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Preventing Bacterial Infections: One Plant Extract at a Time

Abstract

More than 80% of bacterial infections are caused by the formation of biofilms in the body (Sharma et al., 2019). These infections are becoming increasingly more difficult to treat or eradicate because of bacteria's growing antibiotic resistance to current treatments. Biofilm formation is mediated by quorum sensing, a mechanism of bacterial communication. A new approach for combating biofilm formation, which could be a source for supplementary therapy through vitamins and minerals, targets the steps involved in quorum sensing. One way to inhibit biofilm formation is through the use of plant extracts and naturally occurring peptides (Ghosh et al., 2021). Specifically, our research was aimed at combating canine periodontal disease which is caused by both Gram-negative and Gram-positive bacteria. Current approaches for treating this disease include adding thymoquinone, a seed extract, and nisin, a lantibiotic, to various bacterial strains. In this study, we proposed to research the effects of these naturally derived compounds on the biofilm formation of a model bacterial system called *Vibrio harveyi.* We hoped to understand the inhibiting mechanisms of these compounds and to uncover any synergistic effects when they are added together. We measured bacterial growth, bioluminescence and biofilm formation of *Vibrio Harveyi* in the presence of various concentrations of thymoquinone and nisin.

Introduction

Canine Periodontal Disease (CPD) is an often overlooked but extremely dangerous infection of the mouth that can damage the oral cavity in dogs. Inflammation, abscess formation, and bone loss are all symptoms of Periodontal Disease (PD) that can become fatal and extremely harmful for the pet, especially if left untreated (Niemiec, 2008). The excessive buildup of dental plaque and firm attachment of tartar to the teeth is the leading cause for the development of CPD due to the growth and development of an array of bacteria (Richardson et al., n.d.). Despite the frequency and prevalence of the disease, there are still no sufficient treatment methods. In addition, the total cost for current CPD treatments is high as a result of the extensive veterinary visits and check-ups (Richardson et al., n.d.). Therefore, developing a safe, effective, and inexpensive treatment method can help save almost two-thirds of pet dogs from the pain and suffering caused by CPD (Dogs et al., n.d.).

Background

About Biofilms. Biofilms are a collection of microorganisms bound together and sticking to surfaces causing infection and disease. They are a significant threat because of their prevalence in chronic wound infections, surgical devices, and bacterial infections (Vlamakis, 2011). A shocking 80% of bacterial infections in the body such as bloodstream and urinary infections, cystic fibrosis, and periodontal disease can be attributed to biofilm formation (Sharma et al., 2019). Biofilms start out as free living, planktonic cells that then join together on any surface and form a protective layer known as the extracellular polymeric substance (EPS), which acts as a “bacterial blanket” made of polysaccharide polymers and organic and inorganic materials (Paluch et al., 2020; Costa et al., 2018). The growth stage of biofilms is when bacterial infections are most likely to occur. The steps of biofilm formation are attachment, growth, and

dispersal. Because of the significant role biofilms play in bacterial infections, it is necessary to prevent their formation.

Biofilm Formation Regulated by Quorum Sensing. Quorum sensing is a mechanism of microbial communication between bacteria through the production and reception of signal molecules Autoinducer 1 (AI-1) and Autoinducer 2 (AI-2) (Paluch et al., 2020). AI-1 is the intraspecies signal molecule responsible for signal communication between bacteria of the same species. Conversely, AI-2 is the signal for communication between bacteria of any species that is produced and received by all bacterial species, making it the "universal language" of bacteria. These two communication signals are produced inside the cell and received by signal receptors on the surface of the cell (Mok et al., 2003). The reception of the autoinducers allows for gene expression and thus the expression of virulence factors (Reuter et al., 2016). Biofilms are a virulence factor under quorum sensing control that are especially dangerous because of their complexity and expansiveness (Paluch et al., 2020). Discovering a compound that possesses the ability to inhibit quorum sensing at certain stages involved in biofilm regulation can lead to the prevention of its development.

Antivirulence Therapy and Approach. Antivirulence therapy is the treatment of infection and disease once the host has already been exposed to the bacteria (Cegelski et al., 2008). Due to the low antibiotic susceptibility of biofilms, a new method of antivirulence therapy must be discovered to inhibit biofilm formation and prevent the development of PD. Although quorum sensing can be targeted and inhibited at a number of different stages, a promising approach is to target the bacteria at the communication level by inhibiting AI-2 reception or production. This would prevent contact between all bacterial species and ultimately stop the production of the virulence factor (Brackman & Coenye, 2015).

Model System *Vibrio harveyi* is a Gram-negative species of marine bacteria with the capacity to display bioluminescence through the steps of quorum sensing (Nackerdien et al. 2008). This strain is ideal because once the quorum sensing inhibitor is added to the bacteria, researchers can tell whether or not it was effective by observing its viability and bioluminescence. If the bacteria is alive but does not glow, then the inhibitor works on the quorum sensing mechanism. However, if the bacteria is alive and does glow, the inhibitor has no effect on the quorum sensing pathway. If the bacteria dies, the inhibitor is not viable because it has no effect on quorum sensing; instead, the inhibitor killed the bacteria.

Prior Research on TQ. Thymoquinone (TQ) is a component of *Nigella Sativa*, which is a black cumin seed that scientists have studied to prevent biofilm formation and combat canine periodontal disease (Mekhemar et al., 2020; Tantivitayakul et al., 2020). Previous studies have shown that this extract has a strong inhibitory effect on biofilm formation (50-90%) in both Gram-negative and Gram-positive bacteria even when used at low concentrations (8-512 ug/ml) (Chaieb et al., 2011). These results are significant because they show that thymoquinone's inhibitory action extends to both classifications of bacteria, which differ in membrane and composition (Vollmer et al., 2015). Against *F. nucleatum* (FN) and *P. gingivalis* (PG), two Gram-negative bacteria that play major roles in dental plaque formation, TQ was able to significantly reduce biofilm formation (Tantivitayaku et al., 2020). With a concentration of 6.25 μg/mL, TQ reduced biofilm formation of FN by 76% and by 62% in PG with a concentration of 0.78 μg/mL (Tantivitayaku et al., 2020). In *Vibrio parahaemolyticus*, TQ inhibited biofilm formation by 64.10% with a concentration of 3.2 μg/mL (Guo et al., 2019). Its anti-inflammatory and antioxidant properties are tentatively attributed to TQ's ability to inhibit eicosanoid production and suppressed lipid peroxidation (Mekhemar et al., 2020). Because the

specific mechanism of antimicrobial action by thymoquinone is still unclear, additional studies like our project are necessary.

Research Objectives. Nisin is a bacteriocin produced by *Lactococcus lactis* that presents strong inhibitory activity against Gram-positive bacteria and some Gram-negative bacteria (Cunha et al., 2020b). Through the compound's interaction with lipid II, an important component of cell-wall synthesis, nisin is able to inhibit the production of the cell wall of the bacteria, thereby stopping bacterial growth (Breukink & de Kruijff, 2006; Cunha et al., 2020a). This mechanism displays powerful activity at targeting Gram-positive bacteria involved in CPD. While nisin has not yet been tested on *Vibrio Harveyi*, high concentrations of nisin have been combined with certain antibiotics such as colistin, rifampicin, streptomycin, and penicillin and studied with Gram-negative bacteria (Field et al. 2016). As a result of the possible detriments to using nisin to combat CPD, researchers are exploring a cocktail approach that includes a combination of Nisin, antibiotics, and other compounds with quorum sensing inhibition properties. While no research has been done to pair the combination of nisin and polymyxin B or colistin with quorum sensing inhibitors such as TQ, this approach appears viable (Zhang et al. 2020). A combination of Nising and TQ has strong potential to be more effective at preventing the formation of biofilm than any of the inhibitors on their own. More studies involving combination therapies are necessary as this represents a new area of research.

Importance and Impact. This research is especially important in relation to caring for our pets and ensuring their health and safety. Overlooking a dog’s oral hygiene can lead to canine periodontal disease (CPD), which is a serious inflammatory disease that is often difficult to detect (Cunha et al., 2021). This disease can eventually lead to bone loss, loss of teeth, gum infections, or other serious health problems that can cause death (Mekhemar et al., 2020). This

research could be used to develop a topical or oral treatment for bacterial infections such as canine periodontal disease. In this way, our research could be used to show which quorum sensing inhibitors are most effective and the concentrations at which they are most useful. Using this information from our laboratory experiments, scientists could develop a paste to spread on dogs' teeth in a similar manner as a toothpaste or in a treat. Our research could also be used in medical devices such as prosthetics, heart valves, and catheters to prevent the formation of biofilms. Using this ongoing research, medication could be created to combat the effects of cystic fibrosis which is a dangerous bacterial infection of the lungs. Because of the serious implications of biofilm formation, it is important to research how to disrupt this process and identify a treatment that can save the 9-18% of pets that experience unnecessary suffering and death (Wallis & Holcombe, 2020) as well as the thousands of people who suffer from potentially treatable 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. *Vibrio harveyi* is well known for studying quorum sensing inhibitors. All *Vibrio harveyi* strains (BB120, BB152, BB721) were grown in sterilized Autoinducer Bioassay (AB) and Nutrient Broth (NB) media and were incubated at 30°C while constantly shaking (Vikram et al., 2010). Thymoquinone and nisin were dissolved in ethanol and water, respectively, in order to address solubility issues. The wells in the final assay contained no more than 1% of ethanol to minimize the possibility of its interfering with bacterial growth. Thymoquinone was added at four different concentrations (serial dilutions of 0, 25, 50, 100 μM) according to published methods by Vikram and coauthors (Vikram et al., 2010). Experiments with Nisin have yet to be performed and will follow published protocols (Field et al., 2016;

Chaieb et al., 2011; Cunha et al., 2020a).

Several *VH* mutants were used and their growth, bioluminescence, and biofilm formation in the presence of TQ were monitored. 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. If the BB120 strain produced bioluminescence, we knew that quorum sensing was occurring, and we could therefore begin to test for the viability of thymoquinone for inhibiting this communication. We treated a *VH* strain (BB721) that has steady-state luminescence (constitutively glowing) with varying concentrations of TQ to better understand if TQ acts upstream or downstream from the LuxO protein.

We conducted the experiments using black-walled microtiter plates, and we prepared two plates so that the optical density (OD) of bacterial growth (measured at 595 nm) and luminescence (quorum sensing activity) can be measured in parallel using two spectrophotometers. We conducted our quorum sensing inhibition studies using a microplate reader (Molecular Devices Lmax II 384) that detects the bioluminescence from our bacterial samples before and during incubation with thymoquinone. The microplate reader measured the bioluminescence produced by the samples and the light intensity is expressed in relative light units (RLU). Our data were copied into a spreadsheet program (GoogleSheets) and processed manually. “Media only” blank values were averaged and subtracted from the OD at 595 nm or LUM data to give a corrected value for each well.

Planned Studies The synergistic effects of TQ and nisin, if any, on biofilm formation of *VH* will be explored by May 10th. Concentrations of TQ and nisin will be adjusted based on results from Part 1 of the Project. Identical methods will be used as described above.

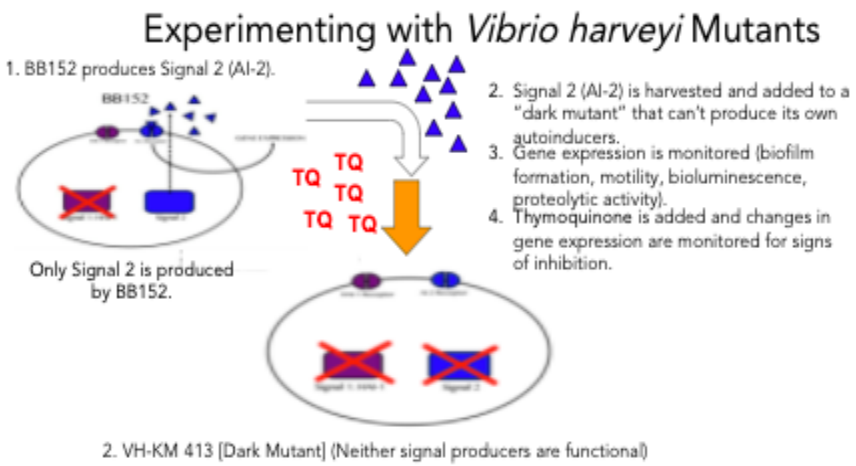
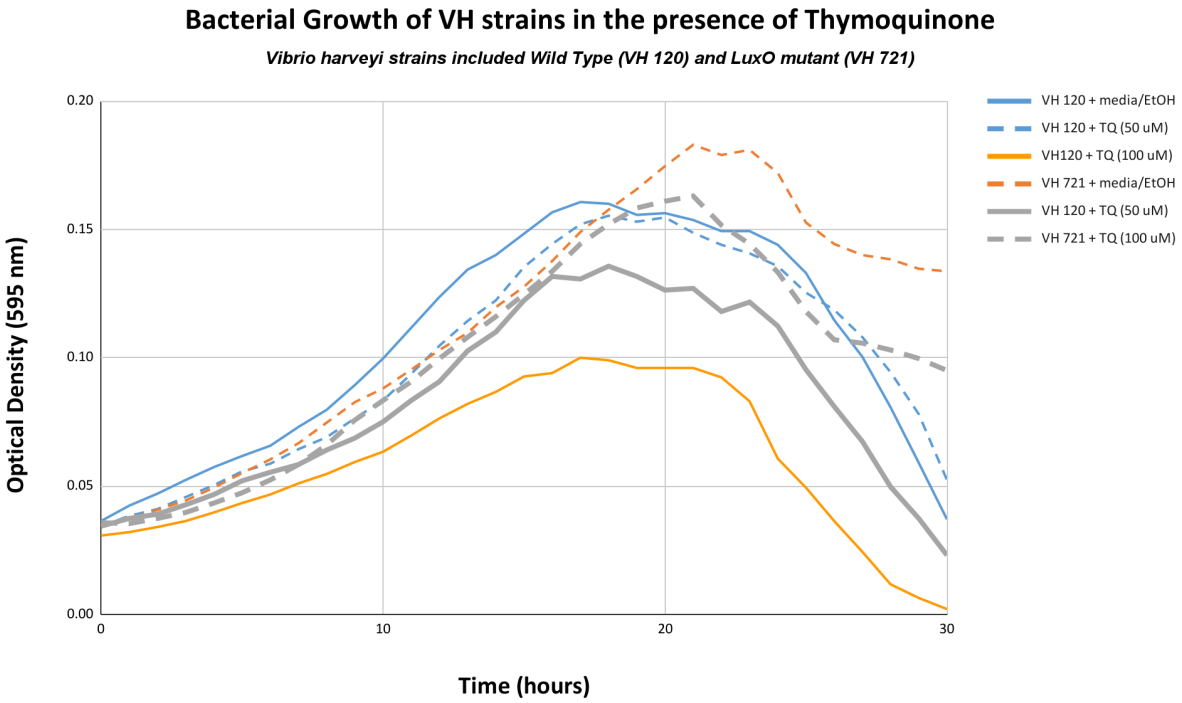


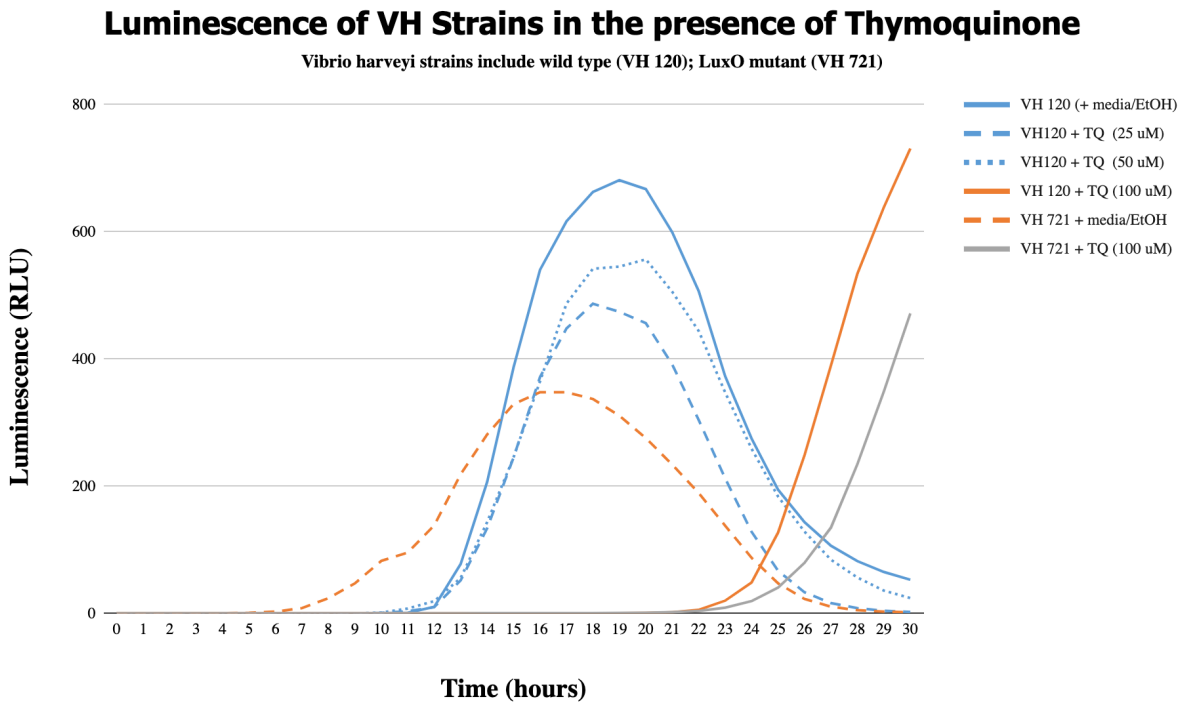
Figure 1: Using BB152 to explore the effect of thymoquinone on AI-2 mediated behaviors. BB152 is a mutant strain of *Vibrio harveyi* capable of producing only AI-2. Without AI-1 present in the population, AI-1 regulated quorum sensing pathway is not turned on. If BB152 does not bioluminesce, then we can suggest that thymoquinone inhibits quorum sensing under the AI-2 pathway.

Results

In these studies, bacterial growth of *Vibrio harveyi* strains in the presence of Thymoquinone was inhibited in a concentration-dependent manner. Concentrations of 25 uM, 50 uM, and 100 uM of thymoquinone were studied. In addition, a control group of media and ethanol was used to see how the bacteria grows without any added inhibitor. This was measured by the optical density at 595 mm over a time of 30 hours as indicated (**Graph 1**). As exhibited in the data (**Graph 1**), as the concentration of TQ increases, the bacterial growth for each strain decreases. Most strains and concentrations with TQ peaked at similar times between 16-20 hours. Additionally in these studies, the presence of TQ inhibits luminescence at lower concentrations (**Graph 2)**. The concentrations of 25 uM, 50 uM, and 100 uM of TQ were applied to each strain and the luminescence was measured in relative light units (RLU) over a period of 30 hours. Because this data is not sufficient for making definite conclusions about the effectiveness of thymoquinone, additional studies are necessary using more bacterial strains and more concentrations of thymoquinone, especially in conjunction with the 721 *VH* bacterial strain.



Graph 1: Concentrations of 50 uM and 100 uM thymoquinone were added to *VH* strains of 120 and 721 as well as two control groups with media and ethanol for both strains. The *VH* 120 control reached a maximum growth at 18 hours while the *VH* 721 control reached a maximum growth at 22 hours. *VH* 120 with TQ at 50 uM and at 100 uM decreased in a dose-dependent manner. Similarly, both *VH* 721 with TQ at 50 uM and at 100 uM experienced a reduction in a non-dose-dependent manner. Both of these strains suggest positive results.



Graph 2: Concentrations of 25uM, 50uM, and 100uM of TQ were added to *VH* 120 and 100 uM was added to *VH* 721 as well as two control groups with media and ethanol in both strains. The graph measures luminescence in the presence of the concentrations of *VH*. The 25uM concentration stopped the luminescence more than the 50uM in the 120 bacterial strain. The 100uM concentration delayed the luminescence by around ten hours but did not inhibit it. For the 721 strain, the addition of TQ also delayed the luminescence by about ten hours.

Discussion and Conclusion

Thus far, we have studied the effects of TQ on bacterial growth and luminescence of *Vibrio Harveyi*. Our data shows instances where the growth was inhibited in all of the studied strains. As depicted in Graph 1, TQ best inhibited the growth of *VH*120 and *VH* 721 at a concentration of 100uM, but also exhibited inhibiting properties on both strains at 50uM. The luminescence data (Graph 2) shows that concentrations of 25 and 50uM of TQ inhibited luminescence while the presence of 100uM of TQ **enhanced** luminescence in both strains of *VH*. This is an unexpected outcome since we predicted that higher amounts of TQ would decrease the luminescence if TQ is indeed a quorum sensing inhibitor. We do not know yet why this enhancement takes place when 100 uM TQ is added. This information suggests that, at lower

concentrations, TQ works as a quorum sensing inhibitor in the tested strains of bacteria because the bacteria was able to grow but the virulence factor (luminescence) was inhibited under these conditions.

There are limitations to our study that require more research and testing. Our studies are usually conducted in a media (Autoinducer Bioassay media) that is designed to promote significant luminescence of the Wild-type strain, but, due to solubility issues with TQ, we needed to conduct our luminescence studies in a different media (Nutrient Broth) that does not promote the same level of glowing. We studied the difference in luminescence when no TQ was added so we could observe the effect of the two media types on the basic level of glowing expected for each strain. When we added TQ to the VH strains prepared in Nutrient Broth, we had a difficult time observing if the reduced glowing was due to the media or to the TQ. Our results are also limited by the quantity of experiments we were able to conduct in the time available, which may not be sufficient for applications beyond this study. We have not yet conducted the planned "synergy" studies that look at luminescence and growth when TQ and Nisin are added together. Nonetheless, our initial hypothesis has so far been supported and the plant extract TQ has reduced the growth and the luminescence of *VH* in multiple experiments using two strains of VH bacteria.

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