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Another Reason to Like Red Wine -- It Might Make Medical Implants Safer

Abstract. Biofilm, a complex collection of bacterial colonies, presents many challenges to current drugs targeting bacterial infections (Barzegari et al., 2020). Resveratrol, a compound found in grape plants, has reduced biofilm formation and interfered with quorum sensing mediated behaviors in several bacterial species (Vestergaard & Ingmer, 2019). In *Vibrio harveyi*, biofilm formation is regulated by quorum sensing, which is a bacterial communication system (Frederix & Downie, 2011). Thus, we propose to study the inhibitory effects of resveratrol on the mechanisms of quorum sensing in *Vibrio harveyi* by observing biofilm formation and bioluminescence. Conclusions were made regarding BB120 (wild-type) and BB721 (constitutively glowing mutant). Our result suggests that resveratrol is a promising quorum sensing inhibitor within a specific concentration range, as it didn't kill the bacteria and yet disabled quorum-sensing-associated phenotypes.

Introduction. You might have heard that daily moderate intake of red wine offers health benefits to adults. Have you wondered why red wine is considered “healthier” than white wine? The answer can be traced back to the fermentation process: white wine is fermented from the juice of the fruit, while red wine is fermented from the juice, seeds, and skin (Brennan, 2020). The grape skin not only gives wine the red hue but also contains many antioxidants, which protect cells from free radicals (Brennan, 2020). Of the naturally-produced antioxidants, resveratrol outshines others with its abundant therapeutic properties and captures the attention of

many current studies on antimicrobial agents (Vestergaard & Ingmer, 2019). It is worthwhile to explore the underlying mechanism of resveratrol's healing potential in an effort to understand the health benefits of red wine. Next time at a family gathering, you can discuss how red wine promotes the well-being of adults and perhaps offer justification for any relatives who want a second glass. Who would have thought drinking red wine long-term might effectively prevent bacterial infection caused by catheters in the urinary tract?

Antibiotic Resistance. Biofilms create complications for traditional antibacterial drugs to work adequately. Antibiotics are commonly used to treat diseases by killing or inhibiting the growth of bacteria. Specifically, the food industry uses antibiotics to treat bacterial infections that damage plants and animals, so as to maximize the yield with limited resources ("Rise to the biofilm challenge", n.d.). However, antibiotics have become less effective as bacteria become more resistant to the drugs, and this is known as antibiotic resistance. This phenomenon has led to more than 10% loss of food production. As farmers try to counter the loss by ramping up the use of antibiotics, resistance genes in bacteria arise at a faster rate to evade more drugs ("Rise to the biofilm challenge", n.d.). Antibiotic resistance can arise genotypically or phenotypically. Genotypic resistance arises from the acquisition of genes or mutations that confer resistance (Corona & Martinez, 2013). Phenotypic resistance involves a change in gene expression to reduce the efficacy of antibiotics (Corona & Martinez, 2013). Since biofilms are organized into layers, cells in the deeper layer have less access to nutrients and oxygen (Yan & Bassler, 2019). These cells have a slower growth rate or prolonged non-growing phase, which makes them tolerant to antibiotics that target newly dividing cells (Yan & Bassler, 2019). Tolerant cells create greater opportunities for resistance genes to arise (Yan & Bassler, 2019). Once the bacteria survive a cycle of antibiotic treatment, they will reproduce to create an entire resistant population

(Yan & Bassler, 2019). Additionally, the matrix protecting the biofilm makes it harder for antibiotics to penetrate and reach the target cells (Yan & Bassler, 2019). The characteristics of biofilms pose many challenges that decrease the potency of current antibiotics.

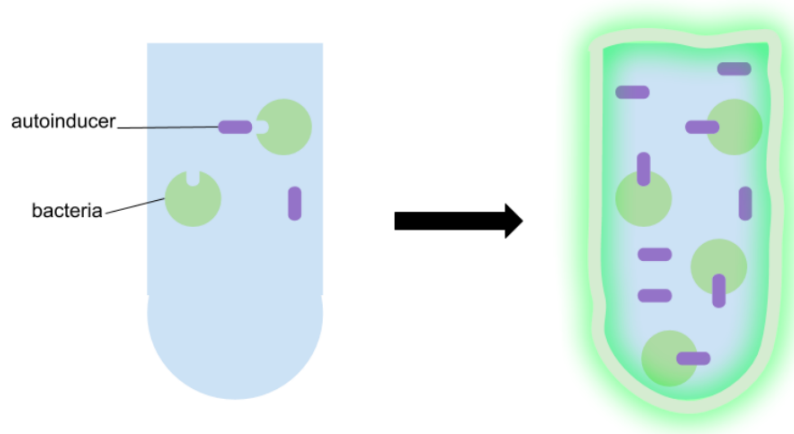


Figure 1: Quorum sensing as a cell density dependent system. When there are more bacterial cells in a population, more autoinducers accumulate extracellularly and are detected by bacterial cells. High levels of reception ultimately trigger group behaviours like bioluminescence, as seen in the test tube on the right. Excerpted from Gomez, E., Manaloto, K., & Partamian, O. (2020). Curbed by clay: stopping bacterial communication in *Vibrio harveyi* with clay minerals. Unpublished manuscript.

Quorum Sensing. The bacterial signaling system used by both Gram-negative and Gram-positive is termed quorum sensing, and it uses chemical signals secreted by bacteria called autoinducers (Waters & Bassler, 2005). The three main steps of quorum sensing are basically signal production, signal reception, and gene expression. As explained in **Figure 1**, at high cell density, the autoinducer concentration increases in the intracellular space accordingly (Kalia, 2013). The autoinducers bind to receptors on nearby bacteria and send signals down a transduction pathway to regulate gene expression (Kalia, 2013). When gene expression is turned on, transcription takes place to produce virulence factors such as bioluminescence (Kalia, 2013). Many virulence factors are under the control of quorum sensing, and there are various assays to detect if quorum sensing is occurring in a bacterial colony (Kalia, 2013). Biofilm formation is commonly measured by the crystal violet assay, which is a staining method. A crystal violet stain

is added to the biofilm cells, and ultimately, the dye concentration of the sample is read with a microplate reader and translated to the volume of formed biofilm mass (Song et al., 2019).

Measuring cell viability of the biofilm is crucial since antivirulence therapy does not intend to kill the cells. Resazurin assay uses a blue dye that turns pink in its reduced form, so pink indicates cell growth, and blue indicates inhibition of cell growth (Welch et al., 2012).

Comparing formed biofilm mass to cell viability can suggest how effective the tested antivirulence compound is.

Resveratrol as an Antimicrobial Agent. Several reports highlight resveratrol as a natural substance that possesses antioxidant, anti-inflammatory, anti-aging, anti-microbial, and other medicinal properties (Kugaji et al., 2019). Resveratrol is a compound found in many seed plants and acts as a defense mechanism against parasites that damage those plants (Frémont, 2000). In several studies, resveratrol reduced multiple virulence factors in various types of bacteria (Vestergaard & Ingmer, 2019). For example, in the Gram-negative bacterium, *Proteus mirabilis*, resveratrol reduced swarming, hemolysis, and urease activity (Wang et al., 2006). In a study conducted by Zhao and his colleagues, resveratrol at 2 µg/mL most effectively reduced the pathogenicity of *Vibrio harveyi* by decreasing the expression of a gene (vhh) that codes for a toxin (hemolysin) produced by the bacteria (Zhao et al., 2020). In other studies on two *Vibrio* species (*Vibrio cholerae* and *Vibrio vulnificus*), resveratrol at several concentrations (30 µg/mL and 30 µM) showed antibiofilm and anti-swarming activity (Augustine et al., 2014; Kim et al., 2010). In a bacterium prominent in periodontal disease (*Porphyromonas gingivalis*), resveratrol at different concentrations (78.12-312.5 µg/mL) reduced the attachment of bacteria to cell surfaces, which is a necessary step in biofilm formation (Kugaji et al., 2019). In addition to reducing virulence factors, resveratrol also disrupted quorum sensing specific mechanisms in two

Gram-negative bacteria, *Escherichia coli* and *Chromobacterium violaceum* (Vestergaard & Ingmer, 2019). Whether or not resveratrol acts on specific quorum sensing steps in our *Vibrio Harveyi* model system, it poses interesting questions as an antibacterial agent.

***Vibrio harveyi*.** *Vibrio harveyi* is a gram-negative bacteria that resides in marine environments, and is also a bioluminescent bacteria once a threshold of bacteria is present and recognized (iBiology, 2010). Like other types of bacteria, it communicates using different autoinducer pathways in order to maintain its life functions (Bassler, 2009). *Vibrio harveyi* contains two possible pathways (AI-1 and AI-2) which can be targeted for quorum sensing inhibition (Bassler, 2009) For the AI-2 pathway, LuxS makes autoinducer-2 while both LuxQ and LuxP are protein receptors (Nackerdien et al., 2008; iBiology, 2010). Both send their received signals to protein LuxU, then it is passed to another protein, LuxO, and eventually the message received and transcribed by LuxR which then serves as a master regulator to luciferase which controls the bioluminescence in *Vibrio harveyi* (Nackerdien et al., 2008). As glowing is an easy indication to whether inhibition has been achieved or not, this, in combination with its relatively simple quorum sensing pathways makes *Vibrio harveyi* good model system for quorum sensing inhibition studies.

Research Objectives. In our study, we aim to study the overall influence of resveratrol on quorum sensing mediated behaviors in *Vibrio Harveyi*, especially biofilm formation. We administered resveratrol at different concentrations to various mutants of *Vibrio Harveyi* that have different aspects of the QS machinery disabled. We observed how QS-regulated virulence factors, such as biofilm formation and bioluminescence, by each strain responds in the presence of resveratrol. We hypothesized that resveratrol would inhibit biofilm formation and

bioluminescence in *Vibrio harveyi* by switching off the gene transcription of those virulence factors regulated by quorum sensing.

Importance and Impact. Biofilms are diverse bacterial colonies that adhere to a surface (Costerton & Stewart, 2001). They constitute a major problem in the medical world because they are the cause of many infectious diseases (Barzegari et al., 2020). Unlike free-floating bacteria which can be easily treated with antibiotics, biofilms persist and evade traditional drugs (Costerton & Stewart, 2001). A strategy to target biofilms is to interfere with bacterial communication because biofilms are formed under quorum sensing regulation (Maura et al., 2016). Resveratrol is a natural compound that has already exhibited anti-virulence properties in *Vibrio harveyi* and multiple other Gram-negative bacteria species. However, only wild-type *Vibrio harveyi* has been used in other studies, and no data of resveratrol on biofilm formation in *Vibrio harveyi* has been reported. Our study which involved various mutants of *Vibrio harveyi* could reveal whether resveratrol inhibits biofilm formation in *Vibrio harveyi* and how. *Vibrio harveyi* causes diseases in marine organisms, so our result could discover a new antimicrobial agent against this bacteria and reduce the significant financial losses to the fish farming industry (Zhao et al., 2020). In addition, we hope to extrapolate our result to other gram-negative bacteria that use the same quorum sensing pathway to cause damage to the host. Some gram-negative bacteria, such as *P. aeruginosa*, commonly form biofilms on medical implants and cause damage to our bodies, and they often adapt to antibiotics quickly (Khatoon et al., 2018). We want to contribute to the understanding of implant-associated bacterial infections.

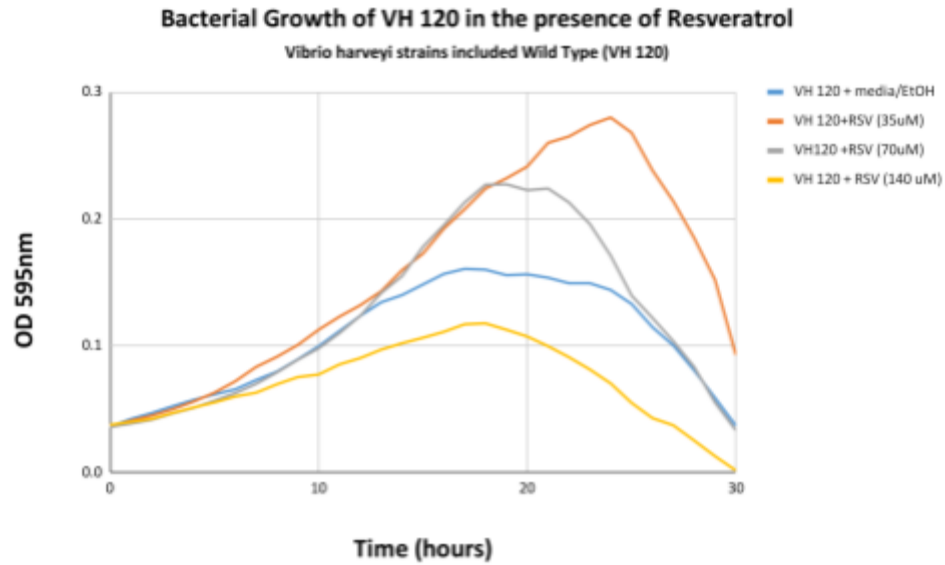
Methods. All studies were conducted using proper aseptic techniques, including disposal of sterilized biological materials. We acknowledge previous students who optimized the methods

below as submitted in previous proposals.

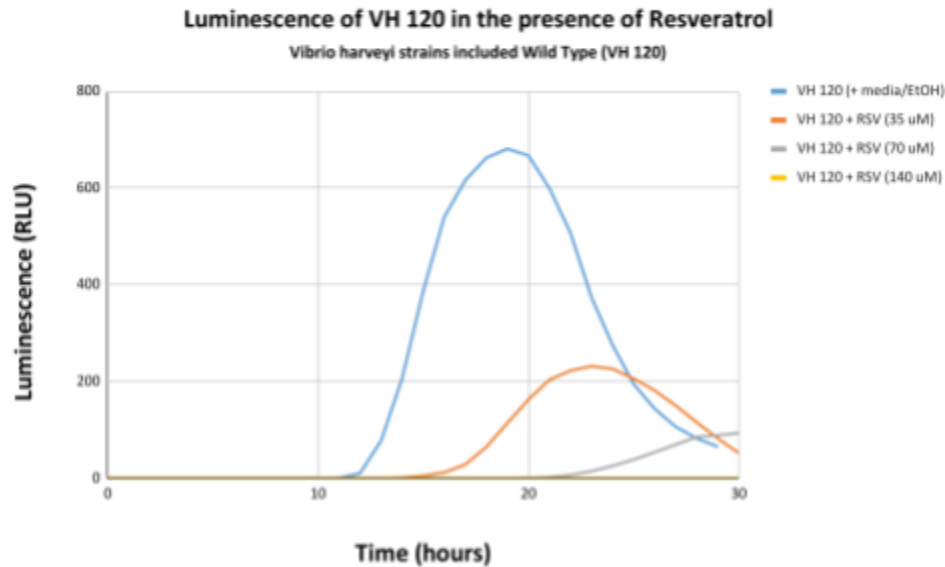
All *Vibrio harveyi* strains (BB120, BB152, BB721) were grown in a sterilized Autoinducer Bioassay (AB) and Nutrient Broth (NB) medium and were incubated at 30°C while constantly shaking (Vikram et al., 2010). These strains were purchased for student projects in previous years and are stored off-site at -80°C. Resveratrol was dissolved in ethanol in order to address solubility issues. The wells in the final assay contained less than 1% of ethanol (as proposed by Vikram et al., 2010) to minimize any interference with bacterial growth. Resveratrol was tested at different concentrations (0-140 uM final concentrations), which is modeled after the study conducted by Zhao and coauthors (Zhao et al., 2020).

To test under what pathway and mechanism resveratrol inhibits the virulence phenotypes, such as luminescence and biofilm formation, in *Vibrio harveyi*, a comprehensive study was conducted using VH mutants. BB120 is the wild-type strain and is capable of producing both AI-1 and AI-2 signal molecules, as well as sensing both autoinducers. BB721 is a mutant strain in which the LuxO protein in charge of luminescence regulation is turned off, meaning that its control phenotype should always remain glowing (Freeman & Bassler, 1999). If resveratrol inhibits the strain's luminescence, it can be concluded that it inhibits the bacteria's luminescence and may prohibit that bacteria's quorum sensing behaviors from occurring (Freeman & Bassler, 1999).

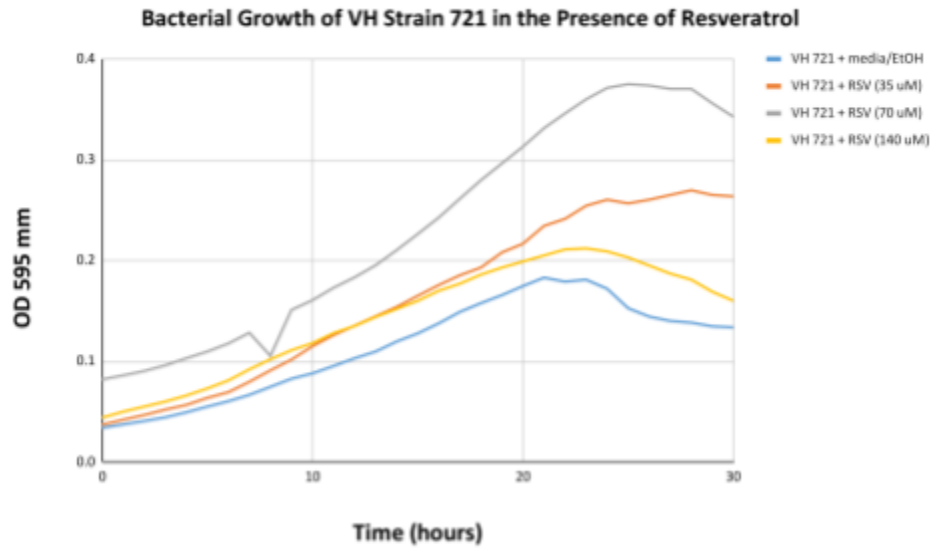
Results. Bacteria growth and luminescence data were collected for three VH strains (BB120, BB152, BB721) in the presence of resveratrol over a period of 30 hours.



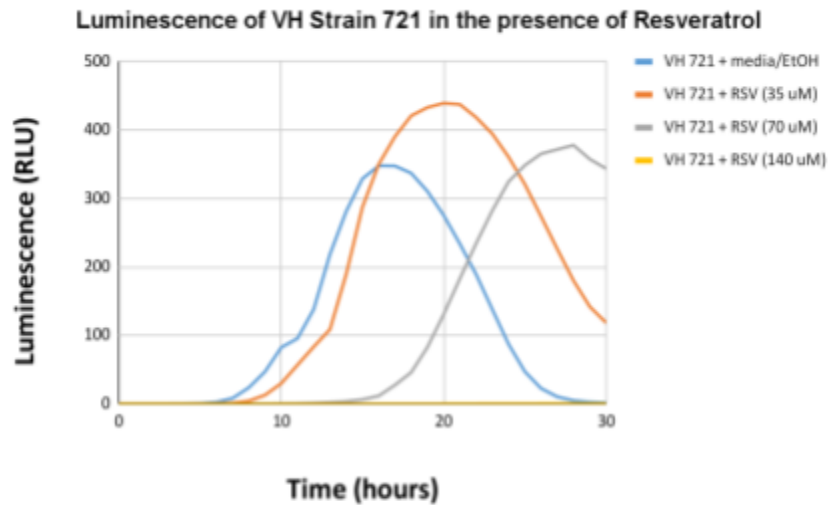
Graph 1. The control group containing the strain, media and EtOH peaked at approximately 17 hours. Additionally, Resveratrol at concentrations of 140 and 70 uM peaked at the same time of 17 hours. The lowest concentration (35uM) yielded the highest peak but with a slight delay compared to the others at approximately 24 hours.



Graph 2. VH120 had luminescence in media/EtOH groups; its luminescence peaked at approximately 19 hours. There is delayed glowing in VH 120 with 35um and 70um resveratrol. Their peaked luminescence levels are also significantly lower than the control groups. In VH120 treated with 140um resveratrol, luminescence is almost entirely inhibited from time=0 to 30 hours. Overall, resveratrol inhibited glowing in wild-type VH in a dose-dependent manner.



Graph 3. The bacterial growth of VH721 appear to peak after 25 hours. The untreated strain and the strain containing the highest concentration of resveratrol (140uM) peak at approximately 23 hours, while the other sample containing concentrations of 35 uM and 70 uM of resveratrol peak at approximately 28 hours. The amount of bacterial growth to be inhibited or enhanced does not appear to be dose dependent as the bacteria with a concentration of 70uM grew more than that of its counterpart with the higher concentration 140uM.



Graph 4. The luminescence of the bacteria was recorded periodically over a period of 30 hours for each group. Each group peaked at different hours, the highest peak being the bacteria treated with 35uM of resveratrol around the 20th hour.

The results from the experiments involving BB120 as shown in **Graph 1** and **2** are varied.

Resveratrol's effect on bacterial growth does not follow a singular trend. Concentrations of resveratrol at 35uM and 70uM appear to enhance bacterial growth while at 140uM, resveratrol

inhibits growth (**Graph 2**). Conversely, the effectiveness of resveratrol when inhibiting this strains' bacterial luminescence appears to be dose dependent (**see Graph 2**), with the highest concentration of resveratrol (140uM) yielding the greatest reduction in luminescence. Results from the experiments with BB721 provide a set of similar results as they too were varied. As demonstrated in **Graph 3**, all three concentrations of resveratrol enhanced bacterial growth. The luminescence study involving this strain did not suggest a singular trend like its counterpart, but it did demonstrate that at concentrations of 35uM and 70uM, resveratrol causes a slight increase in bacterial luminescence but also causes a shift in the maximum luminescence over time (**Graph 4**). Resveratrol at a concentration of 140uM completely inhibits bacterial luminescence.

Planned Experiment

A final biofilm experiment will be conducted using all three strains. Resveratrol will be added in varying concentrations to all three strains, and the extent of biofilm formation will be observed. The amount of bacteria forming a biofilm and the live/dead composition of the biofilm will be measured using resazurin and crystal violet assays. Similar results such as those demonstrated in **Graphs 1-4** are expected. Concentrations at 35uM and 70um should allow most bacteria to be alive while less biofilm to form. At a concentration of 140uM, it is anticipated that more bacteria will die therefore further reducing biofilm formation. The control wells should contain a lot of biofilm formation and large amounts of living bacterial cells.

Discussion & Conclusion

The results of our experiments confirm our hypothesis. The findings in our experiment help us conclude that resveratrol is a possible candidate for treating bacterial infections; however, whether resveratrol directly inhibits quorum sensing behaviors is dependent on concentration.

According to **Graphs 1** and **2**, resveratrol appears to be a viable quorum sensing inhibitor. Concentrations of resveratrol at 35uM and 70uM allow the bacteria to grow, while at the same concentrations they do not glow a lot. These concentrations are not killing the bacteria, but they do appear to prevent a phenotype regulated by quorum sensing. Because of this action, it can be concluded that at those concentrations, resveratrol can possibly prevent quorum sensing. It is also important to note that in experiments involving the wildtype (BB120), the concentration of resveratrol at 70uM presents itself as the ideal amount as growth is allowed, but luminescence is heavily inhibited. Concentrations of 140 uM appear to have a bactericidal effect on the bacteria as it inhibits growth and little to no luminescence occurs. This indicates that at 140uM, resveratrol might instead be killing the bacteria rather than interfering with quorum sensing.

Graphs 3 and **4** help to further designate where, if at all, resveratrol is targeting quorum sensing behavior. The *Vibrio Harveyi* strain BB721 used contains a null version of LuxO, and therefore will always glow. If resveratrol inhibits glowing, it can be concluded that it is targeting a quorum sensing mechanism that is downstream of LuxO in the phosphorylation chain. However, it could also indicate that resveratrol might be targeting the luciferase protein itself and would not be targeting a quorum sensing mechanism. In both **Graphs 3** and **4**, resveratrol at a concentration of 140 uM could be an example of this, as it slightly depletes the bacterial growth but does not allow for any luminescence. At concentrations 35uM and 70uM, bacteria growth occurs and luminescence is delayed but not inhibited. This indicates that these concentrations of the resveratrol could be slowing down or disrupting a mechanism of quorum sensing directly following the LuxO protein. Since glowing is still occurring, it is less plausible that luciferase is being targeted and more likely that a quorum sensing mechanism leading up to its activation is being delayed.

While research on three strains of *Vibrio harveyi* were performed, the experiments involving strain BB152 were purposely left out of our significant findings. It was discovered that BB152 did not properly grow or glow in the Nutrient Broth (NB) media used during our experiment. NB was the only medium in which resveratrol could fully solubilize. A secondary experiment was conducted in order to confirm that the lack of bacterial luminescence in this strain was a media related issue. Without any inhibitor present, all three strains were added to both a NB and Autoinducer Bioassay (AB) medium. While growth and luminescence for all three strains were reduced in NB, it was extremely so in the BB152 strain. Conversely, the AB media promoted better growth and luminescence than its counterpart, NB media. Despite this finding, resveratrol was only soluble in the NB medium, therefore requiring a compromise and limiting them to only include BB120 and BB721 strains. Due to a limited lab schedule, type of equipment used, and disruptions involving the media's interaction with our bacterial strains, our results on this topic are limited. Granted more time and resources, more quorum sensing studies involving a wider range of concentrations and different media should be conducted.

Based on our results, it can be concluded that at concentrations of 35uM and 70uM resveratrol appears to be a good quorum sensing inhibitor in our model system *Vibrio harveyi*. In our last experiment to further discover resveratrol's effect on quorum sensing, if biofilm formation and luminescence are inhibited at 35uM and 70uM resveratrol concentrations, this experiment would further confirm that resveratrol is targeting quorum sensing mechanisms as it is preventing quorum sensing behaviors from happening. Which quorum sensing mechanisms are targeted and whether or not resveratrol at higher concentrations could work as a quorum sensing inhibitor are still unknown.

Acknowledgements

We would like to thank the Science Department at Flintridge Sacred Heart, our mentor Dr. Elizabeth Krider, Manji at Oak Crest for storing our bacterial samples, and Dr. Eva Cunha for her insightful comments about working with specific bacterial inhibitors.

Works Cited

Augustine, N., Goel, A. K., Sivakumar, K. C., Kumar, R. A., & Thomas, S. (2014).

Resveratrol—a potential inhibitor of biofilm formation in *Vibrio cholerae*. *Phytomedicine*, 21(3), 286-289.

Bassler, B. (2009, February). *How Bacteria "talk"* [Video]. TED.

https://www.ted.com/talks/bonnie_bassler_how_bacteria_talk.

Barzegari, A., Kheyrolahzadeh, K., Khatibi, S. M. H., Sharifi, S., Memar, M. Y., & Vahed, S. Z.

(2020). The battle of probiotics and their derivatives against biofilms. *Infection and drug resistance*, 13, 659.

Brennan, D. (2020, September 29). *Are there health benefits to drinking red wine? pros and cons, nutrition information, and more*. WebMD. Retrieved January 7, 2022, from

<https://www.webmd.com/diet/health-benefits-red-wine#1>

Corona, F., & Martinez, J. L. (2013). Phenotypic resistance to antibiotics. *Antibiotics*, 2(2), 237-255.

Costerton, J. W., & Stewart, P. S. (2001). Battling biofilms. *Scientific American*, 285(1), 74-81.

Frederix, M., & Downie, J. A. (2011). Quorum sensing: regulating the regulators. *Advances in microbial physiology*, 58, 23-80.

Freeman, J. A., & Bassler, B. L. (1999). A genetic analysis of the function of *luxo*, a

two-component response regulator involved in quorum sensing in *vibrio harveyi*.

Molecular Microbiology, 31(2), 665–677.

<https://doi.org/10.1046/j.1365-2958.1999.01208.x>

Frémont, L. (2000). Biological effects of resveratrol. *Life sciences*, 66(8), 663-673.

iBiology. (2010, March 27). *Bonnie Bassler (Princeton) Part 1: Bacterial Communication via*

Quorum Sensing [Video]. Youtube. <https://www.youtube.com/watch?v=saWSxLU0ME8>.

Kalia, V. C. (2013). Quorum sensing inhibitors: an overview. *Biotechnology advances*, 31(2), 224-245.

Khatoun, Z., McTiernan, C. D., Suuronen, E. J., Mah, T. F., & Alarcon, E. I. (2018). Bacterial biofilm formation on implantable devices and approaches to its treatment and prevention. *Heliyon*, 4(12), e01067.

Kim, J. R., Cha, M. H., Oh, D. R., Oh, W. K., Rhee, J. H., & Kim, Y. R. (2010).

Resveratrol modulates RTX toxin-induced cytotoxicity through interference in adhesion and toxin production. *European journal of pharmacology*, 642(1-3), 163-168.

Kugaji, M. S., Kumbar, V. M., Peram, M. R., Patil, S., Bhat, K. G., & Diwan, P. V. (2019). Effect of Resveratrol on biofilm formation and virulence factor gene expression of *Porphyromonas gingivalis* in periodontal disease. *Apmis*, 127(4), 187-195.

Maura, D., Ballok, A. E., & Rahme, L. G. (2016). Considerations and caveats in anti-virulence drug development. *Current opinion in microbiology*, 33, 41-46.

Nackerdien, Z. E., Keynan, A., Bassler, B. L., Lederberg, J., & Thaler, D. S. (2008). Quorum sensing influences *Vibrio harveyi* growth rates in a manner not fully accounted for by the marker effect of bioluminescence. *Plos one*, 3(2), e1671.

PA_Consulting. (n.d.). *Rise to the biofilm challenge: Harmful microbes present opportunities for*

- innovators*. PA Consulting. Retrieved January 7, 2022, from <https://www.paconsulting.com/insights/rise-to-the-biofilm-challenge/>
- Song, Y. J., Yu, H. H., Kim, Y. J., Lee, N. K., & Paik, H. D. (2019). Anti-biofilm activity of grapefruit seed extract against *Staphylococcus aureus* and *Escherichia coli*.
- Vestergaard, M., & Ingmer, H. (2019). Antibacterial and antifungal properties of resveratrol. *International Journal of Antimicrobial Agents*, *53*(6), 716-723.
- Vikram, A., Jayaprakasha, G. K., Jesudhasan, P. R., Pillai, S. D., & Patil, B. S. (2010). Suppression of bacterial cell–cell signaling, biofilm formation and type III secretion system by citrus flavonoids. *Journal of applied microbiology*, *109*(2), 515-527.
- Wang, W. B., Lai, H. C., Hsueh, P. R., Chiou, R. Y. Y., Lin, S. B., & Liaw, S. J. (2006). Inhibition of swarming and virulence factor expression in *Proteus mirabilis* by resveratrol. *Journal of medical microbiology*, *55*(10), 1313-1321.
- Waters, C. M., & Bassler, B. L. (2005). Quorum sensing: cell-to-cell communication in bacteria. *Annu. Rev. Cell Dev. Biol.*, *21*, 319-346.
- Waters, C. M., Lu, W., Rabinowitz, J. D., & Bassler, B. L. (2008). Quorum sensing controls biofilm formation in *Vibrio cholerae* through modulation of cyclic di-GMP levels and repression of *vpsT*. *Journal of bacteriology*, *190*(7), 2527-2536.
- Welch, K., Cai, Y., & Strømme, M. (2012). A method for quantitative determination of biofilm viability. *Journal of Functional Biomaterials*, *3*(2), 418-431.
- Yan, J., & Bassler, B. L. (2019). Surviving as a community: antibiotic tolerance and persistence in bacterial biofilms. *Cell host & microbe*, *26*(1), 15-21.
- Zhao, X., Guo, Y., Ni, P., Liu, J., Wang, F., Xing, Z., & Ye, S. (2020). Resveratrol inhibits the

virulence of *Vibrio harveyi* by reducing the activity of *Vibrio harveyi* hemolysin.

Aquaculture, 522, 735086.