



RESEARCH ARTICLE

REVISÉ Enhancement of pyocyanin production by subinhibitory concentration of royal jelly in *Pseudomonas aeruginosa* [version 4; peer review: 3 approved, 1 approved with reservations]

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V4 First published: 11 Jan 2021, 10:14
<https://doi.org/10.12688/f1000research.27915.1>
 Second version: 13 Jul 2021, 10:14
<https://doi.org/10.12688/f1000research.27915.2>
 Third version: 28 Sep 2021, 10:14
<https://doi.org/10.12688/f1000research.27915.3>
 Latest published: 07 Dec 2021, 10:14
<https://doi.org/10.12688/f1000research.27915.4>

Abstract

Background: *Pseudomonas aeruginosa*, a multidrug-resistant Gram-negative bacterium, produces pyocyanin, a virulence factor associated with antibiotic tolerance. High concentrations of royal jelly have an antibacterial effect, which may potentially overcome antibacterial resistance. However, in some cases, antibiotic tolerance can occur due to prolonged stress of low-dose antibacterial agents. This study aimed to investigate the effect of subinhibitory concentrations of royal jelly on bacterial growth, pyocyanin production, and biofilm formation of *P. aeruginosa*.

Methods: *Pseudomonas aeruginosa* ATCC 10145 and clinical isolates were cultured in a royal jelly-containing medium to test the antibacterial activity. Pyocyanin production was observed by measuring the absorbance at 690 nm after 36 h culture and determined using extinction coefficient 4310 M⁻¹ cm⁻¹. Static microtiter plate biofilm assay performed to detect the biofilm formation, followed by scanning electron microscopy.

Results: Royal jelly effectively inhibited the viability of both strains from a concentration of 25%. The highest production of pyocyanin was observed in the subinhibitory concentration group 6.25%, which gradually decreased along with the decrease of royal jelly concentration. Results of one-way ANOVA tests differed significantly in pyocyanin production of the two strains between the royal jelly

Open Peer Review

Reviewer Status

	Invited Reviewers			
	1	2	3	4
version 4 (revision) 07 Dec 2021				 report
version 3 (revision) 28 Sep 2021		 report	 report	 report
version 2 (revision) 13 Jul 2021	 report	 report	 report	
version 1 11 Jan 2021	 report	 report		

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3. **Shizuo Kayama** , National Institute of

groups. Tukey HSD test showed concentrations of 12.5%, 6.25%, and 3.125% significantly increased pyocyanin production of ATCC 10145, and the concentrations of 12.5% and 6.25% significantly increased production of the clinical isolates. Concentrations of 12.5% and 6.125% significantly induced biofilm formation of *P. aeruginosa* ATCC 10145, in line with the results of the SEM analysis.

Conclusions: The royal jelly concentration of 25% or higher inhibits bacterial growth; however, the subinhibitory concentration increases pyocyanin production and biofilm formation in *P. aeruginosa*. It is advisable to determine the appropriate concentration of royal jelly to obtain beneficial virulence inhibiting activity.

Keywords

royal jelly, antibacterial effect, *Pseudomonas aeruginosa*, pyocyanin

Infectious Diseases, Higashimurayama, Japan

4. **Philip S. Bird**, The University of Queensland, Gattton, Australia

Any reports and responses or comments on the article can be found at the end of the article.

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Author roles: **Amy DA:** Data Curation, Investigation, Writing – Original Draft Preparation; **Hajardhini P:** Data Curation, Investigation; **Jonarta AL:** Formal Analysis, Supervision, Writing – Review & Editing; **Yulianto HDK:** Data Curation, Investigation, Supervision, Writing – Review & Editing; **Susilowati H:** Conceptualization, Data Curation, Funding Acquisition, Investigation, Project Administration, Resources, Supervision, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: Part of this research was supported by funding from the Ministry of Research, Technology and Higher Education, Republic of Indonesia, The Excellence Basic Research [Grant No. 26023/ UN1.DITLIT/DIT.LIT/LT/2019] to Heni Susilowati. *The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*

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How to cite this article: Amy DA, Hajardhini P, Jonarta AL *et al.* **Enhancement of pyocyanin production by subinhibitory concentration of royal jelly in *Pseudomonas aeruginosa* [version 4; peer review: 3 approved, 1 approved with reservations]** F1000Research 2021, 10:14 <https://doi.org/10.12688/f1000research.27915.4>

First published: 11 Jan 2021, 10:14 <https://doi.org/10.12688/f1000research.27915.1>

REVISED Amendments from Version 3

We submitted manuscript version 4 in response to suggestions from Dr. Philip S Bird. Changes to the manuscript version 4 include:

- 1) Revision of the statement of *P. aeruginosa* as a transitional pathogen. 2) Additional explanations regarding bee products and the therapeutic potential of royal jelly as written in the Discussion section.
- 3) Revised explanation of the origin of royal jelly, written in the Method section.
- 4) An explanation of the production of royal jelly and its benefits are written in the Introduction. Due to these changes, three new references have been added to the bibliography.

Any further responses from the reviewers can be found at the end of the article

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is transitional pathogen. This Gram-negative bacillus can be involved in seriously opportunistic-systemic-infections, mainly in immunocompromised patients¹⁻³. These bacteria are often found in the dental unit waterlines which allows the transmission of these bacteria into the oral cavity⁴. As an opportunist pathogen, *P. aeruginosa* is also frequently involved in oral infections, such as necrotizing ulcerative gingivitis, periodontitis, and mandibular osteomyelitis⁵⁻⁷. Although the mechanism is not clear yet, its presence in the oral cavity has been shown to result in systemic infections, such as nosocomial pneumonia⁸.

Based on the reports from several clinical cases, the infection caused by *P. aeruginosa* bacteria can be fatal. Treatment of *P. aeruginosa* infection is sometimes ineffective, which is closely related to the number of virulence factors possessed by the bacteria⁹. The bacterial cell surface components and some secretory products are important virulence factors of *P. aeruginosa*, one of which is pyocyanin¹⁰. Pyocyanin is a cytotoxic pigment from the Phenazine group of compounds that can facilitate biofilm development, cause pro-inflammatory effects, and result in host cell death¹¹.

The resistance of *P. aeruginosa* to various spectrums of antibiotics creates difficulties in handling the infection it causes¹². It has been reported recently that the administration of antibiotics below the minimum inhibitory concentration (MIC) can cause specific bacterial responses, such as an increase in pyocyanin production in *P. aeruginosa*. PAO1 and P14 are the attempts by the bacteria to survive under antibiotic stress¹³. This certainly motivates researchers to further analyse the infection they cause, and find the appropriate antibiotic concentration or dose to overcome the problem.

Royal jelly is a natural bee product produced by hypopharyngeal and mandibular glands of worker bees. This substance is a “superfood” for the queen bee. It is widely used as a nutrient-rich supplement to overcome various health conditions such as inflammation, bacterial infection, tumor, allergy, and antioxidant and immunomodulatory effects^{14,15}. Royal jelly

proteins, such as Jelleine, major royal jelly protein-1 (MRJP1), and royalicin are known to have antibacterial effects against *P. aeruginosa*. Major royal jelly protein-1 and Jelleine can interfere with the permeability of the outer membrane of the cell, causing the loss of vital contents of bacterial cells, which in turn causes cell death. Cationic antimicrobial peptides, such as royalicin, are known to also interfere with cell membrane permeability in various Gram-positive and Gram-negative bacteria, such as *P. aeruginosa*¹⁶⁻¹⁸. Results of previous studies have shown that royal jelly can inhibit the growth of *P. aeruginosa*. In this study, royal jelly showed inhibition of the growth of *P. aeruginosa* ATCC 27853¹⁹. In addition, it has also been known that royal jelly in various concentrations can inhibit the nonspecific attachment of *P. aeruginosa* ATCC 27853²⁰, but so far, the effect of the subinhibitory concentration of royal jelly against these bacteria is unknown. Furthermore, as pyocyanin is an indicator of the pathogenicity of *P. aeruginosa* strains, the aim of this study was to determine the effect of subinhibitory royal jelly concentration on pyocyanin production and biofilm formation in representative strains of a high level pyocyanin-producer *P. aeruginosa* ATCC 10145 and clinical isolate.

Methods

This *in vitro* laboratory experimental research was done at the Integrated Research Laboratory of the Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta. All research procedures have been approved by the Ethics Committee of the Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta (No. 00393/KKEP/ FKG-UGM/EC/2020).

Royal jelly used in this study was obtained from Nusukan, Surakarta, Central Java, Indonesia and harvested in May–October 2019. Royal jelly produced by *Apis mellifera* bees live in kapok tree (*Ceiba pentandra*) and sono wood (*Dalbergia latifolia*). *Apis mellifera* bee species have been identified through previous research²⁰. Royal jelly 5.5 grams was dissolved in 10 ml of cold phosphate buffered saline (PBS), then homogenized using a magnetic stirrer (24 hours, 4°C). The royal jelly solution was centrifuged (12,000 g, 45 minutes, 4°C), then the supernatant was taken and checked the pH. The results of pH measurements with a pH meter showed a pH of 3.79. Furthermore, the supernatant was filtered using 0.45 µm Millipore to produce 55% royal jelly and stored at a temperature of 4–8°C²¹.

Pseudomonas aeruginosa ATCC 10145 (Thermo Scientific) was obtained from the Integrated Research Laboratory of the Faculty of Dentistry, Universitas Gadjah Mada. A clinical isolate of *P. aeruginosa* derived from patient sputum was obtained from the Laboratory of Microbiology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada. Identification of the clinical isolate was carried out using API 20NE (bioMérieux, Inc). Both of these strains were each inoculated in Luria Bertani broth (LB; Himedia Laboratories) and incubated at 37°C for 24 hours. After that, the culture was centrifuged at 3000 rpm for 15 minutes and then resuspended using 0.98% NaCl to obtain a bacterial concentration equivalent to 1.5 × 10⁵ CFU/ml.

Measurement of the effect of royal jelly on the viability of *P. aeruginosa*

A sterile 55% w/v royal jelly solution was diluted in brain heart infusion (BHI; Himedia Laboratories) broth to obtain a concentration of 50% and then serial dilution was performed in 96 well microplates. A total of 5 μ l of the *P. aeruginosa* ATCC 10145 suspension or clinical isolate bacteria (1.5×10^5 CFU/ml) was inoculated in all groups, except the groups that had been determined as blanks. The culture was then incubated at 37°C for 18 hours. After that, the microplate was scanned using the Spark® Multimode Microplate Reader (Tecan trading AG) to measure optical density (OD) using a 600 nm wavelength. The percentage of bacterial viability inhibition was determined based on the OD value of the treatment group against the control. The bacterial viability assay experiments were carried out in quadruplicate.

Analysis of the effect of royal jelly on pyocyanin production

Royal jelly solution was diluted into sterile BHI broth to get the concentration of 12.5%, 6.25%, 3.125%, 1.56%, 0.78%, 0.39%, 0.19%, and 0.098% w/v. Both strains of *P. aeruginosa* were cultured on BHI broth containing various concentrations of royal jelly as treatment and BHI broth only as a blank. The cultures were incubated at 37°C for 36 hours, then the pyocyanin production of each strain was observed visually, which appeared green in the culture supernatant. The pyocyanin concentration was further quantified using previously published methods²². Briefly, after 36 hours of incubation, the culture supernatant was transferred to a sterile tube and centrifuged at a rate of 10,000 g for 30 minutes. The supernatant was filtered using a 0.45 μ m Millipore filter and transferred to fresh 96-well microplates. The absorbance value of the supernatant containing pyocyanin was measured at a wavelength of 690 nm, then the pyocyanin concentration was calculated using the following equation²². The pyocyanin experiments were performed in triplicate.

Concentration of pyocyanin = $A_{690} \text{ nm} (A_{690} \text{ nm of sample} - A_{690} \text{ nm of blank}) / \epsilon * d$

ϵ = extinction coefficient (pyocyanin at $A_{690} \text{ nm} = 4310 \text{ M}^{-1}\text{cm}^{-1}$)

d = path length (0.23 cm for 96 well microplate)

Analysis of the effect of royal jelly on biofilm formation

Static microtiter plate biofilm assay with crystal violet staining was performed to detect the association between pyocyanin production and biofilm formation. We have used *P. aeruginosa* ATCC 10145 as this strain is more responsive in producing pyocyanin when compared to the clinical isolate strains we previously used. Experiments were carried out on a 96-well U bottom microtiter plate (Iwaki, Japan). *P. aeruginosa* ATCC 10145 (1.5×10^8 CFU/ml) inoculated in 100 μ l of LB broth as negative control and on culture media containing royal jelly with final concentration at 25%, 12.5%, 6.25%. The cultures were incubated aerobically at 37°C for 24 hours. After completing the incubation period, the culture rinsed three times using 200 μ L of 1x PBS, followed by staining using 200 μ L of 0.1% crystal violet for 15 minutes at room temperature. Following incubation, 1x PBS was used to rinse the biofilm, followed

by exposure of the biofilm mass with 200 μ l of absolute methanol for 15 minutes at ambient temperature. Afterward, 100 μ l of the biofilm mass solution was transferred to a flat bottom 96-well microplate and the absorbance was read using a microplate reader at a wavelength of 540 nm (Multiskan Sky, ThermoScientific). The percentage change in biofilm mass of each treatment group was calculated from the absorbance value using the formula we previously used²¹. The experiments were carried out in triplicate.

Analysis of biofilm architecture

Scanning electron microscopy was performed to analyse the architecture of the biofilm mass of *P. aeruginosa* ATCC 10145. The bacterial suspension (1.5×10^8 CFU/ml) was inoculated into a sterile tube containing LB broth culture media mixed with royal jelly with a final concentration of 25%, 12.5%, 6.25%. Chlorhexidine gluconate 0.2% was used as a positive control. Meanwhile, for the negative control group, the bacteria were inoculated into LB broth only. Five sterile composite resin discs with a 5 mm diameter and 2 mm height were prepared by using an aseptic method as a medium for adhering to bacteria. The discs were immersed in each tube containing culture media mixed with royal jelly and incubated aerobically at 37°C for 24 hours to provide an opportunity for bacterial biofilm growth. On the following day, each resin disc was taken and processed for qualitative analysis using SEM (JSM – 6510LA, JEOL Ltd, Japan) to investigate the biofilm-mass development on the surface of discs for respective royal jelly concentration.

Statistical analysis

The data in this study were presented as the percentage of bacterial viability, biofilm mass, and pyocyanin concentration in the *P. aeruginosa* culture supernatant. Data were analysed by using the Shapiro-Wilk for normality, followed by Levene's Test for homogeneity using SPSS Statistic v20. Furthermore, one-way ANOVA and Games-Howell parametric analysis were performed for bacterial cell viability and biofilm mass formation data; and parametric one-way ANOVA followed by Tukey HSD on pyocyanin concentration data.

Results

Antibacterial activity of royal jelly against *P. aeruginosa*

The antibacterial activity of royal jelly against the two strains of *P. aeruginosa* is shown in Figure 1. Data on the percentage of bacterial growth inhibition shows normal distribution data ($p > 0.05$), but has a non-homogeneous variant ($p < 0.05$). One-way ANOVA showed a significant difference in the percentage of growth inhibition in *P. aeruginosa* ATCC® 10145™ ($p = 0.000$) and *P. aeruginosa* clinical isolate ($p = 0.000$) between royal jelly treatment groups and negative control. In this study, it was proven that royal jelly can inhibit the viability of both *P. aeruginosa* strains starting from a concentration of 25%. The results of the multi-comparison analysis showed that there was no significant difference between the concentrations of 25% and 50% and significant differences were identified between the concentrations of 25% and 50% with 12.5% to 0.098% in both strains. In clinical isolate, 25% royal jelly caused 60% death in the bacterial population; meanwhile, in *P. aeruginosa* ATCC 10145 this concentration caused growth inhibition of

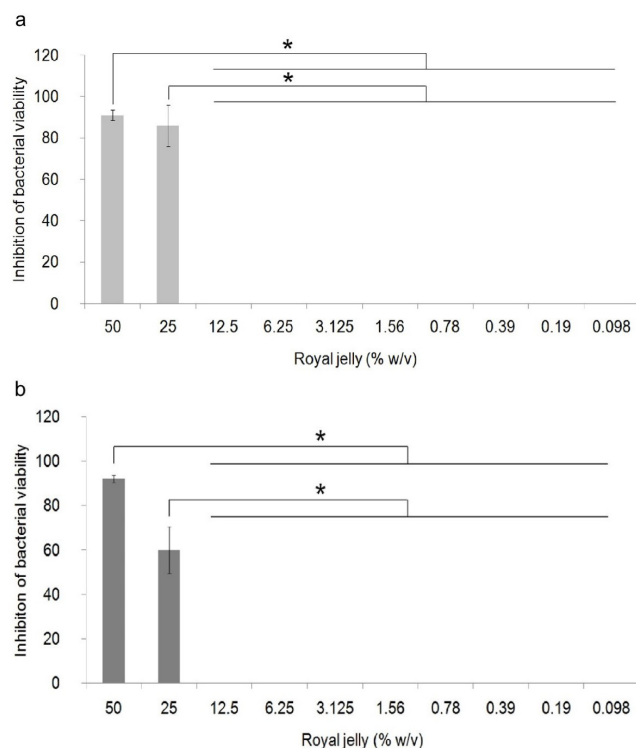


Figure 1. Percentage of inhibition of growth of *P. aeruginosa* bacteria (1.5×10^5 CFU/well). Bacterial cultures were incubated with varying concentrations of royal jelly for 18 hours at 37°C. Royal jelly 50% and 25% inhibit bacterial growth. The difference is based on the results of the Games-Howell analysis at the significance value (*) $p < 0.05$. ATCC 10145 (a) strain; clinical isolate (b).

85%. We therefore categorized royal jelly concentrations below 25% as subinhibitory concentrations.

Exposure to subinhibitory royal jelly concentrations induced increased pyocyanin production in *P. aeruginosa*

Pyocyanin was identified as greenish colour in culture supernatant *P. aeruginosa* ATCC 10145 and clinical isolate. After 36 hours of incubation, pyocyanin production was increased in the stimulated culture group with subinhibitory concentrations below 25%. The intensity of green colour in the culture medium increased with the increase in the concentration of royal jelly (Figure 2). The change in the colour intensity of the culture supernatant was consistent with the results of the pyocyanin concentration measurement.

Pyocyanin concentration data in each royal jelly treatment group and negative control were the results of experiments on triplicate cultures. Figure 3 shows the average pyocyanin concentration for each group. The highest average pyocyanin concentration was identified in *P. aeruginosa* ATCC 10145 induced by royal jelly with a concentration of 6.25%, which was 23.59 μM , while the lowest mean was identified in clinical isolate of *P. aeruginosa* without exposure to royal jelly, which was 0.7 μM . The pyocyanin concentration of *P. aeruginosa* ATCC 10145 was seen to be higher than clinical isolate in the same concentration in all treatment groups.

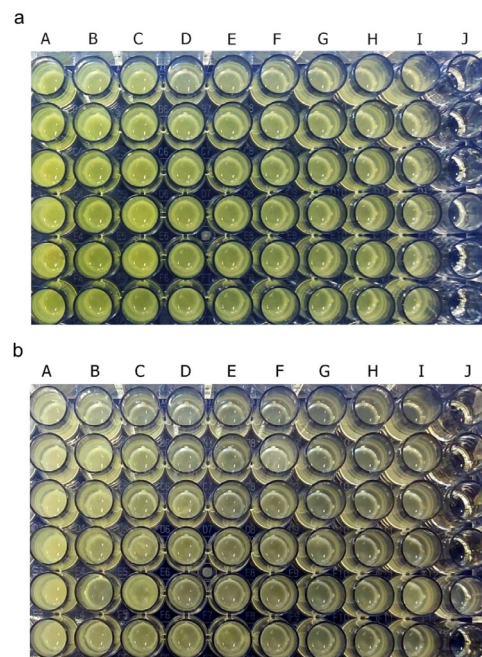


Figure 2. Pyocyanin was identified from the greenish colour of the *P. aeruginosa* culture supernatant after 36 h incubation at 37°C. *P. aeruginosa* bacteria (1.5×10^5 CFU/well) ATCC 10145 (a); clinical isolate (b). Royal jelly 12.5% (A); 6.25% (B); 3.125% (C); 1.56% (D); 0.78% (E); 0.39% (F); 0.19% (G); 0.098% (H); 0% (I); No treatment (J).

Pyocyanin concentration data both on ATCC 10145 and clinical isolate in all groups were normally distributed ($p > 0.05$) and homogeneous ($p > 0.05$). There was a significant difference in the concentration of pyocyanin ATCC 10145 ($p = 0.000$) and clinical isolate ($p = 0.000$) between the treatment groups. The results of multiple comparison analysis of Tukey-HSD on *P. aeruginosa* cultures of ATCC 10145 showed a significant difference between the royal jelly groups with concentrations of 0% with 12.5%, 6.25%, and 3.125%. In addition, a significant difference in pyocyanin concentrations in clinical isolate was found between the 0% royal jelly group with 12.5% and 6.25%.

Subinhibitory royal jelly concentrations increased biofilm formation in *P. aeruginosa*

The results of the biofilm mass test using a static microtiter plate biofilm assay were presented as the relative percentage value of the biofilm mass in the treatment group to the negative control (Figure 4).

Figure 4 illustrates the peculiarity of 12.5% royal jelly that induced biofilm mass formation up to four times as much (434.06%) than bacteria not exposed to royal jelly. The lower concentration of royal jelly (6.12%) also increased biofilm formation but was lower than 12.5% royal jelly (234.35%). Meanwhile, the concentration of 25% suppressed the bacterial growth so that less biofilm compared to the other groups, around 5.85% relative to the negative control group. Welch ANOVA analysis has shown significant differences across groups ($p < 0.05$).

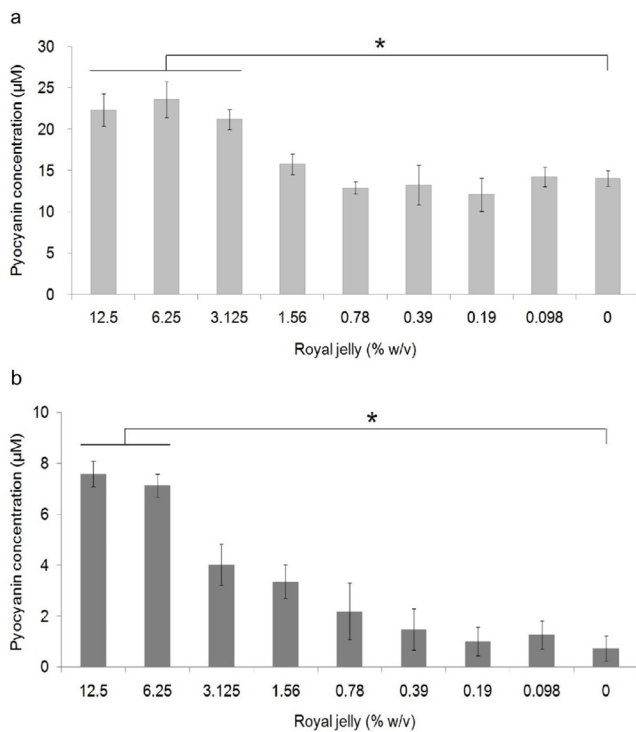


Figure 3. Pyocyanin production in *P. aeruginosa* bacteria (1.5×10^5 CFU/well) after 36 hours of exposure to various concentrations of royal jelly. Pyocyanin production increased due to exposure to sub-inhibitory royal jelly concentrations of 6.25% and decreased at lower royal jelly concentrations. The difference was based on the results of the Tukey-HSD analysis at the significance value (*) $p < 0.05$. ATCC 10145 (a) strain; (b) clinical isolate.

In addition, the results of multiple comparisons using Games-Howell showed a significant difference between royal jelly 12.5% and 25%, 6.25%, and the negative control group ($p < 0.05$).

The effect of royal jelly on *P. aeruginosa* biofilm architecture

Observation of biofilm mass architecture using scanning electron microscopy has been carried out to complement the data from the measurement of biofilm quantity. The results indicated an effect of differences in royal jelly concentrations on the biofilm characteristics of *P. aeruginosa* ATCC 10145 (Figure 5).

The x1000 magnification of the 6.25% royal jelly specimen showed a specific appearance of the fern-like bacterial colony. The x5000 magnification gives a clearer picture of the colony's shape. Meanwhile, the biofilm mass was thicker in the specimens treated with 12.5% royal jelly. Bacterial colonies covered all surfaces of the attachment media, significantly different from bacterial biofilms treated with 6.25% royal jelly. However, the biofilm mass formation inhibited in the group treated with 25% royal jelly. The subinhibitory concentrations of royal jelly increased biofilm formation; the thickest biofilm mass was in the 12.5% royal jelly treatment group.

Discussion

The antibacterial effect of royal jelly has been widely reported by previous researchers¹⁶⁻¹⁸. The ability of royal jelly to inhibit the growth of *P. aeruginosa* is thought to be related to the variety and concentration of its antibacterial protein. Royal jelly components that have been identified as having antibacterial activity are major royal jelly protein-1 (MRJP-1), Jelleine I-III, royalicin, and 10-hydroxy-2-decenoic (10-HDA)^{16,18,23}.

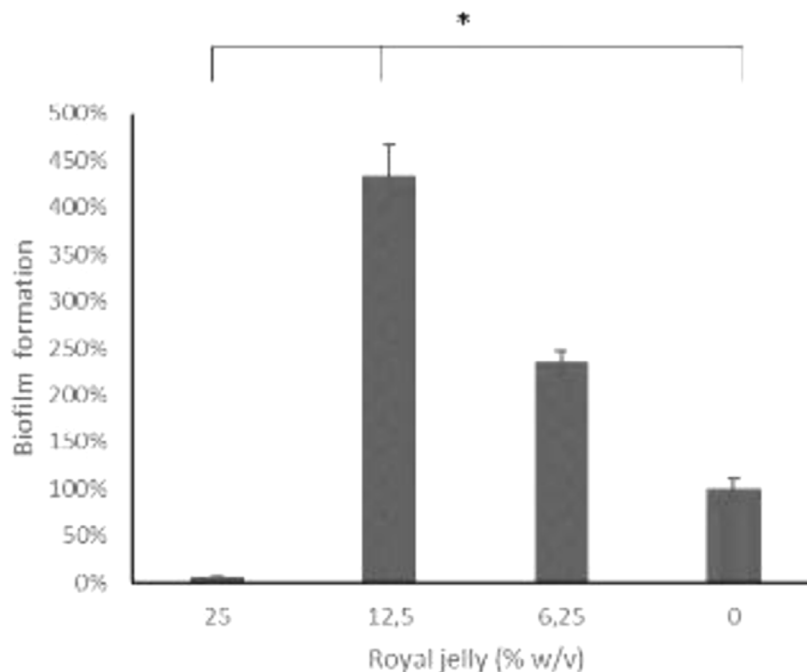


Figure 4. The 12.5% royal jelly increased the formation of *P. aeruginosa* biofilm stronger than 25% and 6.25% royal jelly. ATCC 10145.

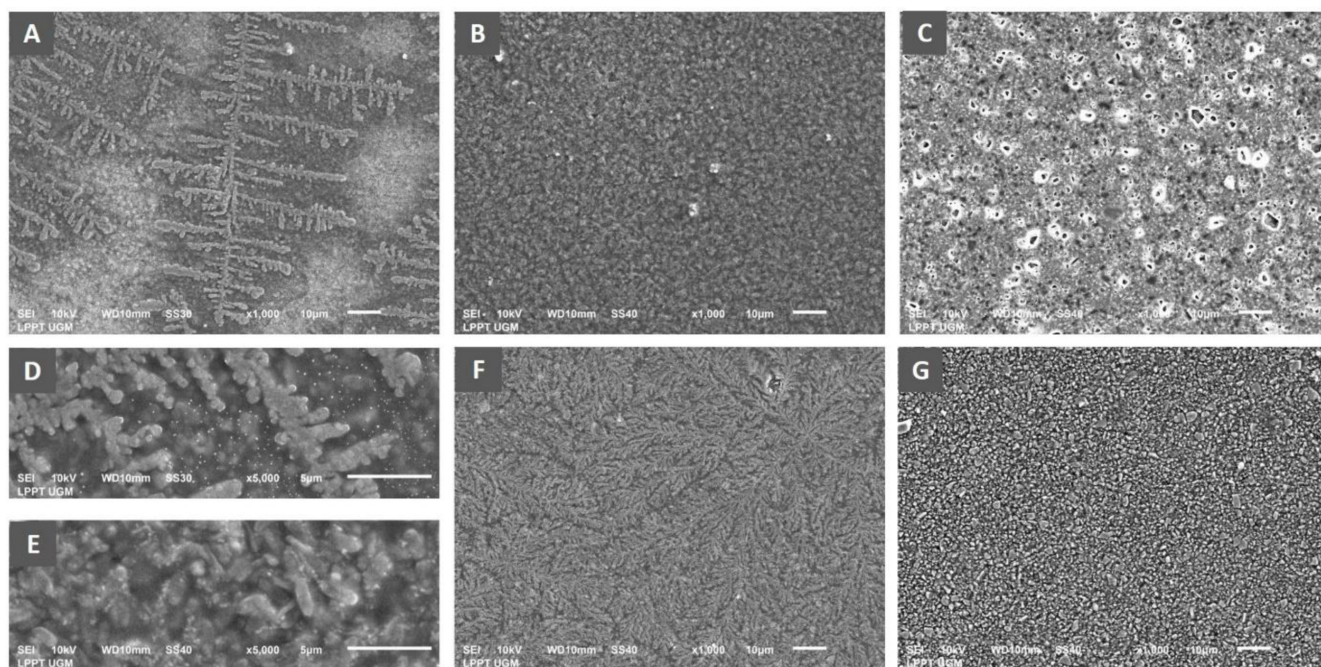


Figure 5. Scanning electron micrograph of *P. aeruginosa* biofilm showed the formation of specific bacterial colonies on the adhesion surface with extensions indicating bacterial motility in the group exposed to royal jelly 6.25% (A), complete coverage of the adhesion surface by the bacterial biofilm mass in the treatment group with royal jelly 12.5% (B). Adhesion surface was free from bacterial biofilm in the bacterial group treated with 25% royal jelly (C). Figure D shows the biofilm architecture of the 6.25% royal jelly group, and E is that of the 12.5% royal jelly group at a magnification of x5000. The negative control group showed evenly distributed bacterial colonies on the adhesion surface (F), while the positive control group (chlorhexidine) showed complete inhibition of biofilm formation (G). Overall, the SEM results presented the highest increase in biofilm mass formation by 12.5% royal jelly. ATCC 10145 strain.

This study performed the extraction method using phosphate buffer saline²⁴ that was chosen in order to dissolve MRJP1, the active components that are antibacterial or anti-adhesion. It is known that MRJP1 is a hydro soluble protein dissolved in PBS, which is higher in supernatants than in pellets²⁵. After centrifugation there were indeed pellets, and it has been found that the pellets contain more liposoluble proteins than supernatants. To ensure that the compounds contained in the PBS royal jelly extract supernatant, further research is needed. However, from the study of Furusawa *et al.*, it was found that MRJP1 was contained in the supernatant of PBS royal jelly extract in quite a large amount, reaching 27.6%. The active components that are antibacterial or anti-adhesion in the supernatant of royal jelly extract in this study cannot be explained with certainty, but the royal jelly extract used in this study was thought to mainly contain MRJP1²⁶.

This study showed that royal jelly concentrations of 25% and 50% had antibacterial activity against *P. aeruginosa* ATCC 10145 and clinical isolate. The results of this observation are different from previous studies that showed *P. aeruginosa* growth could be inhibited at concentrations >50%¹⁹. This difference is thought to be closely related to differences in geographical location, botanical origin, climate, and storage conditions of royal jelly, which affect the antibacterial component of royal jelly²⁷. Previous studies have shown that royal jelly originating from

different geographic and botanical locations affects the quantity of 10-HDA. Royal jelly originating from tropical climates is reported to contain lower concentrations of 10-HDA than cold climates²⁷. The higher temperature and longer storage time also resulted in a significant reduction in the quantity of MRJP1²⁸. However, the bacterial strains studied probably also had an effect, as previously reported there was a variable response between clinical isolates and standard bacteria^{19,20}.

Pyocyanin is an indicator of the pathogenicity of *P. aeruginosa*. To our knowledge, this study report is the first to demonstrate a dualism effect of royal jelly on *P. aeruginosa*. The subinhibitory concentration of royal jelly amplify the effect of an autoinducer. It was able to increase the production of pyocyanin in ATCC 10145 and clinical isolate to protect and maintain their survival¹³. The pyocyanin concentration in the ATCC 10145 strain appeared to be significantly higher than the clinical isolate. This observation is in accordance with previous studies that found the ATCC 10145 strain produced more pyocyanin than the clinical isolate strains from active ulcerative keratitis patients²⁹. The presence of *phzM* and *phzS* genes was thought to affect the concentration of pyocyanin produced³⁰. This was proven by previous studies that the *phzM* and *phzS* gene expression of multidrug resistance (MDR) clinical isolate *P. aeruginosa* was lower leading to less pyocyanin production than non-MDR isolates and PAO1 strains³¹. Some clinical

isolates were also reported not to have the genes so that these bacteria cannot produce pyocyanin³⁰. Other research results also showed that the pyocyanin concentration of ATCC 10145 strains is higher than that of PAO1 and PA14 strains after incubation for 60 hours³². It is estimated that ATCC 10145 is one of the strong pyocyanin producing strains. However, other virulence factors possessed by this strain were lower than the clinical isolate strains so that they were considered less virulent²⁹.

Various virulence factors, including pyocyanin are generally associated with the quorum sensing mechanism³³. Quorum sensing refers to the communication process between microbial cells using autoinducer molecules³⁴. One of the autoinducer molecules that plays an important role in the regulation of pyocyanin production is the *Pseudomonas* quinolone signal (PQS). Mutation of the PQS gene results in reduced pyocyanin production³⁵. When bacterial cells are exposed to exogenic stress, such as an antibacterial agent that can threaten their survival, the bacteria immediately respond to the stimulus by inducing the production of PQS which is responsible for activating various genes involved in the production of virulence factors, including pyocyanin^{33,36}. Although the effect of royal jelly subinhibitory concentration on this autoinducer molecule is not yet known, several studies have reported that the increase in pyocyanin production is closely related to the effect of subinhibitory antibiotics that increase PQS gene expression³⁷. It is thought that this is the cause of increased pyocyanin production at subinhibitory concentrations.

The increase in pyocyanin production in *P. aeruginosa* bacteria will have implications for the mechanism of bacterial attachment and biofilm formation. Apart from its production, which is closely related to the quorum sensing mechanism, pyocyanin is also a signalling factor in the quorum sensing process itself. This was identified from the results of research on *P. aeruginosa* PAO1 and PA14³⁸. We have found that subinhibitory concentration of royal jelly increases pyocyanin production. It has also increased biofilm mass formation. It confirmed in the strain producing high levels of pyocyanin, *P. aeruginosa* ATCC 10145. In addition, the increase in pyocyanin is likely to have an impact on the activity of bacteria to produce extracellular DNA (eDNA). Extracellular DNA is an important part of extracellular polymeric substance (EPS) which is the main component of the biofilm matrix. The increase in EPS production is very beneficial for the bacterial attachment process and subsequently the formation of biofilms. Pyocyanin can induce eDNA production in low level pyocyanin-producer strains, PAO1 and pyocyanin-deficient strains, PA14. In this study, it was proven that pyocyanin caused an increase in the production and release of eDNA, which is the main component in forming and stabilizing bacterial biofilms³⁹.

The regulation of biofilm formation and pyocyanin production involves the regulatory genes of the PQS system and the Rhl system. The mechanism of the PQS system in regulating pyocyanin synthesis has been confirmed in several strains of *P. aeruginosa* bacteria but may vary depending

on each strain⁴⁰. The mechanism of the effect of royal jelly on these systems is not yet known. In this regard, in the future, it is important to analyse the effect of royal jelly on the expression of genes related to PQS and Rhl regulation in *P. aeruginosa* bacteria.

The increase in pyocyanin production induced by subinhibitory royal jelly concentrations in *P. aeruginosa* ATCC 10145 and clinical isolate in this study is an interesting phenomenon. Although, subinhibitory royal jelly concentrations were not effective in inhibiting the growth of these bacteria, on the other hand, they increased production of pyocyanin virulence factors. The known bee products are honey, propolis, and royal jelly. All three have antibacterial and antibiofilm properties, in addition to other therapeutic potentials in the process of tissue healing, reproduction, and infectious diseases¹⁵. Manuka honey has been widely studied and proven to be antibacterial and inhibit the formation of biofilms in *P. aeruginosa*; however, bacteria derived from biofilms experienced a decrease in sensitivity to manuka honey due to the presence of variants in the bacterial population⁴¹ or due to subinhibitory concentrations of manuka honey⁴². Our results indicate that the sensitivity of *P. aeruginosa* bacteria to royal jelly may also vary, depending on the concentration of royal jelly exposed to the bacteria. At sub-inhibitory concentration, we found the increase of the pyocyanin production and biofilm growth. This has inspired the alleged biphasic nature of royal jelly which has antibacterial potential, but at different exposure concentrations, it can induce the production of *P. aeruginosa* bacteria virulence factors. This phenomenon leads us to think that researchers, as well as medical practitioners, should be careful in determining the concentration of royal jelly for its antibacterial research purposes or its therapeutic potential. This of course requires further research on the mechanisms associated with bacterial response to subinhibitory concentrations of royal jelly.

Conclusions

Royal jelly at a concentration of 25% was able to inhibit the growth of *P. aeruginosa* bacteria, but at subinhibitory concentrations, it could increase pyocyanin production in *P. aeruginosa* strain ATCC 10145 and clinical isolate. The subinhibitory concentration of royal jelly also increased biofilm formation in *P. aeruginosa* ATCC 10145. Based on the results of this study, we suggest selecting the appropriate dose or concentration for the purpose of inhibiting the growth and production of *P. aeruginosa* virulence factors.

Data availability

Underlying data

Figshare: *Pseudomonas aeruginosa* pyocyanin, <https://doi.org/10.6084/m9.figshare.13247429.v1>⁴³.

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](https://creativecommons.org/licenses/by/4.0/) (CC-BY 4.0).

Acknowledgements

Researchers appreciate the help of Bunga Artika as a laboratory assistant who helped in this research.

References

1. Corrin B, Nicholson AG: **Pathology of the Lungs**, 3rd ed. London: Churchill Livingstone. Elsevier. 2011; 177.
[Publisher Full Text](#)
2. Migiyama Y, Yanagihara K, Kaku N, et al.: ***Pseudomonas aeruginosa* bacteremia among immunocompetent and immunocompromised patients: relation to initial antibiotic therapy and survival.** *Jpn J Infect Dis.* 2016; **69**(2): 91–96.
[PubMed Abstract](#) | [Publisher Full Text](#)
3. Wolska K, Kot B, Jakubczak A: **Phenotypic and genotypic diversity of *Pseudomonas aeruginosa* strains isolated from hospitals in Siedlce (Poland).** *Braz J Microbiol.* 2012; **43**(1): 274–282.
[PubMed Abstract](#) | [Free Full Text](#)
4. Ouellet MM, Leduc A, Nadeau C, et al.: ***Pseudomonas aeruginosa* isolates from dental unit waterlines can be divided in two distinct groups, including one displaying phenotypes similar to isolates from cystic fibrosis patients.** *Front Microbiol.* 2015; **5**: 802.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
5. Jandial A, Mishra K, Panda A, et al.: **Necrotising ulcerative gingivitis: a rare manifestation of *Pseudomonas* infection.** *Indian J Hematol Blood Transfus.* 2018; **34**(3): 578–580.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
6. Vieira Colombo AP, Magalhães CB, Hartenbach FA, et al.: **Periodontal-disease-associated biofilm: a reservoir for pathogens of medical importance.** *Microb Pathog.* 2016; **94**: 27–34.
[PubMed Abstract](#) | [Publisher Full Text](#)
7. Pappalardo S, Tanteri L, Brutto D, et al.: **Mandibular osteomyelitis due to *Pseudomonas aeruginosa*. Case report.** *Minerva Stomatol.* 2008; **57**(6): 323–329.
[PubMed Abstract](#)
8. Rivas Caldas R, Boisramé S: **Upper aero-digestive contamination by *Pseudomonas aeruginosa* and implications in Cystic Fibrosis.** *J Cyst Fibros.* 2015; **14**(1): 6–15.
[PubMed Abstract](#) | [Publisher Full Text](#)
9. Macin S, Akarca M, Sener B, et al.: **Comparison of virulence factors and antibiotic resistance of *Pseudomonas aeruginosa* strains isolated from patients with and without cystic fibrosis.** *Rev Rom Med Lab.* 2017; **25**(4): 327–334.
[Publisher Full Text](#)
10. Ben Haj Khalifa A, Moissenet D, Vu Thien H, et al.: **[Virulence factors in *Pseudomonas aeruginosa*: mechanisms and modes of regulation].** *Ann Biol Clin (paris).* 2011; **69**(4): 393–403.
[PubMed Abstract](#) | [Publisher Full Text](#)
11. Hall S, McDermott C, Anoopkumar-Dukie S, et al.: **Cellular effects of pyocyanin, a secreted virulence factor of *Pseudomonas aeruginosa*.** *Toxins (Basel).* 2016; **8**(8): 236.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
12. Nguyen L, Garcia J, Gruenberg K, et al.: **Multidrug-resistant *Pseudomonas* infections: hard to treat, but hope on the horizon?** *Curr Infect Dis Rep.* 2018; **20**(8): 23.
[PubMed Abstract](#) | [Publisher Full Text](#)
13. Zhu K, Chen S, Sysoeva TA, et al.: **Universal antibiotic tolerance arising from antibiotic-triggered accumulation of pyocyanin in *Pseudomonas aeruginosa*.** *PLoS Biol.* 2019; **17**(12): e3000573.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
14. Fratini F, Cilia G, Mancini S, et al.: **Royal jelly: an ancient remedy with remarkable antibacterial properties.** *Microbiol Res.* 2016; **192**: 130–141.
[PubMed Abstract](#) | [Publisher Full Text](#)
15. Pasupuleti VR, Sammugam L, Ramesh N, et al.: **Honey, propolis, and royal jelly: A comprehensive review of their biological actions and health benefits.** *Oxid Med Cell Longev.* 2017; **2017**: 1259510.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
16. Brudzynski K, Sjaarda C, Lannigan R: **MRJP1-containing glycoproteins isolated from honey, a novel antibacterial drug candidate with broad spectrum activity against multi-drug resistant clinical isolates.** *Front Microbiol.* 2015; **6**: 711.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
17. Fontana R, Mendes MA, de Souza BM, et al.: **Jelleines: a family of antimicrobial peptides from the royal jelly of honeybees (*Apis mellifera*).** *Peptides.* 2004; **25**(6): 919–928.
[PubMed Abstract](#) | [Publisher Full Text](#)
18. Bilikova K, Huang SC, Lin IP, et al.: **Structure and antimicrobial activity relationship of royalisin, an antimicrobial peptide from royal jelly of *Apis mellifera*.** *Peptides.* 2015; **68**: 190–196.
[PubMed Abstract](#) | [Publisher Full Text](#)
19. Boukraa L: **Additive activity of royal jelly and honey against *Pseudomonas aeruginosa*.** *Altern Med Rev.* 2008; **13**(4): 330–333.
[PubMed Abstract](#)
20. Hartono SK, Haniastuti T, Susilowati H, et al.: **The effect of *in vitro* royal jelly provision on adhesion of *Pseudomonas aeruginosa*.** *Maj Kedokt Gigi Indones.* 2019; **5**(1): 1–5.
[Publisher Full Text](#)
21. Susilowati H, Murakami K, Yumoto H, et al.: **Royal jelly inhibits *Pseudomonas aeruginosa* adherence and reduces excessive inflammatory responses in human epithelial cells.** *Biomed Res Int.* 2017; **2017**: 3191752.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
22. Price-Whelan A, Dietrich LEP, Newman DK: **Pyocyanin alters redox homeostasis and carbon flux through central metabolic pathways in *Pseudomonas aeruginosa* PA14.** *J Bacteriol.* 2007; **189**(17): 6372–6381.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
23. Han B, Fang Y, Feng M, et al.: **In-depth phosphoproteomic analysis of royal jelly derived from western and eastern honeybee species.** *J Proteome Res.* 2014; **13**(12): 5928–5943.
[PubMed Abstract](#) | [Publisher Full Text](#)
24. Hu FL, Biliková K, Casabianca H, et al.: **Standard methods for *Apis mellifera* royal jelly research.** *J Apic Res.* 2019; **58**(2): 1–68.
[Publisher Full Text](#)
25. Gismondi A, Trionfera E, Canuti L, et al.: **Royal jelly lipophilic fraction induces antiproliferative effects on SH-SY5Y human neuroblastoma cells.** *Oncol Rep.* 2017; **38**(3): 1833–44.
[PubMed Abstract](#) | [Publisher Full Text](#)
26. Furusawa T, Arai Y, Kato K, et al.: **Quantitative analysis of Apisin, a major protein unique to royal jelly.** *Evid Based Complement Alternat Med.* 2016; **2016**: 5040528.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
27. Wei WT, Hu YQ, Zheng HQ, et al.: **Geographical influences on content of 10-hydroxy-trans-2-decenoic acid in royal jelly in China.** *J Econ Entomol.* 2013; **106**(5): 1958–1963.
[PubMed Abstract](#) | [Publisher Full Text](#)
28. Li JK, Feng M, Zhang L, et al.: **Proteomics analysis of major royal jelly protein changes under different storage conditions.** *J Proteome Res.* 2008; **7**(8): 3339–3353.
[PubMed Abstract](#) | [Publisher Full Text](#)
29. Sewell A, Dunmire J, Wehmann M, et al.: **Proteomic analysis of keratitis-associated *Pseudomonas aeruginosa*.** *Mol Vis.* 2014; **20**: 1182–1191.
[PubMed Abstract](#) | [Free Full Text](#)
30. Samad A, Ahmed T, Rahim A, et al.: **Antimicrobial susceptibility patterns of clinical isolates of *Pseudomonas aeruginosa* isolated from patients of respiratory tract infections in a Tertiary Care Hospital, Peshawar.** *Pak J Med Sci.* 2017; **33**(3): 670–674.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
31. Fuse K, Fujimura S, Kikuchi T, et al.: **Reduction of virulence factor pyocyanin production in multidrug-resistant *Pseudomonas aeruginosa*.** *J Infect Chemother.* 2013; **19**(1): 82–88.
[PubMed Abstract](#) | [Publisher Full Text](#)
32. Rada B, Lekstrom K, Damian S, et al.: **The *Pseudomonas* toxin pyocyanin inhibits the dual oxidase-based antimicrobial system as it imposes oxidative stress on airway epithelial cells.** *J Immunol.* 2008; **181**(7): 4883–4893.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
33. Lin J, Cheng J, Wang Y, et al.: **The *Pseudomonas* Quinolone signal (PQS): not just for quorum sensing anymore.** *Front Cell Infect Microbiol.* 2018; **8**: 230.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
34. Zhang W, Li C: **Exploiting quorum sensing interfering strategies in gram-negative bacteria for the enhancement of environmental applications.** *Front Microbiol.* 2016; **6**: 1535.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
35. Bala A, Kumar L, Chhibber S, et al.: **Augmentation of virulence related traits of *pqs* mutants by *Pseudomonas* quinolone signal through membrane vesicles.** *J Basic Microbiol.* 2015; **55**(5): 566–578.
[PubMed Abstract](#) | [Publisher Full Text](#)
36. Bru JL, Rawson B, Trinh C, et al.: **PQS produced by the *Pseudomonas aeruginosa* stress response repels swarms away from bacteriophage and antibiotics.** *J Bacteriol.* 2019; **201**(23): e00383–19.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
37. Cummins J, Reen FJ, Baysse C, et al.: **Subinhibitory concentrations of the cationic antimicrobial peptide colistin induce the *Pseudomonas* quinolone signal in *Pseudomonas aeruginosa*.** *Microbiology (Reading).* 2009; **155**(Pt 9): 2826–2837.
[PubMed Abstract](#) | [Publisher Full Text](#)
38. Dietrich LE, Price-Whelan A, Petersen A, et al.: **The phenazine pyocyanin is a terminal signalling factor in the quorum sensing network of *Pseudomonas aeruginosa*.** *Mol Microbiol.* 2006; **61**(5): 1308–1321.
[PubMed Abstract](#) | [Publisher Full Text](#)
39. Das T, Manfield M: **Pyocyanin promotes extracellular DNA release in *Pseudomonas aeruginosa*.** *PLoS One.* 2012; **7**(10): e46718.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
40. García-Reyes S, Soberón-Chávez G, Cocotl-Yanez M: **The third quorum-sensing system of *Pseudomonas aeruginosa*: *Pseudomonas* quinolone signal and the enigmatic PqsE protein.** *J Med Microbiol.* 2020; **69**(1): 25–34.
[PubMed Abstract](#) | [Publisher Full Text](#)

41. Camplin AL, Maddocks SE: **Manuka honey treatment of biofilms of *Pseudomonas aeruginosa* results in the emergence of isolates with increased honey resistance.** *Ann Clin Microbiol Antimicrob.* 2014; **13**: 19.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
42. Lu J, Cokcetin NN, Burke CM, *et al.*: **Honey can inhibit and eliminate biofilms produced by *Pseudomonas aeruginosa*.** *Sci Rep.* 2019; **9**(1): 18160.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
43. Susilowati H, Amly DA, Yulianto HDK, *et al.*: ***Pseudomonas aeruginosa* pyocyanin.** *figshare.* Figure. 2020.
<http://www.doi.org/10.6084/m9.figshare.13247429.v1>

Open Peer Review

Current Peer Review Status: ✓ ✓ ✓ ?

Version 4

Reviewer Report 20 January 2022

<https://doi.org/10.5256/f1000research.79877.r102187>

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Philip S. Bird

School of Veterinary Science, Faculty of Science, The University of Queensland, Gatton, Qld, Australia

I still have a few issues with the latest revision.

First paragraph of Introduction:

"*Pseudomonas aeruginosa* (*P. aeruginosa*) is **transitional** pathogen. This Gram-negative bacillus can be involved in seriously opportunistic-systemic-infections, mainly in immunocompromised patients¹⁻³. These bacteria are often found in the dental unit waterlines which allows the transmission of these bacteria into the oral cavity⁴. As an opportunist pathogen, *P. aeruginosa* is also **frequently involved** in oral infections, such as necrotizing ulcerative gingivitis, periodontitis, and mandibular osteomyelitis⁵⁻⁷. Although the mechanism is not clear yet, its presence in the oral cavity has been shown to result in systemic infections, such as nosocomial pneumonia⁸."

My comment was to illustrate that Pa is a transitional bacterium in the oral cavity. It is an opportunistic pathogen. It is not a frequent oral pathogen as the references refer to case studies. A better reference to cite to illustrate Pa infections is Klochgetter and Tümmeler (2017¹), for example.

Methods: "Royal jelly used in this study was obtained from Nusukan, Surakarta, Central Java, Indonesia and harvested in May–October 2019. **Royal jelly produced by *Apis mellifera* bees live in kapok tree (*Ceiba pentandra*) and sono wood (*Dalbergia latifolia*). *Apis mellifera* bee species have been identified through previous research²⁰. Royal jelly 5.5 grams** was dissolved in 10 ml..."

1. I'm not sure what the tree species have to do with the production of RJ - maybe say that RJ was harvested from feral bee colonies found in kapok trees etc. - but usually to collect royal jelly hives as set up so that bees produce a number of queen cells are from those royal jelly is collected. Maybe the authors could look at how royal jelly is produced, cited in reference Fratini *et al.* (2016) #14.

2. I'm not sure what the sentence with cited reference #20 means.
3. Was the RJ freeze-dried or a raw fresh extracted solution?

My comments in the review:

Just as an addendum, while this paper is mainly a technical paper I thought it would be **of interest to readers with a few sentences on bee products** as well as royal jelly. Something on how royal jelly is produced by bees. **How and what would royal jelly be used for in treatment and what infections?**

1. It is good to see that some discussion on other bee products was included in the last paragraph of the discussion. Bee products appear to be used currently for treatment of wounds. How does this research lead to royal jelly treatments of Pa infections? Are there any current papers that can be cited?

References

1. Klockgether J, Tümmler B: Recent advances in understanding *Pseudomonas aeruginosa* as a pathogen. *F1000Res.* 2017; **6**: 1261 [PubMed Abstract](#) | [Publisher Full Text](#)

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Version 3

Reviewer Report 18 October 2021

<https://doi.org/10.5256/f1000research.77925.r92435>

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Philip S. Bird

School of Veterinary Science, Faculty of Science, The University of Queensland, Gatton, Qld, Australia

Introduction - Pa is not frequently involved in oral infections. Pa is a transitional pathogen and can be involved in opportunistic infections. Please change to indicate that. Please change throughout the article 'isolates' to 'isolate' (delete "s"). There is nothing in the introduction or discussion on the other products from bees such as honey, propolis. Why only introduce royal jelly and not these products which have anti-bacterial properties? References Pasupuleti *et al.* (2017¹) and Camplin

and Maddocks (2014²) would be useful additions. Was Royal jelly freeze-dried? The royal jelly came from bees, not beekeeper, and what race of bees?

Just as an addendum, while this paper is mainly a technical paper I thought it would be of interest to readers with a few sentences on bee products as well as royal jelly. Some thing on how royal jelly is produced by bees. How and what would royal jelly be used for in treatment and what infections?

References

1. Pasupuleti VR, Sammugam L, Ramesh N, Gan SH: Honey, Propolis, and Royal Jelly: A Comprehensive Review of Their Biological Actions and Health Benefits. *Oxid Med Cell Longev*. 2017; **2017**: 1259510 [PubMed Abstract](#) | [Publisher Full Text](#)
2. Camplin AL, Maddocks SE: Manuka honey treatment of biofilms of *Pseudomonas aeruginosa* results in the emergence of isolates with increased honey resistance. *Ann Clin Microbiol Antimicrob*. 2014; **13**: 19 [PubMed Abstract](#) | [Publisher Full Text](#)

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Oral microbiologist and beekeeper

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 27 Nov 2021

Heni Susilowati, Faculty of Dentistry, Universitas Gadjah Mada, Sleman, Indonesia

Reviewer comment 1:

Introduction - Pa is not frequently involved in oral infections. Pa is a transitional pathogen and can be involved in opportunistic infections. Please change to indicate that.

Response:

We have changed the first sentence of the first paragraph in the introduction as follows: *Pseudomonas aeruginosa* (*P. aeruginosa*) is transitional pathogen. This Gram-negative bacillus can be involved in seriously opportunistic-systemic-infections, mainly in immunocompromised patients ¹⁻³.

Reviewer comment 2:

Please change throughout the article 'isolates' to 'isolate' (delete "s").

Response:

Correction was done on paragraph 4 of Introduction, paragraph 1 and 3 of Results, paragraph 4 and 8 of the Discussion section.

Reviewer comment 3:

There is nothing in the introduction or discussion on the other products from bees such as honey, propolis. Why only introduce royal jelly and not these products which have anti-bacterial properties? References Pasupuleti et al. (2017) and Camplin and Maddocks (2014) would be useful additions.

Response:

Additional information about honey products in the 6th paragraph of the Discussion section, written as 4th-7th sentences.

The increase in pyocyanin production The known bee products are honey, propolis, and royal jelly. All three have antibacterial and antibiofilm properties, in addition to other therapeutic potentials in the process of tissue healing, reproduction, and infectious diseases. (Pasupuleti et al, 2017) Manuka honey has been widely studied and proven to be antibacterial and inhibit the formation of biofilms in *P. aeruginosa*; however, bacteria derived from biofilms experienced a decrease in sensitivity to manuka honey due to the presence of variants in the bacterial population (Camplin and Maddocks, 2014) or due to subinhibitory concentrations of manuka honey. (Lu et al, 2019). Our results indicate that the sensitivity of *P. aeruginosa* bacteria to royal jelly may also vary, depending on the concentration of royal jelly exposed to the bacteria. At sub-inhibitory concentration, we found the increase of the pyocyanin production and biofilm growth. of royal jelly.

Reviewer comment 4:

Was Royal jelly freeze-dried?

Response:

The royal jelly preparation used in this study was a fresh extract in PBS as described at the end of the first paragraph of the Methods section.

Reviewer comment 5:

The royal jelly came from bees, not beekeeper, and what race of bees?

Response:

The royal jelly we use is produced by *Apis mellifera* bees. We have revised it according to the reviewer's suggestion, which can be read in the 4th sentence of the first paragraph of the Methods section.

Reviewer comment 6:

Just as an addendum, while this paper is mainly a technical paper, I thought it would be of interest to readers with a few sentences on bee products as well as royal jelly. Something on how royal jelly is produced by bees. How and what would royal jelly be used for in treatment and what infections?

Response:

Additional information regarding royal jelly is written at the beginning of paragraph 4 of the Introduction section as follows:

Royal jelly is produced by hypopharyngeal and mandibular glands of worker bees. This substance is a "superfood" for the queen bee. It is widely used as a nutrient-rich supplement to overcome various health conditions such as inflammation, bacterial infection, tumor, allergy, and antioxidant and immunomodulatory effects. [Fratini et al., 2016; Pasupuleti et al., 2017]

Response to the reviewer comments on statistical analysis:

We have consulted statistical analysis of the results of this study with statisticians and stated that no changes were needed.

Additional references:

41. Pasupuleti VR, Sammugam L, Ramesh N, et al: Honey, propolis, and royal jelly: A comprehensive review of their biological actions and health benefits. *Oxid Med Cell Longev*. 2017;2017:1259510. doi: 10.1155/2017/1259510

42. Camplin AL and Maddocks SE: Manuka honey treatment of biofilms of *Pseudomonas aeruginosa* results in the emergence of isolates with increased honey resistance. *Ann Clin Microbiol Antimicrob*. 2014;13:19

43. Lu J, Cokcetin NN, Burke CM, et al.: Honey can inhibit and eliminate biofilms produced by *Pseudomonas aeruginosa*. *Sci Rep*. 2019; 9(1):18160

Competing Interests: I declare that I have no conflict of interest with this peer review report.

Reviewer Report 06 October 2021

<https://doi.org/10.5256/f1000research.77925.r95642>

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Shizuo Kayama 

Antimicrobial Resistance Research Center, National Institute of Infectious Diseases,
Higashimurayama, Japan

The revised manuscript has been improved compared to the previous version. I have no more comment.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: bacteriology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 28 September 2021

<https://doi.org/10.5256/f1000research.77925.r95641>

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Theerthankar Das

Department of Infectious Diseases and Immunology, School of Medical Science, The University of Sydney, Camperdown, NSW, Australia

I approve this manuscript.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 2

Reviewer Report 07 September 2021

<https://doi.org/10.5256/f1000research.54720.r89549>

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Theerthankar Das

Department of Infectious Diseases and Immunology, School of Medical Science, The University of Sydney, Camperdown, NSW, Australia

I approve this manuscript and recommend for indexing.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 31 August 2021

<https://doi.org/10.5256/f1000research.54720.r92437>

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Shizuo Kayama 

Antimicrobial Resistance Research Center, National Institute of Infectious Diseases, Higashimurayama, Japan

Amy *et al.* investigated that 25% royal jelly was able to inhibit *P. aeruginosa*, but it could increase pyocyanin and biofilm production at subinhibitory concentrations. This bilateral reaction of *P. aeruginosa* against royal jelly is interesting. However, it seems that this manuscript is constructed according to mainly observation. Your estimation of the molecular mechanism brought from your observation is detail. But I am interested in the relationship between the expression of these genes and those phenotypes. I want to know the molecular mechanism more deeply. The reader of your manuscript will also want to know the gene expression related to pyocyanin, or quorum sensing, and so on. If possible, please measure the gene expression.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: bacteriology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 21 Sep 2021

Heni Susilowati, Faculty of Dentistry, Universitas Gadjah Mada, Sleman, Indonesia

We are very grateful for your invaluable input. We agree that an analysis of the effect of royal jelly on the expression of quorum sensing-related genes that play important roles in the regulation of biofilm formation and *P. aeruginosa* pyocyanin production is urgently needed. Accordingly, we add this discussion to the manuscript version 3, paragraph 7 of the discussion section. We have also added one related reference to the bibliography (no. 39).

Competing Interests: I hereby declare that neither of us has a conflict of interest with the reviewer.

Reviewer Report 22 July 2021

<https://doi.org/10.5256/f1000research.54720.r89548>

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Ariadna A. Djais 

Department of Oral Biology, Faculty of Dentistry, University of Indonesia, Jakarta, Indonesia

I confirm that I have read this submission and its revisions, and I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 04 February 2021

<https://doi.org/10.5256/f1000research.30869.r77077>

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Theerthankar Das

Department of Infectious Diseases and Immunology, School of Medical Science, The University of Sydney, Camperdown, NSW, Australia

The intention of this study is good and to enlighten the antibacterial and anti-virulence effect of royal jelly in *P. aeruginosa*.

To be more conclusive this study needs more work:

1. Its better to use chloroform-HCl (standard and more popular) assay to quantify pyocyanin yield - its more rigorous than just taking absorbance at 690nm of pyocyanin (broad peak).
2. Biofilm (Crystal violet for biomass quantification) study needs to be done to ensure impact of royal jelly/pyocyanin on biofilm development.

3. Confocal microscopy will be of great help to show biofilm architecture.

Specific comments:

- Why solubilized royal jelly is centrifuged and supernatant is only taken? What happens with pellets (are there any pellets found after centrifugation?). If yes - then how can we know that supernatant has all royal jelly?
- At 25% royal jelly we see only 60% growth inhibition for both bacterial strains. Its not MIC - you can call it LC50 (lethal dose to reduce at least 50% growth). MIC is when there is zero or 1-2% growth only.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Biofilms, antimicrobial agents

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 07 Jun 2021

Henri Susilowati, Faculty of Dentistry, Universitas Gadjah Mada, Sleman, Indonesia

We thank you for the thorough and careful review of our manuscript. We apologize for our delay in revisions due to the various obstacles we face regarding the availability of laboratory facilities and access during the Covid-19 pandemic.

The following are our responses to the suggestions and specific comments.

The response to comment 1 (quantification of pyocyanin)

We measured the concentration of pyocyanin contained in the culture supernatant of *P. aeruginosa*. This condition is in accordance with the previous research conducted by Price-Whelan et al. (2007). Indeed, we only measured the absorbance value of the supernatant filtrate of the bacterial culture at a wavelength of 690 nm, but the pyocyanin concentration was then known by calculations using a formula published by previous researchers (Price-Whelan et al, 2007). The constant value of $4310 \text{ M}^{-1} \text{ cm}^{-1}$ for the absorbance at 690 nm has been determined based on the research of Dietrich et al. 2006. Since our experimental conditions match the experimental conditions in both studies, both of which are published in outstanding journals, we feel it is sufficient to refer to their methods.

The response to comment 2 (Biofilm [Crystal violet for biomass quantification] study)

We have conducted additional experiments to detect the association between pyocyanin production in bacteria exposed to royal jelly and biofilm formation using the static microtiter plate biofilm assay (crystal violet staining) method. To analyze the relationship between pyocyanin production and biofilm mass production, we have used *P. aeruginosa* ATCC 10145 as this strain is more responsive in producing pyocyanin when compared to the clinical isolate strains we previously use. The results showed that the detectable biofilm mass significantly increased in bacterial cultures exposed to 12.5% royal jelly extract. Meanwhile, cultures treated with 25% royal jelly did not show any biofilm formation. The 6.25% royal jelly extract and the lower concentrations induced biofilm mass formation but at a lower quantity than 12.5% royal jelly. This evidence suggests a relationship between royal jelly concentration, pyocyanin production, and biofilm mass formation. The subinhibitory concentration (12.5%, 6.23%) of the royal jelly extract induced more pyocyanin production and biofilm mass formation rather than the higher concentrations. *Related changes were in the Methods, Results, and Conclusion of the Abstract, paragraph 6 of the Methods, paragraph 5 of Results, Figure 4, and line 5 of paragraph 6 on the Discussion section in the manuscript.*

The response to comment 3 (microscopy of biofilm architecture)

In order to complement the data regarding the effect of royal jelly on the mass formation of biofilms, we have carried out observations of the microscopic architecture of *P. aeruginosa* ATCC 10145 biofilms using scanning electron microscopy, since we could not access the confocal microscopy method at our university. We chose secondary electron (SE) mode rather than a backscattered electron (BSE) to investigate biofilm in a three-dimensional perspective. The results of observations on representative samples showed that in the culture exposed to 25% royal jelly extract there was no biofilm mass deposition, while the group exposed to 12.5% royal jelly showed a larger density of biofilm than the 6.25% royal jelly group. Chlorhexidine 0.2% control showed total inhibition of biofilm mass formation. Methods, results, discussion, and related references have been added to the manuscript on Methods, Results, and Conclusion in the Abstract, paragraph 7 of the Methods section, paragraph 6-7 of the Results section, and line 5 in paragraph 6 of the Discussion section.

Specific comments:

1. Why solubilized royal jelly is centrifuged and supernatant is only taken?

Answer: The extraction method using phosphate buffer saline (Hu et al, 2019) was chosen in order to dissolve major royal jelly protein 1 (MRJP1), the active components that are antibacterial or anti-adhesion. It is known that MRJP1 is a hydrosoluble protein dissolved in PBS, which is higher in supernatants than in pellets (Gismondi et al., 2017).

What happens with pellets (are there any pellets found after centrifugation?). If yes - then how can we know that supernatant has all royal jelly?

After centrifugation, there were indeed pellets, and it has been found that the pellets contain more liposoluble proteins than supernatants. To ensure that the compounds contained in the PBS royal jelly extract supernatant, further research is needed; However, from the research of Furusawa et al. (2016), it was found that MRJP1 was contained in the supernatant of PBS royal jelly extract in quite a large amount, reaching 27.6%.

The active components that are antibacterial or anti-adhesion in the supernatant of royal jelly extract in this study cannot be explained with certainty, but the royal jelly used in this study was thought to mainly contain MRJP1.

We add this explanation to paragraph 2 of the Discussion section. Additional sources of literature have been written in the reference list numbers 37, 38, and 39.

2. At 25% royal jelly, we see only 60% growth inhibition for both bacterial strains. It is not MIC - you can call it LC50 (lethal dose to reduce at least 50% growth). MIC is when there is zero or 1-2% growth only.

Answer: We agree with the reviewer's comments regarding the MIC score. In clinical isolates, 25% royal jelly extract caused 60% death in the bacterial population; meanwhile, in the standard strain (*P. aeruginosa* ATCC 10145), this concentration caused growth inhibition of 85%.

We have revised the first sentence in Methods of Abstract and two last sentences in the Results section.

Competing Interests: I declare that the authors have no conflict of interest with the reviewer.

Author Response 11 Jun 2021

Heni Susilowati, Faculty of Dentistry, Universitas Gadjah Mada, Sleman, Indonesia

We thank you for the thorough and careful review of our manuscript.
The following are our responses to the suggestions and specific comments.

The response to comment 1 (quantification of pyocyanin)

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Methods, results, discussion, and related references have been added to the manuscript on Methods, Results, and Conclusion in the Abstract, paragraph 7 of the Methods section, paragraph 6-7 of the Results section, and line 5 in paragraph 6 of the Discussion section.

Specific comments

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We have revised the first sentence in Methods of Abstract and two last sentences in the Results section.

Competing Interests: We declare that we have no conflict of interest with this peer review.

Reviewer Report 01 February 2021

<https://doi.org/10.5256/f1000research.30869.r77076>

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Ariadna A. Djais

Department of Oral Biology, Faculty of Dentistry, University of Indonesia, Jakarta, Indonesia

This study examined royal jelly and *Apis mellifera* bees as a base ingredient. These ingredients are antibacterial with pycocyanin as a pigment by the bacterium *Pseudomonas aeruginosa*. The *P. aeruginosa* was selected because these bacteria are known as multidrug-resistant bacterium. This research is interesting and specific. In this study, the data was analyzed as an indicator of the difference in the contrast of the green pigment color, which was carried out by measuring at a wavelength of 690 nm.

In my opinion, this research needs to add more information regarding the methods chapters:

1. It is necessary to add information about the stage of royal jelly making as the test material.
2. It is necessary to add information about the bacterium identification technique which used

by the researcher when confirming clinical isolates as *P. aeruginosa*.

3. Number of replication of independent experiment conducted by the researcher.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 14 Feb 2021

Heni Susilowati, Faculty of Dentistry, Universitas Gadjah Mada, Sleman, Indonesia

Thank you for providing a review and suggestions for improving our manuscript. Below are our revisions to the manuscript according to the review comments.

1. Response to comments 1:

We explain the stages of preparing royal jelly on the manuscript, written in the Methods section (page 3) as follows:

Royal jelly used in this study was obtained from Nusukan, Surakarta, Central Java, Indonesia and harvested in May-October 2019. This product was obtained from the beekeeper *Apis mellifera* who lives in the randu trees (*Ceiba pentandra*) and sono wood (*Dalbergia latifolia*). *Apis mellifera* bee species have been identified through previous research¹⁹. Royal jelly 5.5 grams was dissolved in 10 ml of cold phosphate buffered saline (PBS), then homogenized using a magnetic stirrer (24 hours, 4°C). The royal jelly solution was centrifuged (12,000 g, 45 minutes, 4°C), then the supernatant is taken and checked the pH. The results of pH measurements with a pH meter showed a pH of 3.79. Furthermore, the supernatant was filtered using 0.45 µm Millipore to produce 55% royal jelly.

2. Response to comment 2:

We write an explanation of how to identify bacteria in the Methods section (page 3), which is as follows:

Identification of the clinical isolate was carried out using API 2ONE (bioMérieux, Inc).

3. Response to comment 3:

Each experiment was carried out with 6 replications.

We write the revision on page 3 of the Methods section, on bacterial viability tests and pyocyanin detection.

Competing Interests: I hereby confirm that there I have no competing interest with the reviewer.

Author Response 11 Jun 2021

Heni Susilowati, Faculty of Dentistry, Universitas Gadjah Mada, Sleman, Indonesia

Thank you for providing a review and suggestions for improving our manuscript. Below are our revisions to the manuscript according to the review comments.

1. The response to comment 1

We explain the stages of preparing royal jelly on the manuscript, written in *line 2-6 of paragraph 2 of the Methods* section as follows:

Royal jelly used in this study was obtained from Nusukan, Surakarta, Central Java, Indonesia and harvested in May-October 2019. This product was obtained from the beekeeper *Apis mellifera* who lives in the randu trees (*Ceiba pentandra*) and sono wood (*Dalbergia latifolia*). *Apis mellifera* bee species have been identified through previous research (Hartono et al., 2019). Royal jelly 5.5 grams was dissolved in 10 ml of cold phosphate buffered saline (PBS), then homogenized using a magnetic stirrer (24 hours, 4°C). The royal jelly solution was centrifuged (12,000 g, 45 minutes, 4°C), then the supernatant was taken and checked for the pH. The results of pH measurements using a pH meter showed a pH of 3.79. Furthermore, the supernatant was filtered using 0.45 µm Millipore to produce 55% royal jelly.

2. The response to comment 2

We have written an explanation of how to identify bacteria in *line 5 of paragraph 3 the Methods* section, which is as follows:

Identification of the clinical isolate was carried out using API 2ONE (BioMérieux, Inc).

3. Response to comment 3

The bacterial viability assay experiment was carried out in quadruplicate, meanwhile, the pyocyanin experiments were performed in triplicate.

We write the revision in the last line of related paragraphs in the Methods section.

Competing Interests: We declare that we have no conflict of interest with this peer review.

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