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Spring 5-31-2021

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Kale, Mita, "The Effects of Infliximab on the Fibrosis Response of Three-spine Stickleback" (2021). *Honors Scholar Theses*. 800.
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The Effects of Infliximab on the Fibrosis Response of Three-spine Stickleback

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June 2021

Abstract

This study was conducted to determine if the monoclonal antibody drug infliximab could effectively suppress the fibrosis immune response in three-spine stickleback fish. Successful suppression of this response could allow for further study of cestode infection and growth without the presence of fibrosis. Infliximab's efficacy was investigated through conducting two intraperitoneal injection experiments and examinations of the fibrosis in the body cavity of the euthanized stickleback. We used immune adjuvant alum to induce a fibrosis response without the presence of the *S. solidus* tapeworm parasite. Ultimately, the differences in fibrosis levels between the treatment groups that received alum and the groups that received alum+infliximab were not statistically significant. These findings suggest that infliximab is not a good candidate for attenuating the fibrosis response in three-spine stickleback and other methods of suppressing fibrosis to examine cestode growth should be pursued.

Introduction

Fibrosis is a pathological response characterized by an accumulation of extracellular matrix proteins that plays a major role in a wide variety of diseases in humans. The two major purposes of the fibrosis response are host resistance to a pathogen or wound healing. In some instances, the fibrosis response leads to an excessive amount of extracellular matrix buildup that can cause long-term scarring (Thannickal *et al.* 2014). The excess formation of connective tissue due to fibrosis can result in damage or failure in multiple organs or organ systems. This outcome is so common that an estimated 45% of all mortality in the United States involve some form of fibrotic disorder (Neary *et al.* 2015).

After infection with a pathogen, the fibrosis response is capable of preventing further spread and damage to the host through the formation of fibrotic scars around the infection, although this is not possible with every pathogen. Wound healing can also be aided by fibrosis, a fibrotic scar could help reduce blood loss and preserve the structural integrity of the organ (Thannickal *et al*. 2014). Organisms that exhibit a fibrosis response need a mechanism to attenuate the fibrotic tissue if it accumulates to the point where it could cause damage (Hall *et al* 2017).

The mechanism of fibrosis formation for wound healing in humans happens in four broad stages: initial injury, hemostasis, regeneration, and remodeling (fibrosis). Once the injury occurs in epithelial or endothelial tissue, platelets accumulate to form a protective clot. During regeneration, neutrophils and monocytes remove debris, which allows the remodeling process to proceed. Multiple types of cytokines recruit fibroblast cells, which produce collagen and other extracellular matrix proteins. Epithelial or endothelial cells subsequently transfer to the site of the injury and complete wound healing (Wynn 2004). Articles such as Wynn's are able to go into great detail about fibrosis pathogenesis, but much less information is available regarding the mechanisms underlying the abnormal deposition of extracellular matrix. This gap in our understanding has created a shortage of specific and effective treatments for the harmful forms of fibrosis (Neary *et al.* 2015).

The three-spine stickleback, also known as *Gasterosteus aculeatus*, is a fish that is found in the northern hemisphere. They reside in both freshwater and marine habitats that are often genetically and physically isolated from one another. Their abundance and phenotypic diversity make them a useful model system for the "analysis of evolutionary mechanisms" (Bell & Foster 1994). The host-parasite interactions between three-spine stickleback and the tapeworm *Schistocephalus solidus* are of particular interest. Three-spine stickleback serve as an intermediate

host for the tapeworm, and infection occurs almost entirely in freshwater populations. As a form of defense against *S. solidus*, some populations of stickleback mount an immune response that relies on fibrosis to impede the cestode's growth. The stickleback's peritoneal cavity becomes riddled with fibrotic tissue in severe cases (Hund *et al.* 2020 *in prep*). While this process is capable of resisting the tapeworm infection, there are often serious fitness costs for the fish. For example, the presence of fibrosis decreases the likelihood of successful reproduction in wild populations (De Lisle & Bolnick 2021). Therefore, the fibrosis response must be both swift and reversible in order to prevent long-term damage (Hund *et al.* 2020 *in prep*).

Similar to humans, the pathway and mechanisms of every part of fibrosis formation are not fully studied in stickleback. According to a coexpression network analysis of immune related genes, resistance to a parasitic infection is influenced by the tapeworm's survival and the level of growth arrest (Lohman *et al.* 2017). Other studies use a stepwise approach to identify aspects of the fibrosis pathway by testing marine, resistant, and susceptible populations with immune challenges. All three populations exhibited fibrosis after injection with the immune adjuvant alum, but only the resistant population exhibited fibrosis after injection with proteins from the tapeworm. The findings of this study suggest that the ability to mount a beneficial fibrosis response after infection in the wild is population specific (Hund *et al.* 2020 *in prep*). Overall, the molecular processes of the fibrosis pathway in stickleback are less clear when compared to our range of knowledge for humans.

Examining the interactions between the stickleback host, the *S. solidus* parasite, and the immune system's fibrosis response has the potential to inform our understanding of broader evolutionary trends in hosts' response to infection (Hund *et al.* 2020 *in prep*). Studies that investigate the growth of cestodes within stickleback must consider the interference of fibrosis in

their findings if the population is resistant. While there are therapeutic options for pharmacologically treating fibrosis in humans, these drugs have little to no available efficacy data for stickleback. A drug that is capable of suppressing the fibrosis response for three-spine stickleback could help with the design of experiments examining the growth of *S. solidus*. If it is possible to remove the interference of the fibrosis response, studies could then document whether or not the growth of *S. solidus* can continue after drug-induced suppression.

The drug infliximab, also known as Remicade, is an anti-TNF alpha monoclonal antibody that has been tested in rats and humans to determine if it is capable of suppressing multiple types of fibrosis. The FDA originally approved the drug for Crohn's disease treatment, but it is also applicable in conditions such as rheumatoid arthritis (Multum 2020). It eliminates oxygen free radicals that are meant to activate crucial cytokines within the fibrosis pathway. Ultimately, this results in less overall harm to the tissue because of excessive angiogenesis (Altintas *et al.* 2016). A study of bleomycin-induced lung fibrosis in rats concluded that infliximab was capable of preventing this type of fibrosis in rats. The study came to this conclusion by measuring levels of cytokines such as TNF-alpha and interleukin 6 that play a role in fibrosis activation. The histological findings also supported this because stained tissue from the infliximab treated rats showed less inflammation and excess fibrosis (Altintas *et al.* 2016). An additional study tests infliximab's usefulness in treating human pulmonary fibrosis. Four patients with existing severe pulmonary fibrosis received multiple temporally spaced infliximab treatments, and the patients' symptoms were consistent through the course of the study. The authors posit that infliximab could help stabilize this damaging form of fibrosis, but extensive clinical trials must support these initial findings before infliximab can be considered a good therapy for pulmonary fibrosis (Antoniou *et*

al. 2007). Infliximab also exists in a liquid form that makes it simple to administer intraperitoneally (Multum 2020).

Given the findings of these studies in rats and humans, we chose infliximab as the drug for a pilot study intended to determine if this drug is capable of partially or completely suppressing fibrosis in three-spine stickleback. The immune adjuvant alum can effectively activate the fibrosis response in both susceptible and resistant populations of stickleback, and it often serves as a method of fibrosis activation without the need for infecting fish in an experimental environment (Kool *et al.* 2012) We hypothesized that infliximab could interfere with the fibrosis pathway and ultimately attenuate the alum-induced fibrosis response. Because alum is administered intraperitoneally in stickleback, we chose a series of injection experiments as the experimental strategy for testing infliximab's functioning.

Methods

Injection Experiment One

The first experiment occurred in January 2021 using 39 three-spine stickleback fish from Roselle Lake. It is a freshwater lake located on Vancouver Island in British Columbia, Canada, and its population of three-spine stickleback is resistant to *S. solidus* infection through its strong fibrosis response. We chose Roselle stickleback for this experiment because they exhibit fibrosis after intraperitoneal injection with alum (Hund *et al.* 2020 *in prep*). Collection of Roselle fish occurred in the summer of 2019 and were used to create lab-reared descendants in the animal care facility at the University of Connecticut. We collected these Roselle descendants from vivarium tanks in the animal care facility for use in the injection experiments. We then gave one of four treatments by intraperitoneal injection. The first group served as the control treatment, so it

received 20 µ1 of 1X phosphate buffered saline (PBS). The second group was the alum treatment, which received 10 µ1 alum (2% Alumax Phosphate, OZ Bioscience) and 10 µ1 PBS. We created this group so that we could directly compare the fibrosis levels of the alum treatment and the alum+ infliximab treatment to determine if the infliximab could produce the hypothesized decrease in fibrosis. The third group was the infliximab treatment, these fish received 10 µl infliximab (TNF Alpha antibody cA2, Bio-Rad) and 10 µ1 PBS. This group is not useful for determining the drug's efficacy in reducing fibrosis because it did not receive the alum irritant, but it was necessary to include in the first experiment to ensure that infliximab was not causing any fibrosis in the stickleback on its own. The last group was the alum+infliximab treatment, they received 10 µ1 alum and 10 µl infliximab to test if infliximab could attenuate alum-induced fibrosis. The control, alum, and infliximab treatment groups each had 10 samples, and the alum+infliximab group had 9. We adapted the injection protocol from the novel procedure described in Hund *et al.* (Hund *et al.* 2020 *in prep*).

The solutions for each treatment were prepared and loaded into insulin syringes in a sterile culture hood. The fish were anesthetized with diluted MS-222 and kept on a wet sponge prior to injection. Each fish also received an elastomer dye injection on the top of its head between the eyes to differentiate treatment groups. Injections were given into the peritoneal cavity at an angle parallel to the fish's body to prevent organ puncture. As soon as the injection was complete, the fish were placed in an aerated recovery tank. Injected fish were distributed into five long-term tanks and each tank had one or two samples from each group. The mortality rate was 15% (6 out of 39), four of these deaths occurred multiple days after initial injection. One sample died in the control, alum, and infliximab treatments, and three died in the alum+infliximab group. We excluded dead fish from all data collection.

After 10 days, we euthanized all remaining fish using a lethal dose of MS-222 and assessed their fibrosis by examining the body cavity. A 0-4 scale was used to visually quantify fibrosis: 0 (no fibrosis), 1 (organs are not moving freely), 2 (organs are adhering together), 3 (organs adhering together and to the peritoneal wall), 4 (severe fibrosis, cannot open peritoneal cavity). The protocol was IACUC approved (protocol A18-008).

Injection Experiment Two

The second experiment occurred in April 2021 using 36 three-spine stickleback from Gosling Lake. This lake is also on Vancouver Island in British Columbia, but the stickleback population there is genetically isolated from the Roselle population. Gosling stickleback also exhibit fibrosis, but they are more susceptible to cestode infection (Lohman *et al.* 2017). Gosling fish were collected in the summer of 2019 and subsequently used to breed lab-reared descendants in the animal care facility at the University of Connecticut. We injected these fish that originated in the Gosling lake due to availability constraints for Roselle fish. The injection procedure was performed the same way as the first experiment, but we only used two treatment groups. The previous experiment showed that the alum treatment was inducing fibrosis as expected and the infliximab treatment was not causing fibrosis compared to the saline control. Given these findings, we made these changes to increase the sample size for each group and to continue investigating if there were any statistically significant changes in fibrosis levels. The treatment groups are as follows:

A) 10 µ1 alum + 10 µ1 PBS (alum treatment)

B) 10 µ1 alum + 15 µl infliximab (alum+infliximab treatment)

The mortality rate was 25% (9 out of 36), and the majority of the deaths occurred a few days after the injections. The alum treatment had 4 deaths and the alum+infliximab group had 5 deaths. After 10 days, all of the remaining fish were euthanized.

Analysis of Injection Experiment Results

For both experiments, two-sample Mann-Whitney U tests assuming unequal variances were performed using Microsoft Excel to determine if there were any statistically significant differences in fibrosis between treatment groups. Mann-Whitney U tests are appropriate to analyze this data because fibrosis level is an ordinal value. We also used Excel to determine descriptive statistics such as median and standard error for each treatment group. For the first experiment, we analyzed three sets of data in the U test:

A) Control vs. alum to determine the efficacy of alum

B) Control vs. infliximab to determine the effects of infliximab without alum

C) Alum vs. alum+infliximab to determine the effects of infliximab after alum injection

After the second experiment we only compared the alum and the alum+infliximab groups. We then used R Studio to create violin plots to visualize the distribution of fibrosis level within treatment groups.

Results

In experiment one for the control and alum groups, the medians were 0 and 2 respectively, U= 13.5, $n_1 = n_2 = 9$, $P < 0.05$ two-tailed. The difference in median fibrosis is therefore statistically significant, so the alum was confirmed to be effective. For both the control and infliximab groups, none of the stickleback exhibited fibrosis (value of 0), so the infliximab likely did not cause an increase in fibrosis in this experiment (U=40.5, $n_1 = n_2 = 9$, P >0.05 two tail). The alum and alum+infliximab groups had medians of 2 and 1. The hypothesized outcome was a decrease in fibrosis in the alum+infliximab group, but according to the U-test, this difference is not statistically significant (U=22, $n_1 = 9$, $n_2 = 6$, P >0.05 two tail). The violin plot below visually represents the frequency of each fibrosis level within the groups as well as the mean and standard deviation.

control		*infliximab*	
Mean	0	Mean	0
Standard Error	0	Standard Error	0
Median	0	Median	0
Mode	0	Mode	0
Standard Deviation	0	Standard Deviation	0
Sample Variance	0	Sample Variance	0

control		*alum*	
Mean	0	Mean	1.33333333
Standard Error	0	Standard Error	0.372678
Median	0	Median	2
Mode	0	Mode	2
Standard Deviation	0	Standard Deviation	1.11803399
Sample Variance	0	Sample Variance	1.25

alum		*alum+infliximab*	
Mean	1.333333333	Mean	1
Standard Error	0.372677996	Standard Error	0.36514837
Median	2	Median	1
Mode	2	Mode	1
Standard Deviation	1.118033989	Standard Deviation	0.89442719
Sample Variance	1.25	Sample Variance	0.8

Tables 1-3 Descriptive statistics for experiment one

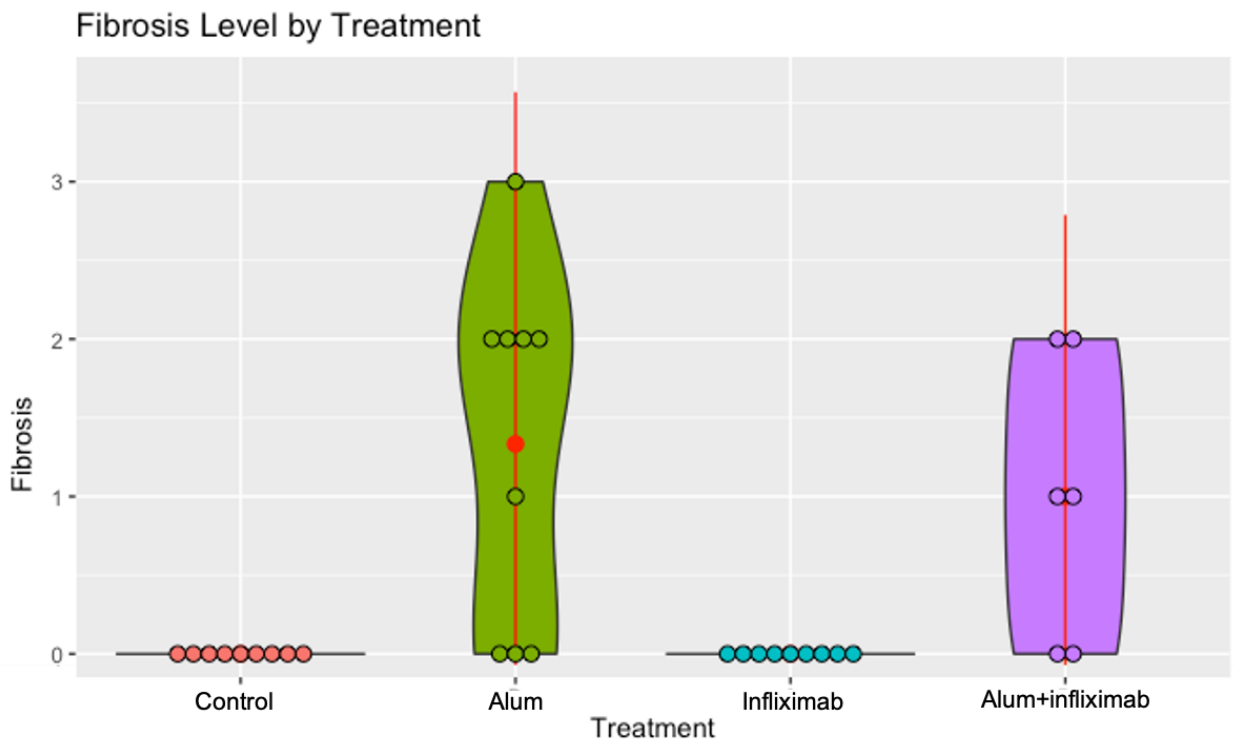

Figure 1 Violin plot showing the distribution of samples within the control, alum, infliximab, and alum+infliximab treatment. Mean and standard deviation are shown. The control, alum, and infliximab groups had 9 samples, and the alum+infliximab group had 6.

In the second experiment, the alum+infliximab group had a median of 1, which is higher than the 0 median value for the alum group. This result contradicts the original hypothesis, but this difference is ultimately not statistically significant (U=63, n_1 = 14, n_2 = 13, P >0.05 two tail). The violin plot below shows the distribution of the collected fibrosis data.

alum		*alum+infliximab*	
Mean	0.5	Mean	1
Standard Error	0.17383837	Standard Error	0.25318484
Median	0	Median	1
Mode	0	Mode	2
Standard Deviation	0.65044364	Standard Deviation	0.91287093
Sample Variance	0.42307692	Sample Variance	0.83333333

Table 4 Descriptive statistics for experiment two

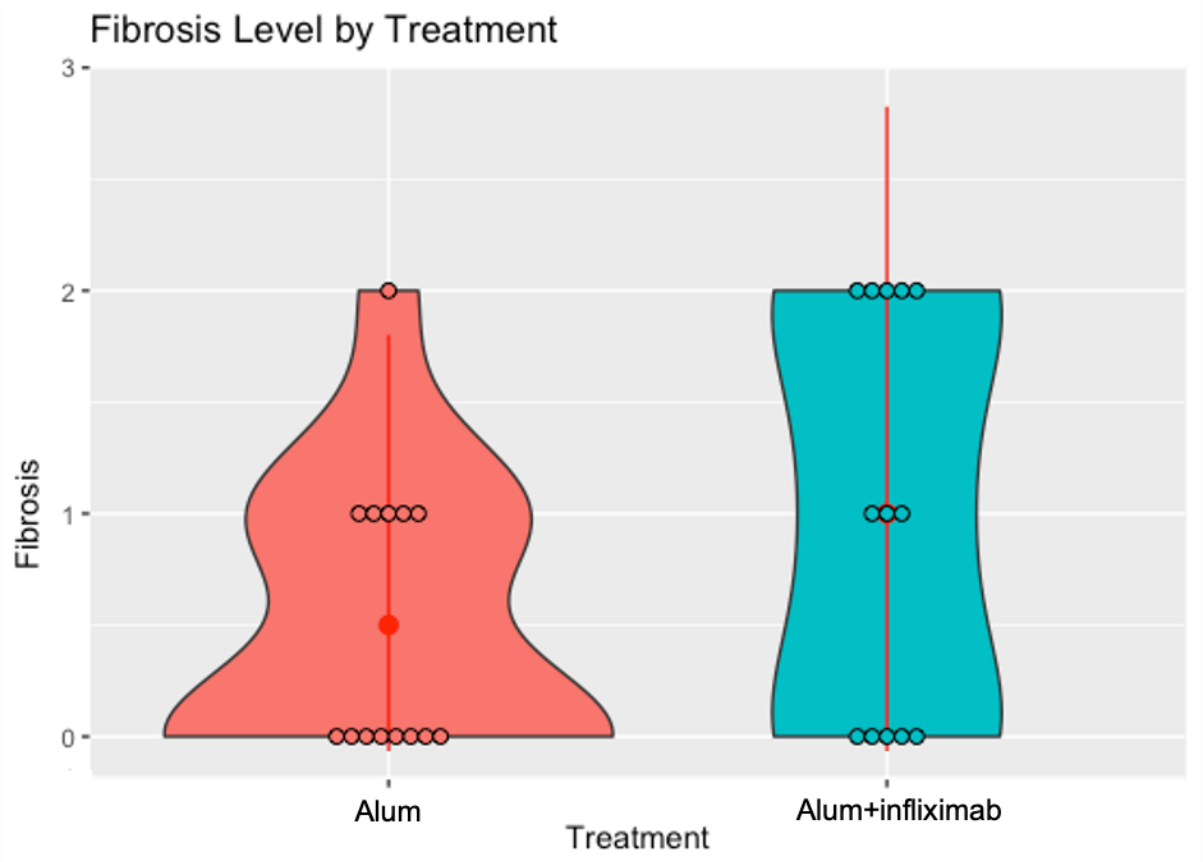

Figure 2 Violin plot showing the distribution of samples within the alum and alum+infliximab treatment groups. Mean and standard deviation are shown. The alum group had 14 samples, and the alum+infliximab group had 13 samples.

Discussion

The lack of a statistically significant change in fibrosis between the infliximab absent and infliximab present groups suggests that infliximab administered at these dosages is not an effective fibrosis suppressant in stickleback from Roselle or Gosling lake. The findings from both experiments support this conclusion. Because of the lack of knowledge regarding the mechanism of the fibrosis pathway in stickleback, there are multiple potential reasons why infliximab was not an effective suppressant. Some of this fish species' genes have been identified as targets in fibrosis, but the ultimate pathway is not known definitively (Fuess *et al.* 2020*).* Because infliximab is a human anti-TNF alpha inhibitor, it is likely that this drug does not chemically interfere with the fibrosis pathway in stickleback (Cerner 2020). Due to the genetic isolation of many populations of stickleback, different populations can significantly vary in their genetic diversity when it comes to their immune responses (Kurtz *et al.* 2004, Bell & Foster 1994). Roselle fish, which were used in the first experiment, have been observed having higher fibrosis than Gosling fish in response to alum injection (Hund *et al.* 2020 *in prep*). The strength of the fibrosis response in a given population could potentially affect the visual observation of the fibrosis in the peritoneal cavity after dissection.

The infliximab dose given to the Roselle fish in the first experiment was 10 µ1, which was chosen due to the dose given in humans, which is 5 mg/kg (Cerner 2020). Since the stickleback from Roselle and Gosling typically have a mass of 2 to 3 grams, a dose of 10 µ1 (5 µ1/gram body mass) was chosen for the sake of simplicity when loading the treatments into the needles. When the infliximab was not effective during the first experiment, a dose of 15 µ1 was given to the Gosling fish to test if a higher dose could produce a statistically significant difference in the

treatments. There was no evidence that the infliximab was the cause of mortality for the Roselle fish because the deaths were similarly distributed among all four treatment groups, so a higher dose was considered to be a reasonable modification to the experimental design. Humans receiving this drug to suppress fibrosis are on it for a prolonged period of time, sometimes ten weeks (Cerner 2020). It is administered intravenously every two weeks, but this strategy to investigate infliximab effectiveness was not chosen since alum is capable of inducing the fibrosis response fairly quickly (Kool *et al.* 2012).

Multiple factors potentially impacted the validity of these results. First, due to this being a pilot study, the sample size for the groups receiving only alum or alum+infliximab ranged from 6 to 14. However, since this experiment was conducted twice and the sample sizes were larger in the second experiment, this does not mean that increasing the number of samples would significantly change the results. In addition, potential infection, organ puncture, or insufficient oxygen intake after the injection are likely causes of death for the stickleback that did not survive the full ten days.

Conclusion and Future Approaches

Due to infliximab's lack of efficacy in this experiment, it cannot be a good candidate for further studies regarding drug-induced suppression. One approach that would have greater potential for fibrosis suppression is interfering with the transcription factor PU.1. Empirical data shows that the heterocyclic diamidine drug DB1976 is able to significantly reverse fibrosis formation in mice through its targeting of the SPI1 gene, which produces PU.1 (Wohlfahrt *et al.* 2019). Sticklebacks have the SPI1 gene, so pharmacological interference with PU.1 could serve as a more direct method of testing drug candidates (Böhne *et al.* 2013).

Acknowledgements

Thank you to Christian Polania for his help with the experimental design and performing the injection experiments. Additionally, thank you to Dr. Daniel Bolnick for his assistance with this thesis.

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