 cm respectively). Moreover, the data set before treatment with gentamicin is left-skewed (where skewness = -0.171). While it is right-skewed (where skewness = 0.438) when gentamicin was present which indicates that the swarming diameter values before the treatment with gentamicin were more than the mean (5.557 cm). Whereas after the treatment with gentamicin, the values were below the mean (0.871 cm). In plain words, the data may not have been normally distributed. Interestingly no outliers were found in both data sets.

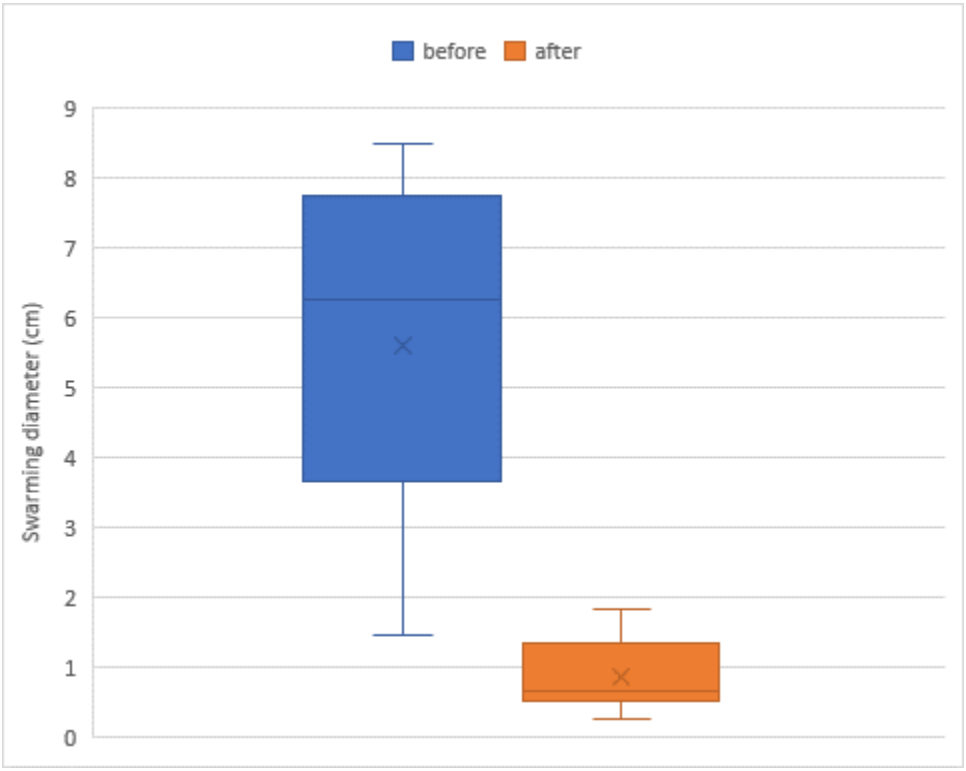

Figure 2-Box plot diagram of gentamicin effects on *Pseudomonas aeruginosa* swarming. Boxes ranged from the 25th to 75th percentile and were intersected by the median line. Asterisks denotes the mean. Whiskers extending below and above the box range represents the maximum and minimum values respectively. Where T test = 8.748×10^{-11}.

Gene Expression

The expression of *rhl* and *amrZ* involving five *P. aeruginosa* isolates was studied using RT-qPCR technique. Based on findings presented in Tables 3-5, these five isolates were the most affected (the lowest being T test level) isolates by gentamicin at sub-MIC.

Gentamicin at sub-MIC affected the *rhl* gene expression variably (Table 4). An upregulation in gene expression was noticed in isolates PA26 and PA55e. Nonetheless, the isolates PA10 and PA76s demonstrated a downregulation in *rhl* expression. Furthermore, isolate PA51p was not affected by the gentamicin stress.

Table 4-Effects of gentamicin on the gene expression of *rhl* in *Pseudomonas aeruginosa*

Isolate code	MIC (µg/ml)	Before treatment with gentamicin			After treatment with gentamicin			ΔΔCt	Fold change
		rhl Ct	*fbp* Ct	ΔCt	*rhl* Ct	*fbp* Ct	ΔCt		
PA10	512	34.73	29.09	5.64	35.89	30.2	5.69	0.05	0.96
PA26	512	34.92	29.3	5.62	32.74	29.69	3.05	-2.57	5.93
PA51p	512	33.7	31.5	2.2	31.63	29.8	1.83	-0.37	1.29
PA55e	64	39.69	29.39	10.3	35.53	30.34	5.19	-5.11	34.53
PA76s	512	31.29	34.53	-3.24	31.29	31.28	0.01	3.25	0.10

Swarming motility controlled through *rhl* and *rhl*-regulated rhamnolipid synthesis, is required for swarming motility in *P. aeruginosa* [20]. Therefore, the downregulation in *rhl* level in this study, perhaps, led to a reduction in the rhamnolipid production, hence reducing the swarming diameter. However, the *rhl* upregulation suggests that the inhibition of swarming by gentamicin is not acted through *rhl.*

Similarly Oura *et al.* [14] reported that rhamnolipid production was equivalent, with or without 1-naphthol and using colorimetric and qRT-PCR analysis, suggesting that the inhibitive effect of 1-naphthol on swarming does not result from defects in rhamnolipid production. Moreover, Caiazza *et al.* [21] showed that *P. aeruginosa* produces extracellular factors capable of modulating tendril movements and genetic analysis revealed that the modulation of these movements was dependent on rhamnolipid biosynthesis. Same authors hypothesized that the rhamnolipid precursor, 3-(3-hydroxyalkanoyloxy) alkanoic acids, intermediate (monorhamnolipids) and the end product (dirhamnolipids) have different roles in swarming, for example, enabling swarming through surface wetting or acting to modulate swarming movement. What's more, Bru *et al.* [22] stated that antibiotic treatment of *P. aeruginosa* abolished swarming motility and repulsed approaching swarms away from the antibiotic-treated area through a *Pseudomonas* quinolone signalling molecule-dependent mechanism.

The results highlighted in Table 5 reveal that three isolates (PA10, PA55e and PA76s) showed an upregulation in the gene expression of *amrZ* due to gentamicin.

Table 5-Effects of gentamicin on the gene expression of *amrZ* in *Pseudomonas aeruginosa*

Isolate code	MIC (µg/ml)	Before treatment with gentamicin			After treatment with gentamicin			ΔΔCt	Fold change
		amrZ Ct	*fbp* Ct	ΔCt	*amrz* Ct	*fbp* Ct	ΔCt		
PA10	512	32.7	29.09	3.61	32.34	30.2	2.14	-1.47	2.77
PA26	512	31.9	29.82	2.08	32.74	29.96	2.78	0.7	0.61
PA51p	512	31.9	29.28	2.62	33.79	30.32	3.47	0.85	0.55
PA55e	64	33.2	29.39	3.81	32.34	30.2	2.14	-1.67	3.18
PA76s	512	33.82	31.53	2.29	32.13	31.28	0.85	-1.44	2.71

MIC= minimum inhibitory concentration

Hou *et al*. [4] stated that the *amrZ* deletion leads to a significant impairment in swarming. Whereas multicopy expression results in the stimulation of *P. aeruginosa* swarming. Mechanistic investigations revealed that the swarming imperfection of an *amrZ* mutant does not include deviations in biosurfactant production but is related to flagellar failure [23].

Conclusion

Swarming of *P. aeruginosa* was highly affected by gentamicin stress. However, *rhl* and *amrZ* were variably affected.

Acknowledgement

The authors would like to thank the University of Baghdad for the partial funding for this study.

References

[1] S. Riedel, S. Morse, T. Mietzner and S. Miller (2019) Jawetz, Melnick, and Adelberg's Medical Microbiology, 28 ed. McGraw-Hill New York.

[2] C. Ayoub Moubareck and D. Hammoudi Halat, "Insights into Acinetobacter baumannii: a review of microbiological, virulence, and resistance traits in a threatening nosocomial pathogen," Antibiotics, vol. 9, no. 3, p. 119, 2020.

[3] S. R. Coleman, T. Blimkie, R. Falsafi, and R. E. W. Hancock, "Multidrug adaptive resistance of *Pseudomonas aeruginosa* swarming cells," Antimicrob. Agents Chemother., vol. 64, no. 3, 2020.

[4] L. Hou, A. Debru, Q. Chen, Q. Bao, and K. Li, "AmrZ regulates swarming motility through cyclic di-GMP-dependent motility inhibition and controlling Pel polysaccharide production in *Pseudomonas aeruginosa* PA14," Front. Microbiol., vol. 10, p. 1847, 2019.

[5] X. Shao, Y. Xie, Y. Zhang, J. Liu, Y. Ding, M. Wu, X. Wang, and X. Deng "Novel therapeutic strategies for treating *Pseudomonas aeruginosa* infection," Expert Opin. Drug Discov., vol. 15, no. 12, pp. 1403–1423, 2020.

[6] G. Soberón-Chávez, A. González-Valdez, M. P. Soto-Aceves, and M. Cocotl-Yañez,

"Rhamnolipids produced by Pseudomonas: from molecular genetics to the market," Microb. Biotechnol., vol. 14, no. 1, pp. 136–146, 2021.

[7] N. A. Khan, S. Ahmed, I. Farooqi, I. Ali, V. Vambol, F. Changani, M. Yousefi, S. Vambol, S. Khan, and A. Khan "Occurrence, sources and conventional treatment techniques for various antibiotics present in hospital wastewaters: a critical review," TrAC Trends Anal. Chem., vol. 129, p. 115921, 2020.

[8] N. T. M. Jaddoa and H. J. F. Al-Mathkhury, "Gentamicin modulates the gene expression of hla in methicillin resistance *Staphylococcus aureus* biofilm," J. Glob. Pharma. Tech, vol. 10, no. 07, pp. 61–70, 2018.

[9] H. T. Ibrahim, "The Dual Role of Gentamicin in Gene Expression of Fibronectin Binding Proteins in *Staphylococcus aureus*," J. Glob. Pharma Technol., 2018.

[10] M. A. R. H. AL-Sheikhly, L. N. Musleh, and H. J. F. Al-Mathkhury, "Gene expression of pelA and pslA in *Pseudomonas aeruginosa* under gentamicin stress," Iraqi J. Sci., vol. 61, no. 2, pp. 295–305, 2020, doi: 10.24996/ijs.2020.61.2.6.

[11] C. H. Teh, W. A. Nazni, A. H. Nurulhusna, A. Norazah, and H. L. Lee, "Determination of antibacterial activity and minimum inhibitory concentration of larval extract of fly via resazurin-based turbidometric assay," BMC Microbiol., vol. 17, no. 1, pp. 1–8, 2017, doi: 10.1186/s12866-017-0936-3.

[12] J. Overhage, M. Bains, M. D. Brazas, and R. E. W. Hancock, "Swarming of *Pseudomonas aeruginosa* is a complex adaptation leading to increased production of virulence factors and antibiotic resistance." Am Soc Microbiol, 2008.

[13] C. J. Jones, C. R. Ryder, E. E. Mann, and D. J. Wozniak, "AmrZ modulates *Pseudomonas aeruginosa* biofilm architecture by directly repressing transcription of the psl operon," J. Bacteriol., vol. 195, no. 8, pp. 1637–1644, 2013, doi: 10.1128/JB.02190-12.

[14] H. Oura, Y. Tashiro, M. Toyofuku, K. Ueda, T. Kiyokawa, S. Ito, Y. Takahashi, S. Lee, H. Nojiri, T. Nakajima-Kambe, U. Uchiyama, H. Futamata, and N. Nomura "Inhibition of *Pseudomonas aeruginosa* swarming motility by 1-naphthol and other bicyclic compounds bearing hydroxyl Groups," Appl. Environ. Microbiol., vol. 81, no. 8, pp. 2808–2818, 2015, doi: 10.1128/AEM.04220-14.

[15] S. Chakravarty, C. N. Melton, A. Bailin, T. L. Yahr, and G. G. Anderson, "*Pseudomonas aeruginosa* magnesium transporter MgtE inhibits type III secretion system gene expression by stimulating rsmYZ transcription," J. Bacteriol., vol. 199, no. 23, pp. e00268-17, 2017.

[16] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method," methods, vol. 25, no. 4, pp. 402–408, 2001.

[17] J. P. Rasigade et al., "Impact of sub-inhibitory antibiotics on fibronectin-mediated host cell adhesion and invasion by *Staphylococcus aureus*," BMC Microbiol., vol. 11, no. 1, pp. 1–9, 2011.

[18] S. Štumpf, G. Hostnik, M. Primožič, M. Leitgeb, J.-P. Salminen, and U. Bren, "The effect of growth medium strength on minimum inhibitory concentrations of tannins and tannin extracts against *E. coli*," Molecules, vol. 25, no. 12, p. 2947, 2020.

[19] M. A. Paduszynska, K. E. Greber, W. Paduszynski, W. Sawicki, and W. Kamysz, "Activity of temporin a and short lipopeptides combined with gentamicin against biofilm formed by *Staphylococcus aureus* and *Pseudomonas aeruginosa*," Antibiotics, vol. 9, no. 9, p. 566, 2020.

[20] Ha D-G, Merritt JH, Hampton TH, Hodgkinson JT, Janecek M, Spring DR, Welch M, and O'Toole GA. (2011). 2-Heptyl-4-quinolone, a precursor of the Pseudomonas quinolone signal molecule, modulates swarming motility in *Pseudomonas aeruginosa*. J Bacteriol 193:6770 –6780

[21] N. C. Caiazza, R. M. Q. Shanks, and G. A. O'toole, "Rhamnolipids modulate swarming motility patterns of *Pseudomonas aeruginosa*," J. Bacteriol., vol. 187, no. 21, pp. 7351–7361, 2005.

[22] J. Bru, B. Rawson, C. Trinh, K. Whiteson, and A. Siryaporn, "crossm PQS Produced by the *Pseudomonas aeruginosa* Stress," vol. 201, no. 23, pp. 1–14, 2019.

[23] P. Dulyayangkul, N. Satapoomin, M. B. Avison, N. Charoenlap, P. Vattanaviboon, and S. Mongkolsuk, "Over-Expression of Hypochlorite Inducible Major Facilitator Superfamily (MFS) Pumps Reduces Antimicrobial Drug Susceptibility by Increasing the Production of MexXY Mediated by ArmZ in *Pseudomonas aeruginosa*," Front. Microbiol., vol. 11, no. January, pp. 1–12, 2021.