Preventing the War on Superbugs: The Powers of Vitamin D

Abstract

As antibiotic resistant bacteria persist as a stagnant issue, researchers and scientists continuously attempt to find new solutions to the problem. The aim of this proposal is to focus on finding preventative measures that will have a longer lasting effect on counteracting infection and antibiotic resistance. Specifically, this proposal homed in on MRSA as the target pathogen and vitamin D as the proposed preventative solution. We chose MRSA because it is a notoriously difficult superbug to treat resulting in around 20,000 deaths each year in the U.S. alone (Kourtis, 2019), and we chose vitamin D because of the significant supporting research that suggests vitamin D increases the body's antimicrobial immune response. Our experiment uses different combinations of MRSA, tissue culture, and vitamin D in vitro to test whether or not vitamin D has a positive effect. Several controls are presented as well to provide comparison. We expect that tissue culture treated with vitamin D will have a significantly stronger immune response to a MRSA infection. Hopefully, the findings of this study will help inform future research. Furthermore, if our hypothesis is supported, it could be important information for the general public, so that they are aware of the importance of maintaining personal vitamin D levels.

Section I. What is your idea?

With the ever-progressing issue of antibiotic resistance and bacterial mutation, it has become increasingly important for both treatments and preventative measures to be developed in an attempt to fix this problem. **This proposal aims to utilize preventative measures as a long-term solution to infection, since development of antibiotics is a short-term solution that bacteria quickly develop resistance to. We propose testing vitamin D on the immune response to MRSA cultures to see if it has any effect on preventing or counteracting the infection.** While there is substantial research on vitamin D and its interactions with the immune system in relation to other superbugs, there has not been

significant research involving methicillin-resistant *Staphylococcus aureus*, or MRSA; exploring this future could prove quite useful considering MRSA is already a very difficult superbug to handle with very little treatment options and a high susceptibility to rapid mutation.

This idea leads us to our research question: How are MRSA levels affected by the increased expression of antimicrobial peptides that are induced by Vitamin D3 supplementation? Furthermore, to get more specific, our sub research question is: How are tissue growth impacted by MRSA levels, and does Vitamin D also directly impact MRSA itself? Having a sub research question will assist us in answering our main research question - understanding tissue growth and vitamin D3 impacts tell us more about the severity and susceptibility of MRSA infections overall. For this proposal, we expect vitamin D3 to have a significant impact on MRSA infections, because of previous research suggesting vitamin D is a direct effect on the antimicrobial innate immune response. We hope that this increased immune response will impact the infection of MRSA, such that fewer cells will be infected, and the overall severity will be decreased. Ultimately, this will combat the increased use of antibiotics, and drastically reduce the development of antibiotic resistance in superbugs. Furthermore, this ties into the One Health guidelines, as it addresses three potential areas of concern - health service delivery, education initiatives, and protection of surrounding ecosystems. For health service delivery, using a preventative measure will reduce the number of infections and their severity, consequently saving many lives and keeping us healthy. The education initiative will serve to both teach the general public of measures they can take to stay healthy, as well as pushing the scientific community to tackle problems from a preventative and proactive standpoint rather than a reactive one. Lastly, the protection of the surrounding ecosystems involves reducing the spread of antibiotic resistant bacteria into various environments through sources such as wastewater. If wild animals or plants come into contact with superbugs, they may not have the necessary defense mechanisms to fight them off, leading to potential ecosystem collapse. If our hypothesis is supported by the data we collect, and vitamin D3 does indeed help against MRSA, this information can be used to simultaneously educate the public, the scientific community, and protect the lives of humans, animals, and plants alike.

Section II. What informs this project?

For decades, antibiotics and antimicrobials have been indispensable parts of cancer treatment, surgery, organ transplantation, and additionally, they have long been used to promote productivity in animal agriculture. While there aren't any efficient and effective alternatives yet, antimicrobials are still the first choice for many medical and non-medical purposes. The excessive use of antibiotics encourages the selective pressure in the organisms and increases the speed of antibiotic resistance. Moreover, many bacteria, fungi, germs even develop resistance to several types of antimicrobials, which the media call superbugs. Some of the infamous and alarming multi-drug resistance strains nowadays include methicillin-resistant *Staphylococcus aureus* (MRSA), clarithromycin-resistant *Helicobacter pylori*, fluoroquinolone-resistant *Salmonella*, etc. These strains do not only pose great threats to human health, but also restrict the effectiveness of treatments and other infection prevention. With the rapid development of new resistance strains and the slow and stagnant process of inventing new methods to treat these infections, it is important to develop new antibiotic-independent agents to fight against this issue (Golpour et al., 2019).

Immunonutrition, which is the regulation of immune system activity through exogenous nutrients, has been often neglected as an alternative solution to prevent illnesses and bacterial infections. It is well understood that proper nutrition will aid in the support of optimal immune function, thus reducing the severity of infections (Pecora et al., 2020). However, major efforts are still focused on developing new antibiotics to fight superbugs, but that has led the issue to remain stagnant. Even some alternatives for antibiotics, for example, using nanotechnology such as silver nanoparticles, will soon be halted as superbugs are also developing resistance to them (Salas-Orozco et al., 2019), (Hemeg, 2017). Therefore, focus should be shifted to preventative measures of illnesses instead.

Vitamin D is a crucial vitamin to the human body. Currently, we are in a worldwide vitamin D deficiency pandemic due to lack of exposure to sunlight and foods rich in vitamin D (Roth et. al., 2018). Extreme deficiency of vitamin D can cause ricket and growth retardation in children. In adults, it can

increase the risk of fracture in bones (Holick & Chen, 2008). Vitamin D3 has also been found to be a direct regulator of antimicrobial innate immune response by inducing antimicrobial peptide gene expression in human keratinocytes, monocytes and neutrophils, and human cell lines, hence, the deficiency of vitamin D3 can cause an increased in susceptibility to infections (Wang et al., 2004), (Aranow, 2011) and (Derbyshire & Calder, 2021). In fact, a recent 2020 study concluded that about nine of the ten deaths caused by SARS-CoV-2 (Covid-19) is due to the lack of vitamin D in patients. If the supplementation of the vitamin D were introduced, the deaths could have been prevented (Brenner & Schöttker, 2020). In addition to this, there is a suspected correlation between increased infection rates with low vitamin D levels during the winter due to less sunlight (Aranow, 2011).

It is important to first understand the preliminary research concerning vitamin D to explain why this is the compound that we chose to explore in relation to superbugs, specifically MRSA. Vitamin D3 itself is inactive when in the body and must be converted into the active form of the vitamin. Vitamin D3 enters the body and is then hydroxylated in the liver to a compound called 25 D, which is still inactive, and then becomes further converted to 1,25 D in the kidneys, which is the final active form of the vitamin. To properly regulate the levels of active vitamin D, another inactive compound comes into play - 1,25,24 D - to create a negative feedback loop, so that the vitamin D levels in the body stay relatively consistent. This information is important for understanding what version of vitamin D we will be applying to our experiment. Since we are using a culture rather than a human test subject, we can't use the external version of vitamin D3 that humans ingest; instead, we must use the active broken down form of vitamin D - 1,25D (Aranow, 2011).

1,25 D binds to the vitamin D receptor (VDR) and combines with the RXR receptor to construct the VDR/RXR complex. This complex can affect the gene expression either through binding to the vitamin D receptor elements of the target genes or association with transcription factors, which eventually prevent binding and activation of target genes. The restriction of the gene expression leads to the reduction of proinflammatory mediators, and overall decreases the inflammatory response within tissues (Yin & Agrawal, 2014). On the other hand, 1,25 D also modulates cathelicidin antimicrobial peptides

(camp) gene expression in innate immune cells, which eventually have broad antimicrobial effects, without provoking inflammation (Golpour et al., 2019).

The history of vitamin D being used as treatments is also a relevant detail for why we think vitamin D could be a potential solution. Historically, vitamin D has been unknowingly used to treat infections, and now it is used more directly, and various studies have shown drastic improvement. Vitamin D injections were shown to decrease influenza infection by 42%. Previously mentioned, a study by Wang et. al. examined the effects of vitamin D on antimicrobial gene expression in the immune system. Not only did the study explore the effects of Vitamin D3 to immune response but it also tested the effects of the immune response against *Escherichia coli* and *Pseudomonas aeruginosa* in the presence of vitamin D (Wang et al., 2004). These are just examples of many that showed decreased infection through the use of vitamin D. Given the fact vitamin D3 can boost the immune system and the idle issue with MRSA, no study has looked at the effectiveness of vitamin D3 in increasing immune response to deal with MRSA. With a widely known ability to boost the immune system against pathogens, we want to investigate the role of vitamin D in reducing the spread of infection in this research.

Section III. How will you test it?

For our methods, we plan on growing multiple different tissue cultures and testing them with or without MRSA and with or without vitamin D. Then using different measurement techniques, we will determine the difference in tissue growth and MRSA growth in each plate to assess whether or not vitamin D actually has a positive effect on the immune response to MRSA.

The table below shows how we'll set up each plate; there are 4 experimental plates and 2 control plates. The two control plates will give us an individual understanding of the growth for the two living aspects of our experiment - one culture (Plate 5) has just the tissue sample with nothing added and the other has just the MRSA culture with nothing added (Plate 6). The experimental controls use every other possible relevant combination of tissue culture, vitamin D, and MRSA culture - Plate 1 has all 3, Plate 2

has just the tissue with vitamin D, Plate 3 has just the tissue with MRSA, and Plate 4 has vitamin D added to the MRSA culture. Assuming that the MRSA culture grows evenly in the tissue culture, we can take a sample from the culture to test both the tissue and MRSA culture. We will be doing this over the course of a week, so we will need 7 versions for each plate (one per day) with a replication of 3 for each plate, so that we can identify consistency. This comes to a total of 126 plates. Plates will be incubated at 37℃.

Set Up

	Plate 1	**Plate 2**	**Plate 3**	**Plate 4**	**Plate 5**	**Plate 6**
Tissue	+	+	+	-	+	-
Vitamin D	+	+	-	+	-	-
MRSA	+	-	+	+	-	+
	Experimental				Control	

To test MRSA levels from our plates, the gram-positive bacteria must first be isolated from the tissue matrix. To do this, Matrix-Lysis will be used, which includes a buffer of 8 M Urea and 1% SDS to solubilize the tissue matrix. Then, the remaining pellet will be concentrated and purified of contaminants through washing and centrifugation steps (Mann et al., 2014). Lastly, the DNA will be extracted from the remaining bacteria and qPCR will be performed to quantify the levels of MRSA using a PCR assay for MRSA screening (Xpert MRSA/SA SSTI from the company Cepheid). One benefit of this method is that it has a high recovery and concentration rate of bacterial cells from a sample mass, and results in a pellet small enough to conduct molecular and microbiological detection methods on it.

Human cell lines can be obtained from the American Type Culture Collection (Manassas, VA). To measure the tissue proliferation, the indicator dye alamarBlue® will be used. alamarBlue® is an assay reagent that contains a weakly fluorescent blue indicator dye called resazurin. The dye will only enter part of the metabolically active part of the cells. When it is reduced by the cell it will become fluorescent. To use the alamarBlue® we would add in the dye to the cell culture before each experiment, incubate it for 2

hours at 37°C between pH 6.8 and pH 7.4. We would measure fluorescence at 530-560 nm excitation wavelength and 590 nm emission wavelength with a spectrometer. We would repeat the process after each individual experiment as well. To indicate whether there was tissue proliferation, growth of tissue would be fluorescence, while inhibition of growth would be non-fluorescence.

Immune response will be measured through gene expression of immune proteins. To do this, a sample of the tissue will be taken from the plate and mRNA will be isolated. Isolating mRNA will be done through a series of buffer additions and pellet formations to wash away cell material from the mRNA. The buffers and protocol to isolate the mRNA can be purchased from New England Biolabs. Next, reverse transcriptase will be added to the mRNA to create DNA from the template. Then primers, that were designed before beginning the experiment, will be used to amplify the mRNA through PCR. The level of amplified DNA will be recorded and used to quantify immune expression. mRNA is only made in the cell when a gene is being expressed, therefore this technique allows expression of a target gene to be detected and measured.

Results

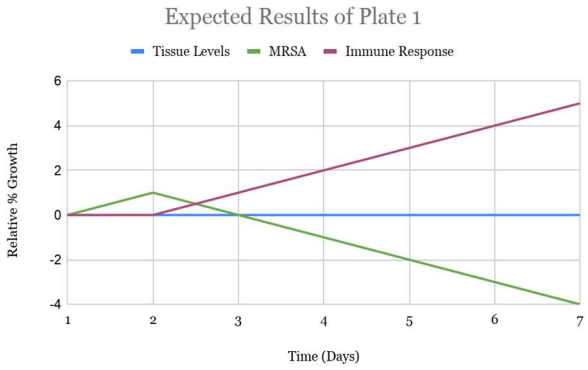
Based on previous studies regarding the effect of vitamin D on innate immune systems against several antibiotic resistant strains, we hypothesized that vitamin D will enhance immune function in vitro and will restrict the growth of MRSA in tissue cultures. Therefore, the table below shows our predicted results after one week of observation.

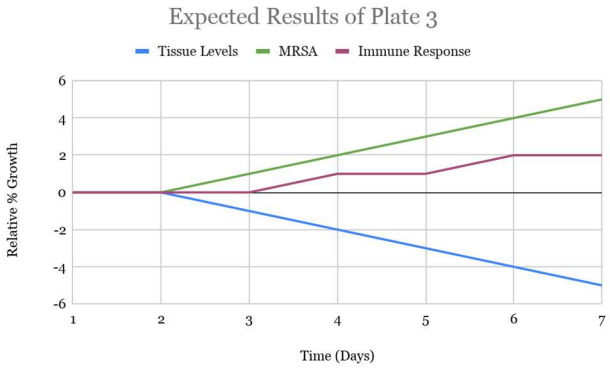
	Plate 1	**Plate 2**	**Plate 3**	**Plate 4**	**Plate 5**	**Plate 6**
Components of the plate	Tissue + VD + MRSA	Tissue + VD	Tissue + MRSA	Vitamin D + MRSA	Tissue	MRSA
MRSA levels	Decrease	-	Increase	Increase	-	Increase
Tissue Growth	Unchanged	Unchanged	Decrease	-	Unchanged	-
Immune response	Strong	Strong (?)	Weak	-	-	-

Plate 5 and plate 6 serve as the control objects, where only tissue cultures or MRSA are put in each type of plate. The tissue growth in plate 6 is expected to be unchanged over time, because the plate condition may not be favorable for the cultures to grow. In plate 6, as MRSA can grow easily at room temperature, MRSA level measured will increase rapidly over time (Missiakas & Schneewind, 2013).

For the sample in plate 4, based on our understanding about the activity of vitamin D, the supplement of vitamin D alone cannot act as antibiotics, and it cannot have any impact on MRSA. Therefore, the bacteria level is also expected to increase as in plate 6. In plate 2, as vitamin D helps induce antimicrobial peptides, we expect to observe a strong immune system activity in the tissue while there will not be any change in tissue growth.

Plate 1 and plate 3 are our focus in the whole experiment, where we want to see whether vitamin D makes any difference in combating against MRSA infection. We predict that MRSA level is





significantly lower in plate 1 than in plate 3, and stronger immune response in plate 1 is also expected to be observed. While the tissue density in plate 1 is unchanged, a decrease in tissue growth in plate 3 may be seen as MRSA affects the normal activity of the tissue. The two figures above show a possible result we may get after 7 days observation.

If our research is successful, the next step would be to reach out to a journal to publish our work, as well as to receive peer review on the article we would write. In addition, we would reach out to hospitals and primary care physicians to let them know about our findings, in the hopes that they enact programs that increase the vitamin D supplementation that their patients take. Based on our results, this

could act as a preventative measure for MRSA infections. Aside from acting on our results, we could also design more experiments that build off this one. For example, testing vitamin D on different antibiotic resistant bacteria. The more superbugs that can be defended against, the less antibiotics will need to be prescribed. Furthermore, we could vary the levels of vitamin D supplementation to determine the most cost-efficient and yet still beneficial dosage to combat infection. The final step would be to let the general public know. Even small steps such as spending more time in the sun would increase vitamin D production in our skin.

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Final Proposal Rubric

Group Name: The Exterminators (Dararath, Ravid, Claire, Ryanne, Quynh)

Criteria	**Key Feedback You Received/Changes You Made**	**Group Self-Assessment**
Title & Abstract (10 points) Title hooks reader in and conveys a succinct understanding of the topic, setting, and important variables Abstract gives a summary of the proposal and key information so that reader gets a snapshot of what is to come (re-visit rhetorical moves and abstract activity week 4 class materials on Moodle) Abstract limit is 250 words	No changes were made. All feedback on this section indicated that this section was at its fullest potential.	10/10

Section I. What is your idea? (20pts) · Indicates in one or two sentences in bold the essence of your idea. · Highlights why this idea is creative/novel/fundable. · Purpose of the study is clearly articulated. · States research questions and any hypotheses or predictions. · Overall structure and flow of this section sells the reader on this as a promising One Health investigation. .	The main feedback we received from this section was to include more about the One Health perspective and how our project related. We ended up elaborating on the One Health perspective more and making it a bigger focus for this section to better explain motivation for our project. We also received a comment suggesting we take out the sub research question and just have a main research question. However, we thought that it was incredibly necessary to have both the main and sub research question, so we decided to keep it as is.	20/20

Section II. What informs this project? (20 pts) · Describes and cites relevant and related studies that inform the project. · Provides necessary background for readers to understand the context of the project. · Identifies the gap: situates the work in what is already known vs. what is still needed. · Uses the literature and the gap to both illustrate and justify a need for the project.	The feedback we received in this section focused on defining a few concepts that might be unclear to the reader. Specifically, we needed to include a definition of "immunonutrition." We got a lot of positive feedback here in regards to how we explained our background information and connected it to our project as well. So, we reread the section to make sure it was clear and defined immunonutrition when the topic was introduced. We also ended up making some cuts to information that was relevant but unnecessary to our specific proposal.	20/20

· **Section III. How will you test it? (20pts)** ● Provides and overview of the project and planned research and experiments ● Gives enough detail and explanation about methodologies to demonstrate the work is feasible ● When existing methodologies are used and/or modified, citations are used ● Describes the types of data that will be generated and how the data will be analyzed and interpreted · Discusses expected outcomes of the project · Describes how any findings in this project will inform next steps: how does this research help move the field/topic/focus forward?	This section had great feedback, and was a very comprehensive section overall. Added graphs to explain results for most important plates (discussed in visuals section of rubric). Methods were clear and the alignment of the procedures with the research question were clear as well. Peer review mentioned that the method of plating was not stated in proposal, but also said this level of detail is not necessary, since the plates themselves were described. Future experiments were also added that could be conducted with the base that would be developed from our experiment.	20/20

Use of visuals (10pts) Uses key figures/tables/diagrams to support proposals that are properly captioned, labeled, and referred to in text.	Added two graphs to better explain our predicted results for plates 1 and 3. The peer reviewers mentioned that another visual could be helpful to break up the text (in addition to the tables we already had). The graphs added help to understand the change in MRSA, tissue, and immune response over time.	10/10
Writing and Structure (10pts) · Proposal is free of typos, spelling, and grammatical errors. · Proposal flows and transitions between sections. · Background literature is synthesized with appropriate paraphrasing and citation.	No grammatical errors were found in peer review. Some transition sentences were added, as well as reformatting (such as changing everything to double spaced) Citations were good. Changed a few that were missing dates or authors (went back into sources and found the necessary information)	10/10

References and Citations (10 pts) · A consistent in-text citation style is utilized throughout the proposal. · Reference list is complete. · Uses APA guidelines for both an in-text citation system and full reference.	We didn't have any negative feedback regarding our references. All necessary references are both included in the works cited page and included in the in text citations where appropriate (in text citations are also hyper linked using zotero). It is also complete in APA format.	10/10
Total		**100/100 pts**