AN EXPLORATORY STUDY ON THE USE OF *GAMBUSIA AFFINIS* AS A MODEL FOR INVESTIGATING GUT INFLAMMATION AND MICROBIOME CHANGES

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by

Eliana Stetco

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DEDICATION

I would like to dedicate this thesis, first and foremost, to my husband, Seth Johnson. If it were not for Seth's tireless support and endless belief in my capabilities, I am not sure I would have made it through the program. My son, Rhett Johnson, was also an integral part to not losing my footing. Because of him, I strive to be better every day. Additionally, my sanity would not have been maintained had it not been for: Jeanette Carlson who made entering a program in which I knew no one and a lab in which I felt supremely intimidated feel like a second home; Chelcy Brumlow who was my rock when I needed to lament how difficult balancing graduate school with family life can be; Stephen Scribner for keeping me laughing and being my lunch date on long days; and Caroline Obkirchner who was writing her thesis at the same time and made me feel less alone throughout the process. Finally, I could not have asked for a more patient, compassionate, and accommodating advisor than Dr. Todd Primm. Thank you.

ABSTRACT

Stetco , Eliana , *Gambusia affinis as a model for the investigation of inflammatory bowel disease, inflammation, and antibiotic usage: An exploratory study* . Master of Arts (Biology), December, 2019, Sam Houston State University, Huntsville, Texas.

A complex relationship exists between gastrointestinal commensal microorganisms and their hosts. While it is not entirely understood how commensal microbiota influence the host immune system, it is evident that the two are largely dependent on one another. Disharmony of the healthy GI tract can result in chronic inflammatory bowel diseases, such as Crohn's disease (CD) or ulcerative colitis (UC). In the healthy GI tract, the lower intestine is largely hypoxic, thus it is expected to be largely dominated by anaerobes. However, inflammation in the large bowel results in dysbiosis of the microflora such that obligate anaerobes decrease in number while the presence of facultative anaerobes increases. As previous literature demonstrates, this could be due to the fact that inflammation in the host generates reactive nitrogen and reactive oxygen species, molecules that facultative anaerobes can use as final electron acceptors in anaerobic respiration. Further, use of antibiotics could result in persistent alterations in the gut microbiome composition that mimic the alterations seen in the inflamed gut, as antibiotic use in humans sometimes improves irritable bowel conditions and sometimes worsens them. Fish were exposed to dextran sodium sulfate (DSS) orally, with gut inflammation monitored using the MPO assay, gut nitrate levels determined, and gut microbiome community analyzed using 16S sequencing. In conclusion, inflammation levels were inconsistent, possibly because these invasive fish are so resilient and DSS levels were insufficient, or the presence of parasites as a confounding factor. Gut community changes were observed but statistical significance not established. These fish are not a good model

for mammalian gut research, as they are normally dominated by facultative anaerobes, while mammalian by obligate anaerobes.In conclusion, it is possible that the amount of DSS used in this explorative study simply is not enough to induce colitis in *Gambusia affinis*, which are known for being rather resilient fish. Furthermore, it is possible that wild caught *Gambusia* have too many inherent confounding factors to be used reliably in gut microbiome research and thus further work is necessary.

KEY WORDS: Inflammation, Gut microbiome, Dextran sodium sulfate, Antibiotics, Fish, Animal model

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CHAPTER I

INTRODUCTION

The complex relationship between the host gut microbiome and the host has been well documented. With approximately 10^{14} microbial cells, which equates to more than ten times the number of human cells (Bull and Plummer, 2014), it is unsurprising that gut bacteria play critical roles in the overall well-being of the host. These microbes supply essential nutrients, synthesize vitamins like vitamin K, metabolize undigested food remnants and are implicated in mood disorders, obesity, allergies, inflammatory diseases, and even cancer (Mohajeri et al., 2018). It is suspected that one of the main factors in the increasing cases of such ailments as inflammatory bowel diseases is the disruption of the gut microbiome.

The Human Gut Microbiome

The healthy human intestinal tract, namely the large intestine where the majority of the gut microbiome resides, is dominated by four major groups: *Firmicutes* and *Bacteriodetes* and, to a much lesser degree, *Proteobacteria* and *Actinobacteria* (Morgan et al., 2012). *Firmicutes* are mostly rod-shaped, all Gram-positive, and divided into two classes, *Clostridia* and *Mollicutes*. *Clostridia* are anaerobes and divided into "clusters." Of note are clostridial clusters IV and XIVa which comprise a substantial amount of the total gut microflora and are made up of different species of bacteria belonging to varying genera (Lopetuso et al., 2013). An example is *Faecalibacterium prausnitzii*, a strict anaerobe whose role is largely functional-to produce butyrate, a short-chain fatty acid produced by anaerobic fermentation of undigested carbohydrates. Butyric acid plays a role in protecting and maintaining the integrity of the intestinal barrier through the

production of mucins. It also has anti-inflammatory functions (Galecka et al., 2013). It has been observed that *F. prausnitzii* are significantly reduced in patients with Crohn's disease and ulcerative colitis as compared to their healthy counterparts (Hippe et al., 2011). . *Bacteriodetes* are Gram-negative, anaerobic, rod-shaped bacteria that, like Firmicutes, comprise a significant portion of the mammalian gut. *Proteobacteria* are facultative or obligate anaerobes that comprise a much smaller portion of the gut microbiome. In fact, Na-Ri Shin et al. describe *Proteobacteria* as a potential marker for gut dysbiosis and suggest that an increased prevalence of this phylum could be used as a diagnostic "signature" of risk for disease (2015). In support of this, the American Gut Project has found that the abundance of *Proteobacteria* in fecal samples can double in the month following antibiotic usage. However, as with other groups, the abundance of *Proteobacteria* in normal healthy donors varies, making conclusions only from abundance data weak. Finally, *Actinobacteria* include the genus *Bifidobacterium*, an anaerobic bacterium that has been well-documented and is sold as a supplement probiotic due to its myriad of benefits including indirect production of butyrate (via lactate), barrier effects, and enhancement of the immune response as is seen in IgA anti-rotavirus antibody activity (Piard et al., 2015).

Host Intestinal Environment and Dysbiosis|

It should be noted that *Firmicute*s and *Bacteriodetes* are obligate anaerobes that rely on fermentation of polysaccharides for growth. In the healthy GI tract, the lower intestine is largely hypoxic, thus it is logical that bacteria belonging to the aforementioned phyla thrive in this environment. In fact, it has been shown that the large intestine is colonized by obligate anaerobes that these microorganisms outnumber their

facultative counterparts (Eckburg et al., 2005; Maier et al., 2015). However, as Winter et al. demonstrate, inflammation in the large bowel results in dysbiosis of the microflora such that obligate anaerobes decrease in number while the presence of facultative anaerobes increases. This, they believe, is due to the fact that inflammation in the host generates reactive nitrogen and reactive oxygen species, molecules that facultative anaerobes can use as final electron acceptors in anaerobic respiration. Thus, they hypothesize that these oxidized by-products, which are a natural component of host inflammation, are utilized by facultative anaerobes in a way that allows them to ultimately overcome fermenting microbes in the gut lumen (2013). Similarly, Rigottier-Gois hypothesizes that the decrease in anaerobes with the subsequent increase in facultative anaerobes or even unusual aerobes may be due to the presence of oxygen in intestinal dysbiosis. He states that in the colonization of infants, there is first the appearance of facultative anaerobes like *Echerichia coli* and *Enterococcus* (2013). Based on supportive evidence from Grutte et al., he believes this is due to the fact that the redox potential immediately after birth is high (or electropositive) and that within a period of a few days, the facultatively anaerobic bacteria consume the available oxygen thus creating an electronegative environment that is hospitable to obligate anaerobes (2013). He thus predicts that dysbiosis in the otherwise healthy intestinal tract leads to a decrease in diversity and a decrease in dominant obligate anaerobes with an increase in facultative or even aerobic bacteria is the result of an increase in oxygen in the gut. This increase in oxygen is selectively advantageous for the facultative anaerobes and the aerobes that then outcompete their oxygen intolerant counterparts. The presence of oxygen in otherwise hypoxic environment, Rigottier-Gois suggests, may be the result of blood entering the

gastrointestinal tract during chronic inflammation and releasing hemoglobin carrying oxygen into the mucosa and lumen where the bacteria reside. Alternatively, the host inflammation itself could lead to both dysbiosis of the microbiota and an influx of reactive oxygen species by neutrophils.

Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is characterized by chronic intestinal inflammation and is mainly diagnosed as either ulcerative colitis (UC) or Crohn's disease (CD) (Frank et al., 2007). Ulcerative colitis, as the name implies, affects the colon while Crohn's disease is marked by inflammation throughout the intestinal tract. While it is not clear what specifically triggers these conditions, a number of susceptibility genes have been identified. However, with the growing incidence of IBD, genetic factors alone cannot explain the increase. For this reason, it is predicted that environmental factors also play a role.

The involvement of the gut microbiota in the pathology of IBD has been highlighted. For example, evidence suggests that a deregulated immune response against commensal bacteria is involved in the pathophysiology of IBD (Becker et al., 2015). For example, it has been shown that end-products produced by commensal microbiota regulate the activation of immune cells and their respective cytokines which protect against pathogenic bacteria and that this regulation is disrupted in patients with IBD . In a study by Kim et al., germ-free IL-10-deficient mice, used because of their lack of both bacterial colonization and the anti-inflammatory cytokine, interleukin-10, were inoculated with *E. coli*, *Enterococcus faecalis*, and *Pseudomonas fluorescens*. *E. coli* induced cecal inflammation, *Enterococcus faecalis* induced distal colitis, and

Pseudomonas fluorescens did not cause cecal inflammation. In this way, the commensal bacteria were able to induce distinct types of colitis in IL-10-deficient mice. This study suggests that even in hosts with the same genetic background, distinct intestinal immune responses can be elicited (2017).

Additionally, patterns in both the emergence and the suppression of certain bacterial families in association with IBD patients versus their healthy counterparts have been documented extensively in the literature. As noted by Hansen and Sartor, in a large study of 447 newly diagnosed pediatric CD patients and 221 non-IBD controls, an increase in *Enterobacteriaceae, Pasteurellaceae, Veillonellaceae, Neisseriaceae, and Fusobacteriaceae* in ileal and rectal biopsies from CD patients vs. controls was observed. Conversely, a decrease in members from *Bifidobacteriaceae*, *Erysipelotrichales, Bacteroidales*, and *Clostridiales* was also observed. It should also be noted that the authors of this study also discovered a marked difference between fecal samples and mucosal samples. In fecal samples there was little bacterial compositional change while significant changes occurred in the tissue biopsies. These findings suggest that perhaps fecal bacterial communities are different from mucosal and play a less critical role in the pathogenesis of CD (2015).

Metagenomic studies have also tracked genes linked to IBD. For example, genes responsible for carbohydrate and amino acid metabolism have been noted to decrease in those with IBD while genes involved in the oxidative stress pathway are increased. This raises the possibility that changes within the gut microbiome that result in certain epigenetic changes could be the cause of intestinal inflammation in IBD patients (Matsuoka et al., 2014).

Use of Antibiotic Therapy

There are a myriad of factors that play into host inflammation: diet, for example, as well as lifestyle, age, and genetics. All of these factors can result in a microbial imbalance that negatively affect the host, causing acute inflammation that, over time, can become chronic. Another potential factor is the use, or overuse, of antibiotic therapies. For example, through the analysis of 8748 patients diagnosed with IBD, Sebastian Sheer et al demonstrate that the use of antibiotics early in life can potentially contribute to inflammatory diseases later in life (2016) while Woldarska et al, using a murine model, show that antibiotics weaken the colonic mucosal layer, thereby predisposing the host to *Citrobacter rodentium*-induced colitis (2009). A study by J. Carlson et al., which utilizes a *Gambusia affinis* model, demonstrates that fish treated with rifampicin experience persistent alterations in their gut microbiome composition. This alteration increases their susceptibility to pathogens and osmotic stress, but not to general high bacterial numbers in the environment or nitrate toxicity (2017). Furthermore, little is known about the ways in which antibiotic use affects the gut microbiome over an extended period of time. Studies have shown, however, that use of antibiotics can result in permanent changes. In a study by Dethlefsen et al., three healthy humans who had not taken antibiotics for at least a year prior to the study underwent treatment with ciprofloxacin (Cp), an antibiotic which is deemed to be relatively benign for the gut microbiota. Using pyrosequencing, the participants had stool samples sequenced before, during, and after treatment. The researchers found that, while the communities among participants remained relatively stable, when comparisons were made across the individuals the relative abundance levels of approximately 30% of the taxa in the gut were affected by Cp treatment. Additionally,

some taxa within a single individual were found to respond to Cp. Of note, while gut function within the communities remained normal, implying functional redundancy among bacteria, the more specialized functions (such as immune regulation) cannot necessarily be implied (2008).

The authors continued this study and published data a few years later. Here, the researchers observed a dramatic shift in community composition 3-4 days post Cp treatment. Upon discontinuation of Cp treatment, the community returned to a state that more closely resembled the pre-treatment state. Surprisingly, this return occurred despite the fact that dominant taxa constituting 25-50% of the community was demolished after exposure to Cp. By the end of the study, community composition differed from what it had been before the first course of antibiotic treatment, but each new state was stable over the final 2 months of the study. Moreover, the communities appeared to display functional redundancy which is perhaps the reason for an absence of negative symptoms experienced by the participants. It should be noted that while this data illuminates potential features of the gut microbiome (such as resilience and functional redundancy) and highlights the intricacies and intravariation between individuals, understanding of this complex ecosystem is limited. In this way, while one individual may not be affected by a particular antibiotic, another may experience community shifts that impact susceptibility to certain pathogens or that result in the expression of genes involved in the oxidative pathway and thus increase the likelihood of inflammatory conditions (2010). .

Current Models of IBD

The most well-established model of IBD is the murine model, primarily the mouse model. Mice have been used to investigate the intricate relationship between the

gut microbiome, immune system, and genetic susceptibility. The type of mouse used depends largely on the investigative question. For example, when investigating the causes of inflammation and their respective pathways, SCID or *RAG1*$^{-/-}$ may be used due to their immune deficiency. Alternatively, when investigating the role of specific cytokines, interleukin deficient mice will be used, such as *IL18-/-* or *IL10 -/-* (Keisler et al., 2015). In this way, the role of immune pathways and the ways in which particular immune cells function can be studied. Due to the similarity of their bacterial composition to humans and their ability to be genetically manipulated, the use of such mice has provided investigators with a robust amount of data and has illuminated aspects of IBD pathogenesis, such as the role of resident microbiota in driving intestinal inflammation, and has led to the discovery of such susceptibility genes as *NOD2* in Crohn's disease (Knights et al., 2013).

The use of these models, while extensive, are not exhaustive and factors such as high cost, imaging limitations, and longer lifespan have led researchers to turn to other models such as fish models. Common fish models include the rainbow trout, common carp, zebrafish, and poecilia. The GI tracts of these models are predominantly comprised of *Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes* and *Fusobacterium* phyla. However, diet and environment play highly influential roles in the composition of the microbiota in fish species. For example, marine fish are dominated by facultative anaerobes including *Vibrio, Pseudomonas, Acinetobacter, Corynbacterium, Alteromonas, Flavobacterium*, and *Micrococcus* while freshwater fish are dominated by *Aeromonas, Pseudomonas,* and *Bacteroides* (Egerton et al., 2018).

In fish, bacterial colonization during the larval stage is significantly impacted by the microbiota of the eggs, water, and feed and a stable indigenous microbiota is established by the post-larval stage. The temperature and salinity of water are two main factors impacting the intestinal microbiota of fish as these two factors affect the relative abundance and type of bacteria present in the water. Additionally, and similarly to humans and mice, diet and the feeding habits also impact the gut microbiota of fish. For example, whether fish feed is plant or fish oil based impacts the dominant phyla present[19]. Moreover, whether fish are omnivores, carnivores, or herbivores also affects the microbiota present, a trend that has been observed in humans(Egerton et al., 2018).. While many fish models are currently being utilized for antibiotic investigation on the gut microbiome, the research is often directed toward fish farming and practices. The use of zebrafish, on the other hand, is showing promise in the realm of IBD research. This is perhaps because zebrafish are easy to cultivate, have a generation time of about 3-4 months, have a gastrointestinal tract that is markedly similar to those of humans, and show orthologs for over 70% of human genes (Hanyang et al., 2017). .

Zebrafish models of IBD include three types: wild type, mutant, and transgenic. In this way, zebrafish can be manipulated in a similar way to murine models. Moreover, zebrafish larvae have been used for imaging purposes due to their transparency. For example, in a study by Renshaw, transgenic zebrafish larvae that expressed GFP under the neutrophil-specific myeloperoxidase promoter was used to visualize and analyze the inflammatory response after tissue injury (2006). In this way, observations were made without the need to kill the fish, which is often necessary with murine models as pathological changes cannot be determined by observation

Currently, *Gambusia affinis* has not been established as a definitive model for investigating IBD. We propose that *Gambusia affinis,* because it has the necessary components of a vertebrate immune system, may be a viable model for the investigation of gut microbial composition as it relates to inflammation and the use of antibiotics. Additionally, *Poecilia* fish (the family to which *Gambusia affinis* belong) are an interesting model for microbiome studies as they are highly adaptable, capable of living on a wide range of diets and under a wide range of environmental conditions, including that of the laboratory environment (Schmidt et al., 2017).

In order to study inflammation in this model, dextran sulfate sodium (DSS) will be used to induce clinical-grade colitis in the animals. A theory by which DSS induces inflammation is that damage to the colonic epithelium results in the invasion of luminal bacteria and their respective antigens into the mucosa which result in proinflammatory responses (Eichele et al., 2017). More specifically, it has been observed that dextran sodium sulfate molecules link with medium-chain-length fatty acids (MCFAs) that are present in the colonic lumen and that these complexes in particular are what activate intestinal inflammatory signaling pathways (Laroui et al., 2012). DSS has been used in numerous studies as an effective way to research inflammatory bowel diseases in animal models, including mice, rats, and zebrafish. Thus, we predict that inflammation induced by DSS will affect the gut microbiome of the *Gambusia affinis* similarly to treatment with rifampicin, a broad spectrum antibiotic, in terms of shifts within bacterial communities and types of bacterial communities present. In this way, we hope to demonstrate that use of antibiotics disrupts the host gut microbiome in a way that can ultimately induce acute inflammation.

Inflammation itself will be gauged using a well-established myeloperoxidase assay. Myeloperoxidase (MPO) is a heme-containing peroxidase expressed mainly in neutrophils in response to tissue damage and microbial invasion. In animal and human studies, MPO is used as a biomarker to measure the amount of inflammation present in tissue as MPO levels often correlate with the number of neutrophils present (Bradley et al., 1982). . For example, Kim et al describe treating a murine model with DSS and measuring the severity of the induced acute colitis using an MPO assay, as an increase in MPO is often correlated with the extent of tissue damage (Kim et al., 2012). Additionally, zebrafish have gained popularity as a reliable model in which to study inflammation. For example, Pase L et al describe a mechanism of healing following tissue injury that involves the downregulation of hydrogen peroxide by myeloperoxidase using wild type, leukocyte-depleted, and myeloperoxidase-deficient zebrafish models (2018). Thus, DSS and the MPO assay are often used as inexpensive and reliable methods to induce and quantify inflammation and investigate such aspects of inflammatory bowel disease as disease progression, relationship to genetic factors, and impact on the gut microbiome. It should be noted, however, that DSS has only been used in zebrafish larvae in order to study mucus production (Fenero et al, 2016). To date, no study has used a DSS fish model to study changes in the gut microbiome.

Hypotheses

Using the *Gambusia affinis* fish model, this study aims to demonstrate the following:

1) Inducing inflammation via DSS in the gut of the *Gambusia affinis* will result in changes in the community composition of the microbiome and specifically raise the abundance of facultative anaerobes, presumably due to the presence of nitrate.

2) Treating *Gambusia affinis* with rifampicin will result in measurable inflammation in the gut tissue, and an emergence of facultative anaerobes, like *Aeromonadaceae*.

CHAPTER II

MATERIALS & METHODS

Part 1. Materials, Equipment, & Techniques

Chemicals

Solutions were prepared in the lab and autoclaved using standard procedures when necessary. Suspensions of antibiotic or biocide compounds were not sterilized when in pure DMSO (dimethyl sulfoxide).

Media

All media for plating were prepared in the lab and autoclaved using standard procedures. Media containing heat-sensitive components, such as dextran sodium sulfate, were heated first and then had the substance added after a brief cooling period of 2-5 minutes for 1.5 mL of solution. All empty petri dishes used were purchased from VWR, sterilized upon purchase, and remained sealed until use.

Equipment, Miscellaneous Items

All equipment was sterilized as necessary using a glassware oven (90°C for >2 days), and items such as alcohol wipes remained sealed until time of use where they were only opened in the BioSafety Cabinet (BSC). All results requiring opening of plates were also performed in the BSC. Gloves were sterilized with 70% ethanol before use. [1]

Part 2. Media & Solutions.

Artificial Pond Water (APW)

APW was made using 3 L of sterile MilliQ water, 0.333 g calcium chloride, 0.333 g magnesium sulfate, and 0.119 g sodium acetate. Ingredients were placed into a large glass jar and autoclaved with a 30-minute sterilization time. APW was used as sterile water in the experiment outlined below and is designed to mimic salt content in natural systems.

Preparation of Phosphate-Buffered Saline with Tween 80% (PBST)

PBST was prepared by adding fibe PBS (phosphate-buffered saline) tablets (Item # E404-100TABS, Amresco©) and 250 µL Tween 80 to 500 mL of MilliQ water. Final concentrations of solutes in PBST solution were 2 mM NaCl, 10 mM KCl, and 137 mM PO_4^{3-} resulting in pH 7.4. The solution was autoclaved at 121°C under pressure with a sterilization time of 15 minutes.

Preparation of Sterile MilliQ Water

Water filtered through a MilliPore system (look up manufacturer) was collected and autoclaved 0.5 L at a time with 15-minute sterilization time, using standard procedures.

Nutrient Agar (NA) Plates

NA was prepared by adding 2.5 g peptone, 1.5 g beef extract, and 7.5 g of agar to 500 mL MilliQ water. Once combined, the solution was placed on a stir plate to ensure mixing and then autoclaved at 121°C for 15 minutes. The solution was removed from the autoclave and allowed to cool on a stir plate for a minimum of 10 minutes before pouring

into sterile petri dishes. Once poured, NA plates were allowed to cool and harden before being sealed and stored at 4°C until use.

Nitrate Agar Plates

Nitrate agar was prepared by adding 9 g of pre-prepared nitrate broth powder and 12 g of agar to 1000 mL of distilled water. Once combined, the solution was placed on a stir plate to ensure mixing and then autoclaved at 121°C for 15 minutes. The solution was removed from the autoclave and allowed to cool on a stir plate for a minimum of 10 minutes before pouring into sterile petri dishes. Once poured, nitrate agar plates were allowed to cool and harden before inoculation conducted in the BioSafety Cabinet (BSC).

Part 3. Sampling & Analysis.

Section 1. Specimen Collection & Maintenance.

Specimen Collection. The fish used in this study, *Gambusia affinis* (*G. affinis*), were collected from the Woodland Hills Lake throughout the length of this study using dip nets. Woodland Hills Lake was chosen due to previous studies also using fish from this location. The lake is pristine and in a neighborhood located in Walker County outside the Huntsville city limits at 30 degrees 49 minutes 23 seconds North by 95 degrees 32 minutes 34 seconds West (derived from Google Maps on 2/10/2019). All fish collected were brought back to the lab in the Lee Drain Building (LDB) room 125A (2017-2018) and the Life Sciences Building (LSB) room 370 (2018-2019) at Sam Houston State University (SHSU), acclimated in the buckets in which they were collected for 3 days, and then placed in aquariums and given a minimum of one week for their microbial flora to homogenize and stabilize. Fish were fed store-purchased fish flakes on an average of 4-5 times per week. All experiments were carried out after the minimum homogenization

time period passed. Aquaria were initially filled with 50% lake water and 50% tap water and kept on a 12-hour light/12-hour dark cycle. The room temperature varied from 21-23°C.

Location. All experiments were performed at SHSU in either LDB 125A or LSB 370 with IACUC approval (ID# 18-10-16-1018-3-01). 16S DNA profiling sequencing was performed by MR DNA in Shallowater, Texas. Data analysis occurred at SHSU.

Fish Handling. Fish were removed from aquaria, buckets, containers, etc. using small fish nets. When handled, fish were grabbed gently but firmly by the tail through the net with gloved fingers for direct removal from the net. All efforts were made to reduce the amount of stress experienced by the fish by handling the fish quickly and efficiently.

Section 2. Acquiring Microbiome Samples.

Gut Microbiome Sampling. To sample the gut microflora of *G. affinis*, fish were individually removed from the aquarium or experiment container using a small net and carefully placed into a sterile petri dish to be weighed. Once weighed, fish were removed from the petri dish, held between the thumb and forefinger of one hand and gently cleaned with an alcohol wipe using the other hand. Once wiped, fish were placed into a new, sterile petri dish. All tools were cleaned with alcohol wipes. Using small forceps and dissecting scissors, the spinal cord was severed between the forward fins and the eyes. Once the spinal cord was severed in this manner, the tools were once again cleaned with alcohol wipes. Once cleaned, the gut was located and removed by cutting the intestines at the esophagus and anus. The gut was then cut into small pieces and placed into either a 1.5 mL centrifuge tube containing HTAB or PBST, depending on the experiment to be carried out.

*Each sample equals one fish. Once a fish was sampled, it was removed from the associated experiment completely. In this way, each fish was only sampled once.

Section 3. Quantitation of Microflora.

***Colony Forming Units (CFU) – Plating*.** Fish intestines were excised, cut into pieces, and placed into a 1.5 mL centrifuge tube containing 1000 µL PBST. The tube was then vortexed for 2 minutes after which a 100 µL aliquot was plated onto nitrate and nutrient agars. Once completed, 100 µL was removed again and placed into a 1.5 mL centrifuge tube containing 900 µL of PBST. In this way, 1:10 serial dilutions were performed with the highest dilution plated being 10^{-3}. The suspensions plated were spread with sterile spreaders, beginning with the most dilute sample to the 1X sample. Plates were then incubated in either aerobic or anaerobic conditions (designation was carefully labeled prior to plating) at room temperature (25°C) for a minimum of 48 hours. After a minimum of 48 hours, all plates were observed for colony counts.

*Because the samples plated were whole-community samples, pure colony counts were not possible; various morphologies were considered to be a single colony.

Section 4. 16S rRNA Sequencing.

Samples for 16S DNA Extraction using MoBio PowerSoil DNA Isolation Kit. Samples for DNA extraction were removed from freezer and thawed on ice. After thawing, 0.010 g – 0.020 g of the sample was added to a MoBio PowerBead tube and vortexed approximately 5 – 15 minutes until the sample was homogenized. Samples were then incubated for 10 minutes at 65°C and then 10 minutes at 95°C in a heat block. Next, tubes were incubated at -80°C overnight. The sample tube was removed from the -80°C freezer and thawed at room temperature for 30 – 60 minutes. After thawing, 60 µL of

solution C1 was added and the tube was vortexed for five seconds. The tube was then vortexed at maximum speed for 10 minutes, and 250 µL of solution C2 was added to a clean Collection Tube. The PowerBead tubes were removed from the vortexer and centrifuged at 10,000 x g for 30 seconds at room temperature. The PowerBead tubes were removed from the centrifuge and the supernatant was transferred to the Collection Tube containing solution C2. The Collection Tube was vortexed for five seconds and incubated at 4°C for five minutes. Next, 200 µL of solution C3 was added to a clean 2 mL Collection Tube. The incubated Collection tubes were removed from the refrigerator and centrifuged for one minute at 10,000 x g. From the centrifuged sample in the first collection tube, 600-650 µL was transferred to the second Collection Tube containing solution C3; the tube was vortexed for five seconds and then incubated at 4°C for five minutes. After incubation, samples were removed from the refrigerator and centrifuged at 10,000 x g for two minutes at room temperature. From the centrifuged sample, a maximum of 750 µL was transferred to a third clean Collection Tube already containing 1 mL of solution C4 and vortexed. Next, 600 – 630 µL of vortexed sample was loaded onto a Spin Filter and centrifuged at 10,000 x g for one minute at room temperature; flow through was discarded and this process was repeated for a total of three times to process the entire sample. Then, 500 µL of solution C5 was added to the Spin Filter and centrifuged at 10,000 x g for 30 seconds at room temperature; flow through was discarded. The Spin Filter was centrifuged again at 10,000 x g for one minute at room temperature. The Spin Filter was then placed into a clean Collection Tube and 55 µL of sterile DNA-Free Molecular Biology Grade water added to the center of the white filter membrane. The tube was centrifuged at 10,000 x g for 30 seconds, the filter was

discarded, and the supernatant was transferred to the Stock-DNA tube. Extraction DNA concentrations were measured and stored at -80°C.

Samples were then mailed to MR DNA in Stillwater Texas.

PCR Amplification and Processing. The primers 515 and 806 were used to amplify the V4 variable region of the 16S rRNA gene in a single-step 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 30 cycles (5 cycle used on PCR products) of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on an Ion Torrent PGM following the manufacturer's guidelines. Sequence data were processed using a proprietary analysis pipeline (MR DNA, Shallowater, TX, USA). In summary, sequences were depleted of barcodes

and primers, then sequences <150bp removed, sequences with ambiguous base calls and with homopolymer runs exceeding 6bp were also removed. Operational taxonomic units (OTUs) were generated and chimeras removed. Operational taxonomic units were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTn against a database derived from RDPII (http://rdp.cme.msu.edu) and NCBI (www.ncbi.nlm.nih.gov).

Part 4. Protocol Development

The experiments listed here are those that were performed to determine the best variables for experimental success such as toxicity levels of chemicals and appropriate treatment conditions.

Section 1. Inflammation.

Preparation of Dextran Sulfate Sodium (DSS) Food Mixture.

To make the modified gelatin feed, fish flakes were crushed into fine power (must be fine so as to avoid clogging the pipette tips) and kept in a sealed container under dry room temperature conditions. Next, 52 mg of gelatin was added to 360 µL of MilliQ water in a 2 mL microcentrifuge tube and placed on a heat block at a temperature range of 60°C – 80°C to fully dissolve. Once the gelatin was dissolved, the mixture was vortexed for approximately 10 seconds. Following thorough mixing, 40 mg of crushed fish food was added along with 20 µL fish oil and vortexed for another 10 seconds to ensure equal distribution. This mixture constitutes the gelatin feed for the control fish. For the experimental fish, the addition of 20 mg dextran sulfate sodium (DSS) was added and mixed well. A P200 pipette was used to make 20ul aliquots of this mixture pipetted onto Parafilm for easy handling. The gelatin feed was then allowed to solidify and served in quarters by cutting with a razor blade (1 serving=4 pieces).

Dextran Sulfate Sodium (DSS) Experimental Group. (*#1*) For the first pre-trial, 3 control fish and 6 experimental fish were used. Fish were individually removed from the aquarium, weighed, and placed into Styrofoam cups filled with 10 mL artificial pond water (APW). Once placed into their individual cups, fish were fed food combined with gelatin for both easy administration and so that the amount eaten by each fish could be tracked: control fish received food containing only fish flakes and fish oil while experimental fish received fish flakes, fish oil, and 2 mg dextran sulfate sodium (DSS). The amount eaten was then recorded every hour for a minimum of 5 hours. 12 hours

post-ingestion, all of the fish were sacrificed in order to obtain myeloperoxidase (MPO) readings and assess level of success at inducing inflammation.

(*#2*) For the second pre-trial, the experiment was carried out exactly as described in the first pre-trial, however, this time the fish were starved for 3 days to ensure hunger. Additionally, the amount of dextran sulfate sodium was increased to 6 mg. The decision to increase the amount of dextran was made due to the apparent success at inducing inflammation in the experimental fish in the first pre-trial. It was also important to test toxicity levels. Again, MPO measurements were recorded.

(*#3*) For the third pre-trial, the experiment was carried out exactly as the first and second, only this time 4 control and 4 experimental fish were used. Additionally, the amount of dextran added to the fish food was increased to 20 mg. The decision to increase the level of dextran to more than three times the amount in the second pre-trial was to, once again, ascertain toxicity levels. As dextran has not yet been studied in *Gambusia affinis*, it was necessary to gauge the amount that the fish could safely ingest. MPO readings were recorded.

Myeloperoxidase (MPO) Assay. The Myeloperoxidase assay is a marker for neutrophils. The MPO is present in neutrophils as they carry out antimicrobial functions. A widely recognized marker of inflammation in a tissue is infiltration by neutrophils. Thus, measuring MPO activity in the gut is a reliable quantitative measure for inflammation. The myeloperoxidase assay functions by the presence of MPO which catalyzes the oxidation of o-dianisidine, a commonly used peroxidase substrate, to generate an orange product which is measured by an increase in absorbance at 450 nm. The colonic pieces were weighed then homogenized in

0.5%hexadecyltrimethylammonium bromide in 50 mM PBS. Samples were placed in an ice bath and bead beaten at maximum capacity for 2 minutes after which they were placed back in the ice bath for 5 minutes. This was repeated 3-5 times until tissue samples were thoroughly homogenized. Following the last beating, samples were centrifuged at 1400 for 3 minutes. MPO activity was measured by adding 10 µL supernatant to a cuvette containing 326 µL phosphate buffered saline (PBS), 67 µL o-dianisidine dihydrochloride (o-danisidine), and 6.7 hydrogen peroxide (H_2O_2). The optical density was measured at 450 nm.

The cuvette was first filled with 426 µL PBS and the spectrophotometer was calibrated. Next, 100 µL of PBS was removed and 67 µL of o-dan was added. A measurement was recorded. Next, 10 µL of lysate was added and a reading recorded. Finally, 6.7 µL of H_2O_2 was added and a reading was recorded from 0 – 120 seconds.

MPO activity is measured in MPO U/mg tissue where one unit of MPO is defined as the amount needed to degrade 1 µmoL of H_2O_2 per minute at room temperature. Considering that one unit (U) of MPO= 1 µmoL of H_2O_2 split and that 1 µmoL of H_2O_2 gives a change of absorbance of 1.13×10^{-2} nm/min, units of MPO in each sample were determined as the change in absorbance $[\Delta A(t2\text{-}t1)]/\Delta min \times (1.13 \times 10^{-2})$. To get units per mg of tissue, a tissue: buffer ratio was utilized. For example, if a tissue: buffer ratio of 7 mg/mL was used, in 10 µL of lysate, there is 0.07 mg of tissue. Therefore, to get units per mg tissue, divide the units of MPO by 0.07.

Nitrate Assay. Another recognized marker of inflammation is the nitrate anion (NO^-_3). To determine proper measurements of reagents, several trials were conducted. First, a standard solution was made containing 6.79 mg sodium nitrate (BA code 2263) in

6790 µL MilliQ. Next, the reagent was made using 29.3 mg diphenylamine, 7032 µL sulfuric acid, and 1758 µL MilliQ. Sample 1 utilized 100 µL of the standard solution, followed by the addition of 450 µL reagent, 425 µL sulfuric acid, and 90 µL MilliQ. Readings were recorded at 597 nm and determined to be much too high (approximately 5 abs). Sample 2 was conducted in exactly the same manner, however, only 10 µL of the standard were added to the mixture. In this case, absorbance was still too great at 2.7. Therefore, 4 tenfold dilutions were made. In this way, Sample 3 contained 100 µL of a 10^{-2} dilution. Here, it was observed that lower concentrations of the standard require longer times to react (0.085 at 10 minutes, 0.13 at 20 minutes, 0.14 at 25 minutes).

It was unclear how the reaction was affected by MilliQ quantities, so for samples 4-7, varying amounts of MilliQ were used. In Sample 4, 25 µL of the standard were added to 450 µL of reagent, 425 µL of sulfuric acid, and 75 µL of MilliQ which resulted in an absorbance reading of 4.3. For Sample 5, 2.5 µL of the standard were added to the same quantities of reagent and sulfuric acid, however, 97.5 MilliQ was added. This resulted in an absorbance reading of 0.64, a reading similar to that noted in the literature.

It was also necessary to ascertain ideal sample volumes. For samples 1-5, sample volumes ranged between 100 – 190 µL. For Sample 6, a sample volume of 400 µL was measured which resulted in an absorbance reading of 0.02. In this way, it was determined that the sample volume should not be changed to greater than 200 µL. As a final consideration, Sample 7 contained 2.5 µL of the standard, 450 µL of the reagent, 325 µL of sulfuric acid, and 197.5 µL of MilliQ. While this sample volume was double that of Sample 5, it nevertheless resulted in similar absorbance readings (0.61 at 4 minutes and 0.71 at 5 minutes). Thus, after numerous trials, it was determined that the standard is best

used in smaller amounts (between 2.5-10 µL), the reaction takes time and can vary between samples, and sample volumes are best kept at 200 µL.

**Upon application of this assay on samples from the experiment, it was determined that 10 µL of a 10^{-2} dilution of sample added to 450 µL reagent, 325 µL sulfuric acid, and 190 µL MilliQ resulted in absorbance readings that remained consistent across samples.*

Once absorbances for each sample were recorded, a standard curve was made and the stable absorbance for each was plotted against the equation obtained from the standard curve in order to obtain the concentration of nitrate anion. The resulting concentration was then divided by the weight of the fish, µg/g.

Histological Assessment of Inflammation. Gut samples utilized for histological evaluation were excised from the control and treatment fish on Days 1, 3, and 7. Samples were spun into tight coils and placed in 2 mL microcentrifuge tubes containing 10% neutral buffered formalin made on 3-17-2015. The tubes were meticulously sealed with parafilm and placed in a small Ziplock bag that was mailed to Dr. Kendra Rumbaugh in the Department of Surgery at Texas Tech University Health Sciences Center.

The slides and staining were done through an automated process in the in-house pathology lab at Texas Tech University Health Sciences Center. The staining followed a basic Haemotoxylin and Eosin (H&E) protocol. First, sections were deparaffinized and Haemotoxylin was added for 4 minutes and then dipped in Acetic H_2O. Then samples were placed into H2O for 1 minute and again for 30 seconds followed by 1% ammonia for 3 seconds, H2O for 40 seconds, 95% ethanol for 10 seconds, and Eosin for 1 minute, followed by quick dehydration of slides in 95% ethanol and absolute ethanol twice for 10

seconds and again for 20 seconds. Lastly, the slides are cleared in several changes of xylene at 30 seconds. Every step is conducted at 65ºC.

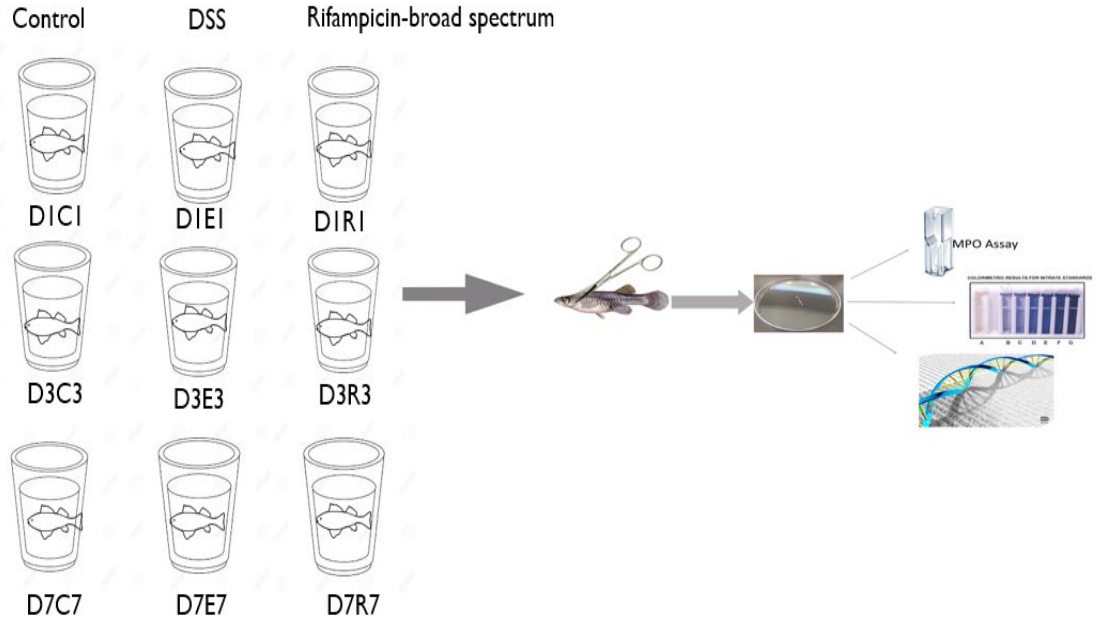
Once slides were stained, Dr. Derek Fleming, post-doctoral research associate under Dr. Rumbaugh at Texas Tech University Health Sciences Center, analyzed the slides with no prior knowledge of which samples the sections represented so that he would not run the risk of subconscious bias. Impressions were documented and sent to our laboratory for further analysis.

Section 2. Antibiotic Treatment.

Rifampicin Experimental Group. Based on previous studies conducted in the laboratory, an established protocol was used to treat fish with rifampicin. A rifampicin stock solution was prepared by adding powdered rifampicin to room temperature dimethyl sulfoxide (DMSO) and vortexed to create a 50 mg/mL stock solution. 500 µL of the stock solution was added per liter of APW (25 µg/mL final concentration). It was calculated that this amount of the antibiotic equates to approximately 25 mg/kg dose while dosages recommended to humans are 10 – 20 mg/kg. In this way, this is a high-dose model.

Fish were placed into individual Styrofoam cups filled with 100 mL of the 25 µg/kg solution. Fish were fed the same feed as the control group and given 24 hours to eat. The following day, the rifampicin treated APW was discarded and replaced with clean APW. In this manner, rifampicin treated fish were only exposed to the antibiotic for 24 hours.

Schematic of Experimental Design:



As illustrated, fish are placed into individualized Styrofoam cups. “D” is for days, “C” is for control, “E” is for DSS-treated group, and “R” is for rifampicin antibiotic-treated group. The timing of 1, 3, and 7 days after treatment was selected because inflammation takes some time to appear and so it is important to capture prior, during, and after inflammation.

CHAPTER III

RESULTS

Part 1. Protocol Development.

DSS and MPO levels. The first dextran sulfate sodium pre-trial revealed that samples taken from fish treated with very low levels of DSS (2 mg) result in absorbance readings that are both inconsistent and too similar to samples taken from control fish (Tables 1a and 1b).

Table 1

Absorbance readings: control vs dextran sodium sulfate treated fish

Control fish (MPO absorbance readings)				Dextran Sulfate Sodium fish (MPO absorbance readings)					
C1	C2	C3		E1	E3	E4	E5	E6	
0.0933	0.0465	0.0161		0.0276	0.0202	0.0237	0.018	0.0467	
0.0838	0.0444	0.0249		0.0318	0.0215	0.0251	0.0198	0.0714	
0.0991	0.0486	0.0347		0.0334	0.0302	0.0290	0.0218	0.0793	
0.1152	0.0544	0.0458		0.0372	0.0349	0.0339	0.0238	0.0898	
0.1433	0.0604	0.0312		0.0416	0.0134	0.0396	0.0256	0.1020	

Note: Myeloperoxidase absorbance readings taken every 30 seconds from 0-120 seconds at 450 nm for control (non-DSS treated) fish and experimental (DSS-treated) fish. E2 not included due to loss of sample.

Table 2

Calculated MPO units and units/gram of control vs dextran sodium sulfate treated fish

Control fish/DSS fish	MPO U	MPO U/g
C1	2.212	2.6
C2	0.615	1.1
C3	0.668	1
E1	0.619	0.6
E3	0	0
E4	0.704	0.8
E5	0.336	0.8
E6	2.447	4.7

Note: Myeloperoxidase activity is measured in units (U) of MPO/g tissue. Numbers are then compared across samples to determine level of inflammation. A "0" indicates a lack of enzyme activity

A second pre-trial, however, revealed that the addition of 6 mg DSS to gelatin fish food resulted in increased absorbance readings (and thus enzyme activity) as well as more consistent readings overall. Here, the control fish samples consistently showed little MPO enzyme activity while the DSS treated fish samples suggested MPO was increased in the experimental group (Tables 2a and 2b).

Table 3

Absorbance readings: control vs dextran sodium sulfate treated fish

Control fish (MPO absorbance readings)				Dextran Sulfate Sodium fish (MPO absorbance readings)			
C1	C2	C3	C4	E1	E2	E3	E4
0.1776	0.1745	0.0513	0.0344	0.0734	0.0579	0.0950	0.0444
0.1644	0.1745	0.0663	0.0384	0.0827	0.0599	0.0950	0.0467
0.1460	0.1439	0.0747	0.0328	0.0927	0.0627	0.1082	0.0461
0.1321	0.1417	0.0873	0.0322	0.0898	0.0673	0.1083	0.0499
0.1261	0.1420	0.0968	0.0287	0.0942	0.0727	0.109	0.0535

*Note:*Myeloperoxidase absorbance readings taken every 30 seconds from 0-120 seconds at 450 nm for control (non-DSS treated) fish and experimental (DSS-treated) fish.

Table 4

Calculated MPO units and units/gram of control vs dextran sodium sulfate treated fish

Control(C)Fish/ DSS(E)Fish	MPO U	MPO U/g
C1	0	0
C2	-0	00
C3	2.013	6.3
C4	0	0
E1	.920	1
E2	.655	1.2
E3	.619	.8
E4	.402	1.2

Note: Myeloperoxidase activity is measured in units (U) of MPO/g tissue. Numbers are then compared across samples to determine level of inflammation. A "0" indicates a lack of enzyme activity.

Based on the results from the second pre-trial, DSS was increased further to 20 mg in an attempt to discover whether larger amounts of this colitogen would result in increased levels of MPO, and thus inflammatory infiltrates of neutrophils within the gut of the fish. It was also not known whether the addition of this amount of DSS would cause lethality

in the fish.

Results from the third pre-trial incorporating 20 mg DSS indicated that the fish could not only survive this dosage, but that in some cases it was sufficient to raise MPO levels slightly above those recorded at 6 mg dosage (Tables 3a and 3b).

Table 5

Absorbance readings: control vs dextran sodium sulfate treated fish

Control fish (MPO absorbance readings)				Dextran Sulfate Sodium fish (MPO absorbance readings)			
C1	C2	C3	C4	E1	E2	E3	E4
0.0595	0.1423	0.1874	0.0659	0.0539	0.047	0.0396	0.0393
0.0566	0.1379	0.1726	0.0588	0.0628	0.0556	0.0485	0.0335
0.0526	0.1288	0.1797	0.0586	0.058	0.0554	0.0519	0.033
0.0538	0.117	0.186	0.0579	0.0649	0.0609	0.0531	0.0367
0.0559	0.1125	0.1789	0.0591	0.0779	0.0647	0.0528	0.0402

Note: Myeloperoxidase absorbance readings taken every 30 seconds from 0-120 seconds at 450 nm for control (non-DSS treated) fish and experimental (DSS-treated) fish

Table 6

Calculated MPO units and units/gram of control vs dextran sodium sulfate treated fish

Control(C)Fish/ DSS(E)Fish	MPO U	MPO U/g
C1	0	0
C2	0	0
C3	0	0
C4	0	0
E1	1.062	1.4
E2	.783	1.7
E3	.584	.8
E4	.040	.1

Note: Myeloperoxidase activity is measured in units (U) of MPO/g tissue. Numbers are then compared across samples to determine level of inflammation. A “0” indicates a lack of activity.

Table 7

Statistics for MPO pre-trial assays. Pre-trial 1

	control	*DSS*
Mean	1.5666667	1.38
Variance	0.8033333	3.552
Observations	3	5
Hypothesized Mean Difference	0	
df	6	
t Stat		
P(T<=t) two-tail	0.8565208	
t Critical two-tail	2.4469119	

Note: When comparing the means of the control versus the DSS-treated fish in the first pre-trial with 2 mg DSS, there is no statistical difference between the three control fish and the five DSS-treated fish.

Table 8

Pre-trial 2

	control	*DSS*
Mean	1.575	1.05
Variance	9.9225	0.036666667
Observations	4	4
Hypothesized Mean Difference	0	
df	3	
t Stat	0.332719151	
P(T<=t) two-tail	0.761240246	
t Critical two-tail	3.182446305	

Note: When comparing the means of the control versus the DSS treated fish in the second pre-trial with 6 mg DSS, there is no statistical difference between the four control fish vs the four DSS treated fish.

Table 9

Pre-Trial 3

	control	*DSS*
Mean	0	1
Variance	0	0.5
Observations	4	4
Hypothesized Mean Difference	0	
df	3	
t Stat	-2.828427125	
P(T<=t) two-tail	0.066275603	
t Critical two-tail	3.182446305	

Note: When comparing the means of the control versus the DSS treated fish in the third pre-trial with 20 mg DSS, there is no statistical difference the four control vs the four DSS treated fish.

Nitrate. Based on Winters *et al.* hypothesis that host derived nitrate increases the growth of facultative anaerobes in the inflamed gut, it was necessary to test nitrate levels within the gut of the sample fish. However, before assessment of the experimental samples could be conducted, standards were made and measured using an established nitrate assay.

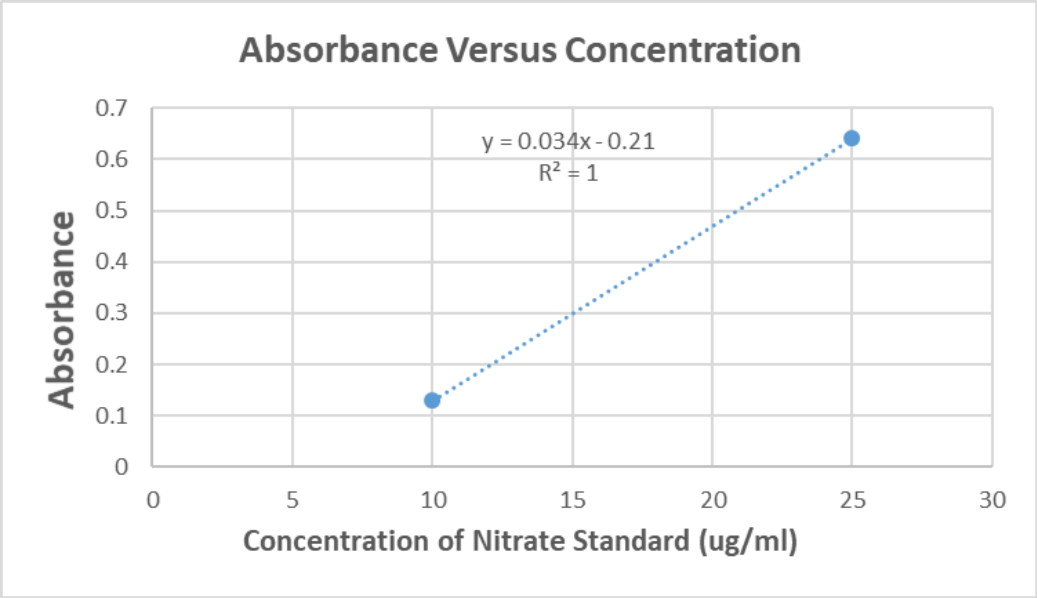


Figure 1. Example of standard curve constructed for successful nitrate anion assays. Sample concentration as micrograms per milliliter with absorbances: 10/.13 and 25/.64. The line having y-axis intercept of 0.034 with $r^2 = 1$.

Part 2. Experimental Results.

DSS and MPO levels. MPO levels in the fish, as indicated by the MPO assay, were inconsistent. The Day 1 and, most notably, the Day 3 control fish revealed higher levels of inflammation than the DSS treated fish. While the Day 7 fish followed more expected patterns across samples, the MPO levels in the DSS treated fish were not markedly pronounced as compared to the control fish. This is suggestive of variables and confounding factors likely inherent in the fish that may play roles in encouraging or attenuating inflammation and that ultimately may interfere with consistent and predictable MPO levels within the *Gambusia*.

Table 10

Calculated MPO units/gram for Experimental Run Day 1

Day 1	MPO U/g
Control	1.46
DSS	2.39
Rif	0.49

Note: Absorbances not shown. MPO activity is measured in units/g tissue. The change in absorbance is divided by time and then divided by 1.13×10^{-2} nm/min (the change of absorbance in 1 µmoL of H_2O_2) and divided by respective tissue to buffer ratio. (based on the tissue to buffer ratio).

Table 11

Calculated MPO units/gram for Experimental Run Day 2

Day 3	MPO U/g
Control	1.02
DSS	0
Rif	0

Table 12

Calculated MPO units/gram for Experimental Run Day 3

Day 7	MPO U/g
Control	2.17
DSS	2.48
Rif	1.01

Nitrate. Based on absorbance values from the standards, it was determined that 10 ul of sample would be necessary and sufficient to run nitrate assays.

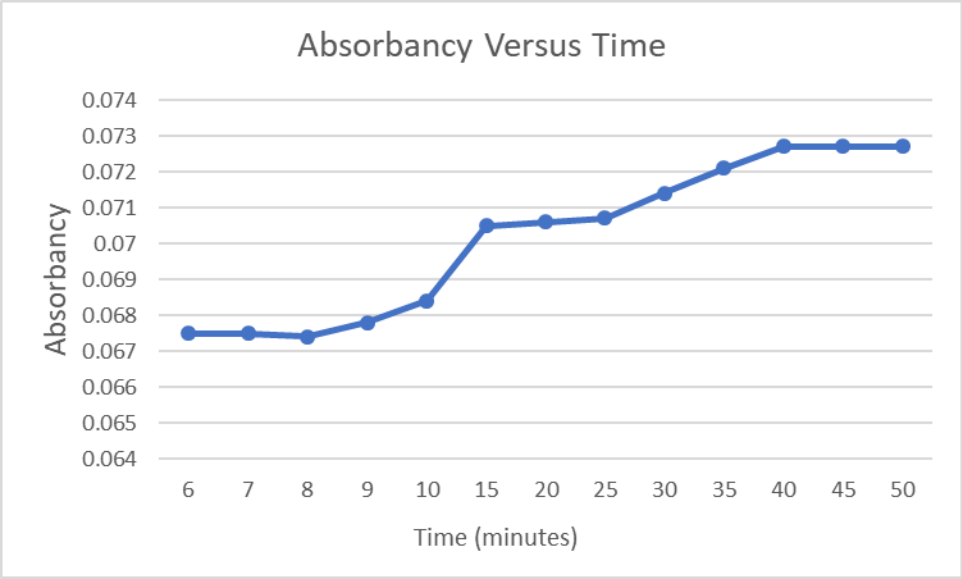


Figure 2. Absorbency versus time in day 1 control sample. Readings started after 5 minutes of reaction time and were read at 597 nm every 1-5 minutes until stabilization occurred. Stable absorbance number was 0.0728.

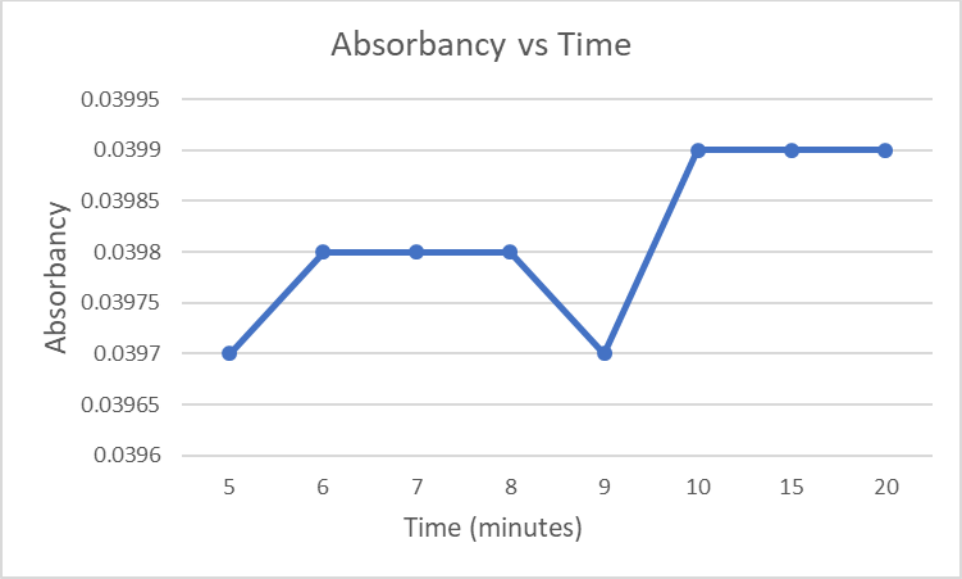


Figure 3. Absorbency versus time in day 1 DSS-treated sample. Readings started after 5 minutes of reaction time and were read at 597 nm every 1-5 minutes until stabilization occurred. Stable absorbance number was 0.0399.

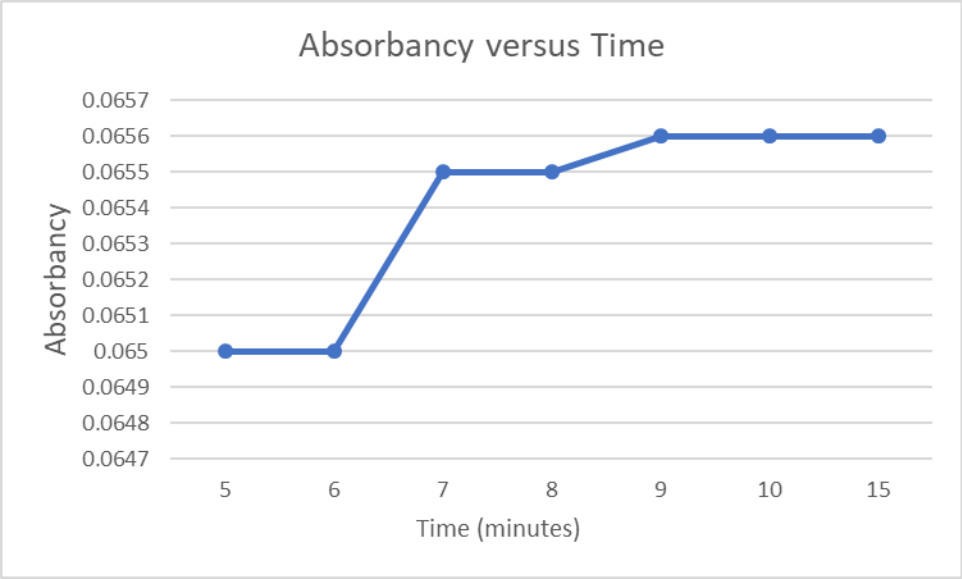


Figure 4. Absorbency versus time of day 1 antibiotic-treated sample. Readings started after 5 minutes reaction time and taken every 1-5 minutes until stabilization occurred. Stable absorbance number was 0.0656.

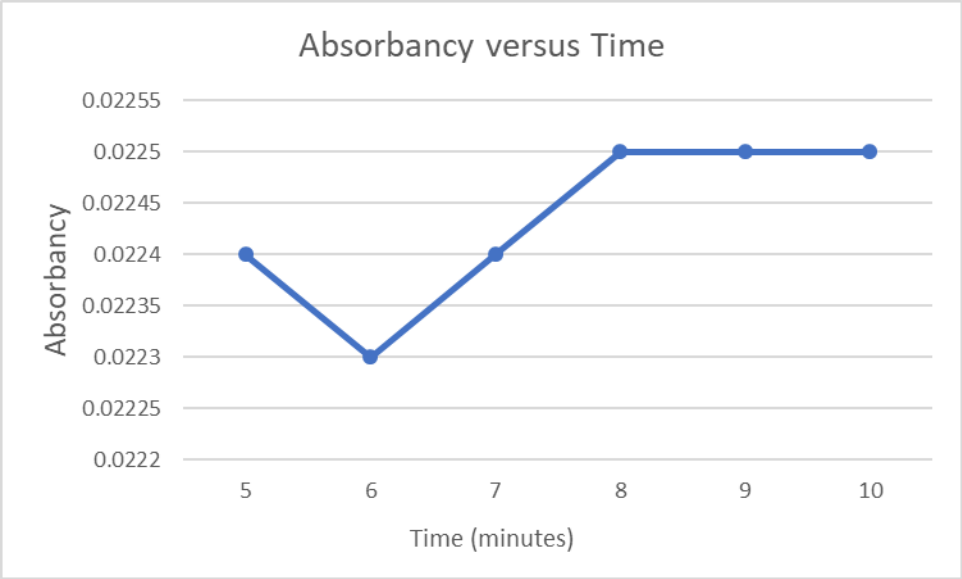


Figure 5. Absorbency versus time of day 3 control sample. Readings started after 5 minutes reaction time and taken every 1-5 minutes until stabilization occurred. Stable absorbance number was 0.0225.

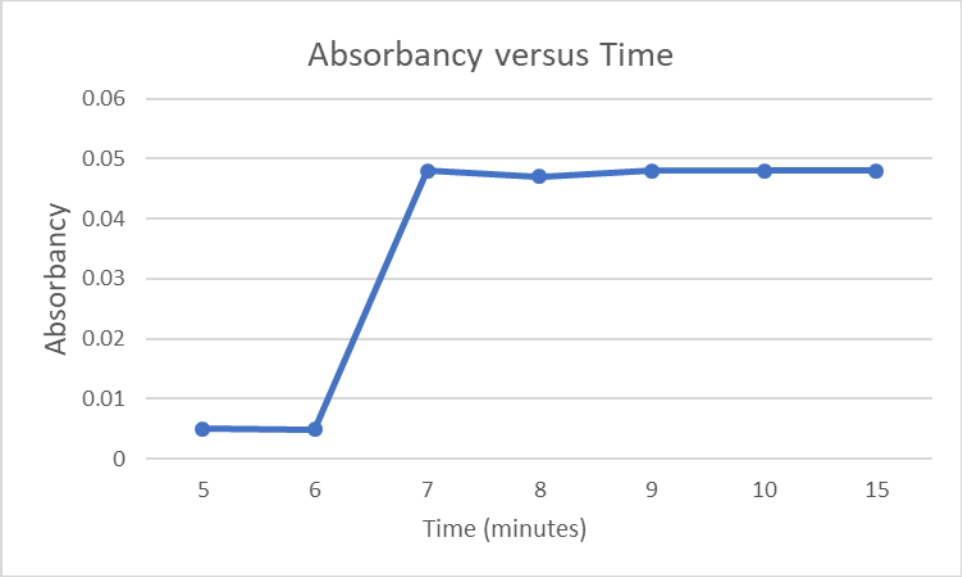


Figure 6. Absorbency versus time of day 3 DSS-treated sample. Readings started after 5 minutes reaction time and taken every 1-5 minutes until stabilization occurred. Stable absorbance number was 0.048.

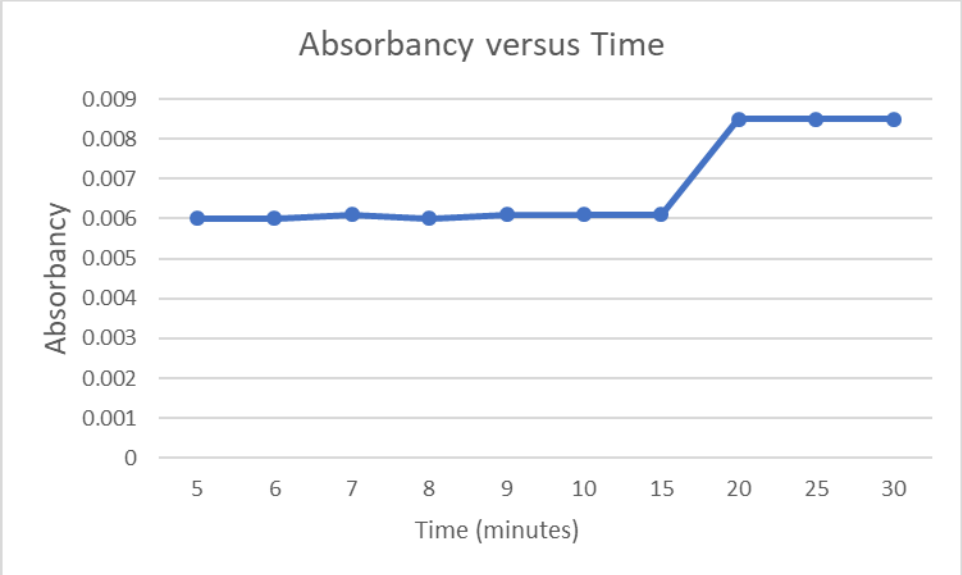


Figure 7. Absorbency versus time of day 3 antibiotic-treated sample. Readings started after 5 minutes reaction time and taken every 1-5 minutes until stabilization occurred. Stable absorbance number was 0.0085.

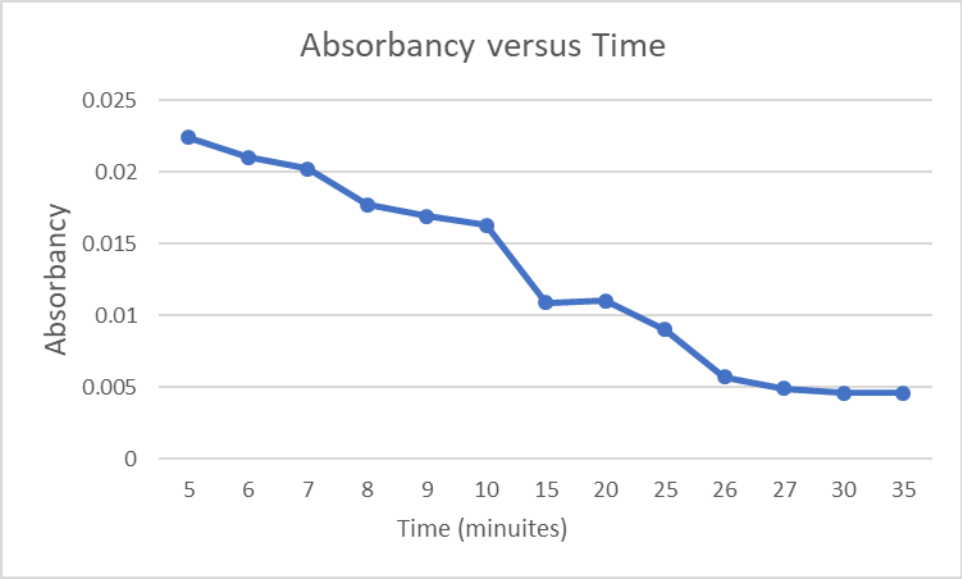


Figure 8. Absorbency versus time of day 7 control sample. Readings started after 5 minutes reaction time and taken every 1-5 minutes until stabilization occurred. Stable absorbance number was 0.0046.

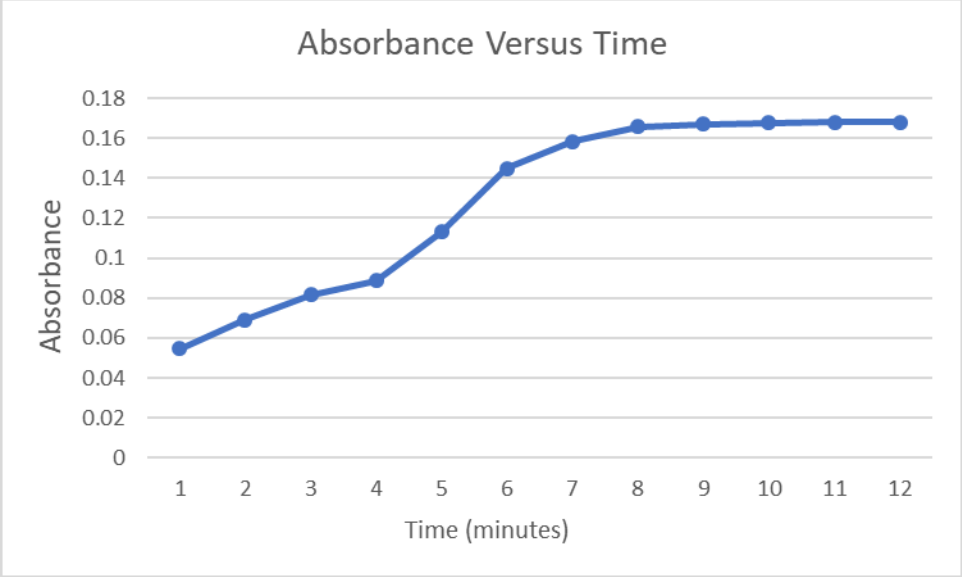


Figure 9. Absorbency versus time of day 7 DSS-treated sample. Readings started after 5 minutes reaction time and taken every 1-5 minutes until stabilization occurred. Stable absorbance number was 0.1680.

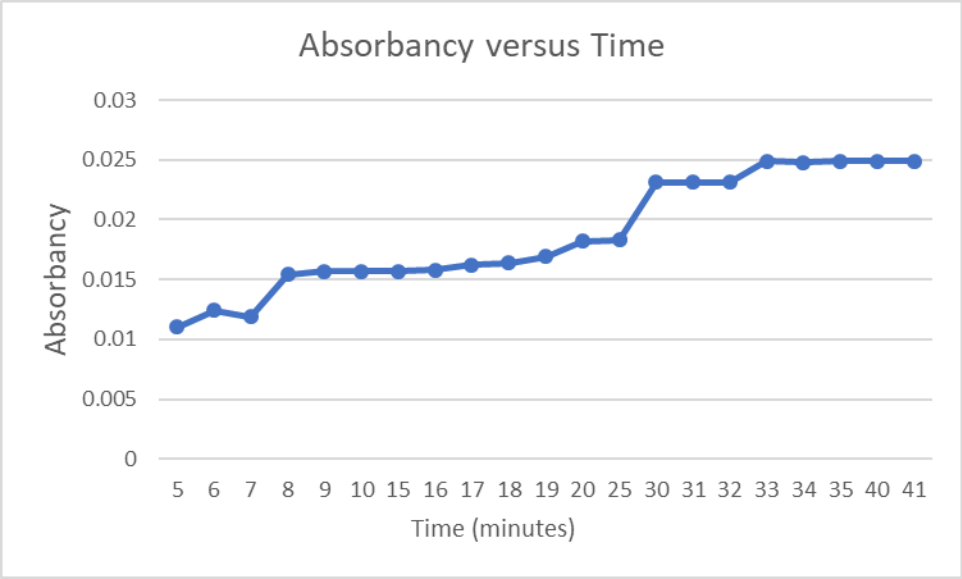


Figure 10. Absorbency versus time of day 7 antibiotic-treated sample. Readings started after 5 minutes reaction time and taken every 1-5 minutes until stabilization occurred. Stable absorbance number was 0.025.

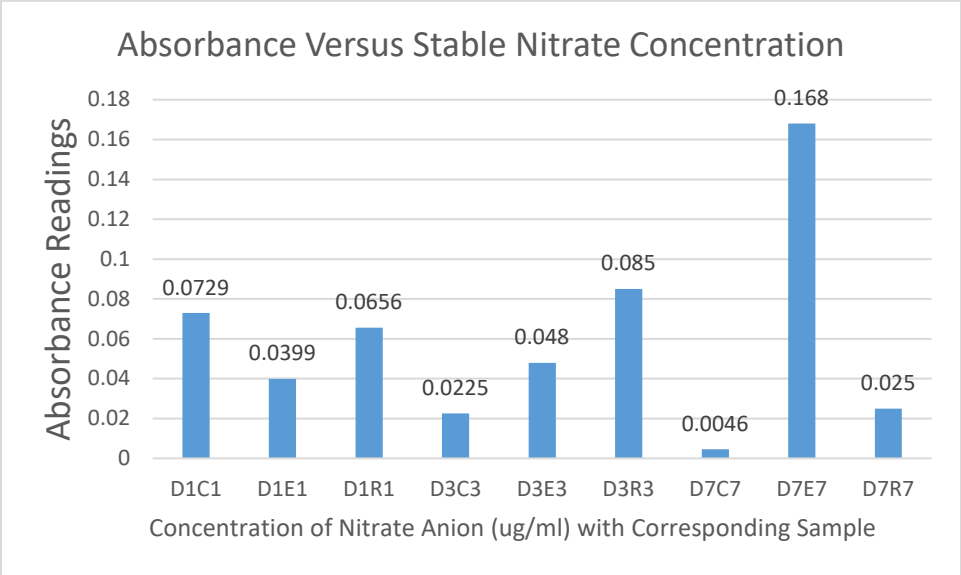


Figure 11. Absorbance readings versus stable concentration of nitrate anion (ug/ml) as depicted in previous graphs. Stable time point was selected from each sample and entered into standard curve equation, $y = 0.034x - 0.21$.

Table 13

Sample with respective nitrate concentration

Sample	D1C1	D1E1	D1R1	D3C3	D3E3	D3R3	D7C7	D7E7	D7R7
Nitrate	0.0182	0.0574	0.0132	0.0077	0.0139	0.08	0.0145	0.0579	0.0195

Note: Sample with normalized measure of nitrate, determined by micrograms of nitrate anion (shown in Figure 11) per gram of fish weight.

16S rRNA Sequencing. Rarefaction curves plot the alpha-diversity (in this case the number of OTUs) found within a given number of observations. The rarefaction curve (Figure 12) is suggestive of sufficient sequencing to to be representative of the total diversity of the sampled communities.

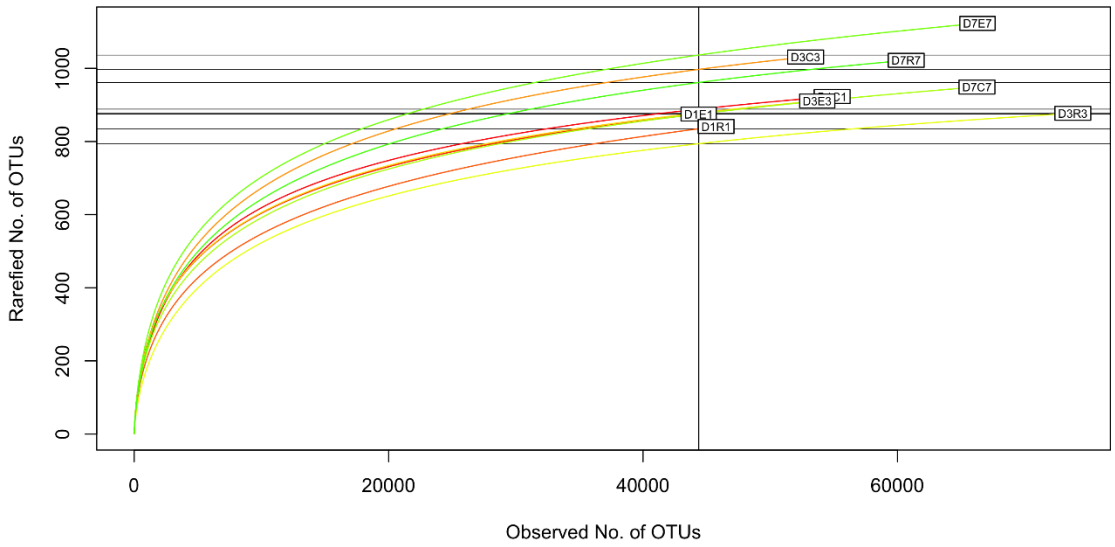


Figure 12. Rarefaction curve generated using RStudio (command rarefy in *vegan* package). Curves are labeled with respective samples and their number of OTUs at corresponding depths.

Table 14

Good's Coverage

Sample	no.sing	no.seqs	goods
D1C1	157	54868	99.71385872
D1E1	173	44374	99.61013206
D1R1	204	45741	99.55401063
D3C3	192	52781	99.63623274
D3E3	171	53693	99.68152273
D3R3	154	73790	99.79129963
D7C7	184	66250	99.72226415
D7E7	215	66289	99.67566263
D7R7	186	60690	99.69352447

Note: Good's coverage estimates of 99.5-99.7% indicate that nearly the full extent of the microbial diversity in the intestines of 9 animals was captured.

Analysis of species across samples revealed that species from the genera *Aeromonas* were the dominant species and made up the top 5 species across samples while *Cetobacterium somerae* was higher abundance in the day 1 antibiotic treated fish (22%). Similarly, species such as *Mycoplasma spp.* were rare across samples with the exception of the day 1 antibiotic treated fish in which there was an increase to 15% of the community. Patterns such as these were seen across antibiotic treated samples and also across day 7 samples.

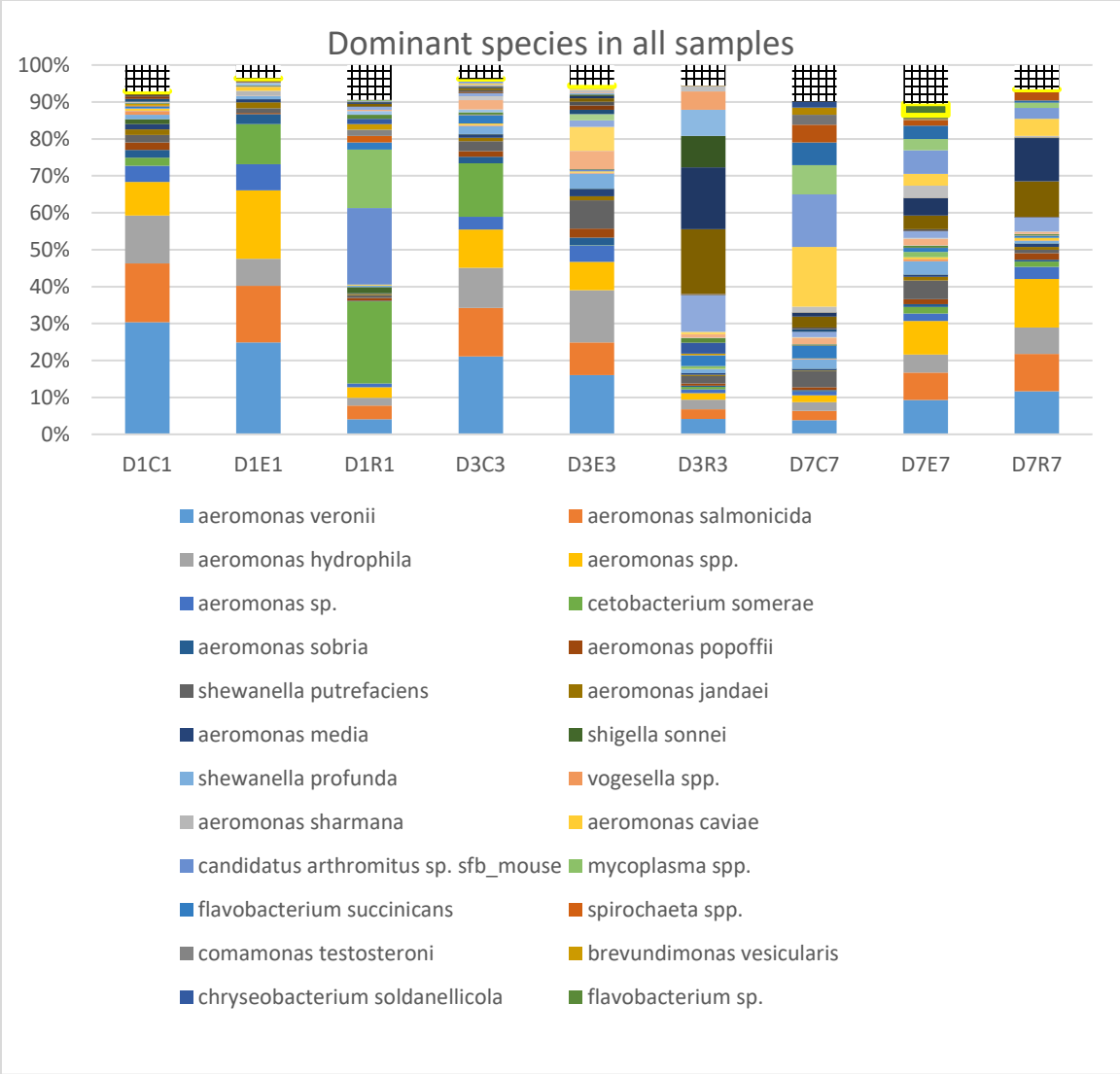


Figure 13. Species present at 1% or greater abundance in at least one of the samples in days 1, 3, or 7 denoted as D1, D3, and D7, respectively. Here, "C," "E," and "R" represent the control, DSS-treated, and rifampicin-treated fish, respectively. "Other" indicates all genera less than 1% combined.

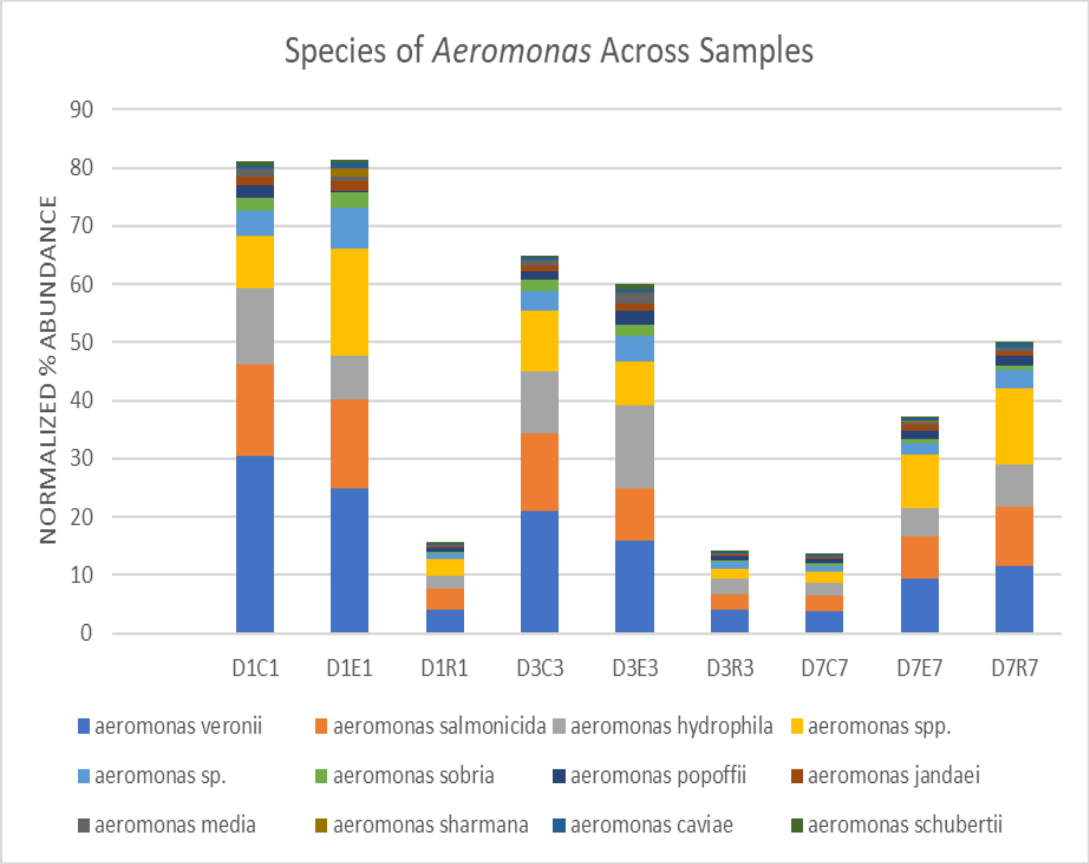


Figure 14. Species of *Aeromonas* present across samples. Abundances shown are average percentage.

Analysis of species of *Aeromonas* across rifampicin-treated samples revealed a lower overall abundance of *Aeromonas* within the community on days 1 and 3 of antibiotic treatment, but that by day 7 the community appeared to re-normalize, recovering overall abundance and similar composition of the individual species. The control and DSS-treated had different patterns of declining abundance from 1 to 3 to 7 days. In all cases, while the overall abundance of the genus *Aeromonas* changes between samples, the proportions of each species within the genus seem relatively stable.

Upon closer inspection of the 16S rRNA sequencing community data, it was observed that certain species of bacteria became more pronounced across the day 7

samples. For example, *Bosea sp.* was in relatively rare abundance (no greater than 0.60% in the day 3 control fish) until day 7 during which there was an increase to 14.20% in the control, 6.38% in the DSS treated fish, and 2.93% in the antibiotic treated fish. Similarly, *Sinorhizobium meliloti* was rare in day 1 and 3 samples (no greater than 0.23% in the day 1 antibiotic treated fish) and increased to 7.92%, 3.06%, and 1.40% in the day 7 control, DSS treated, and antibiotic treated fish, respectively. It should be noted that these species are either aerobic (such as *Bosea sp.* which is a strict aerobe) or nitrogen-fixing (such a *Rhizobium* and *Sinorhizobium meliloti*).

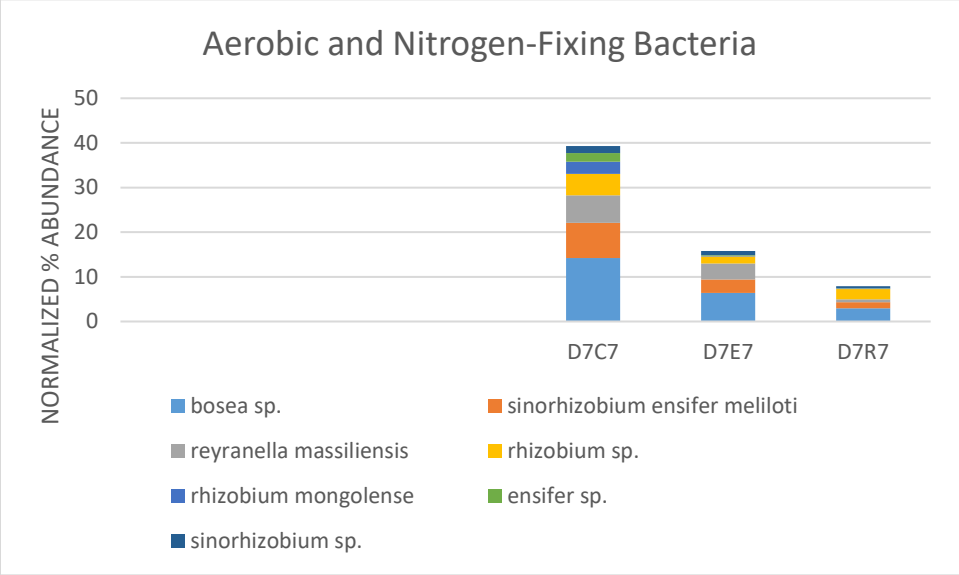


Figure 15. Aerobic and Nitrogen-fixing bacteria that are unique to day 7 samples. **Bosea sp., Reyranella massiliensis,* and *Ensifer* sp. are aerobic ***Rhizobium* are nitrogen-fixing

An analysis of dominant genera across all of the samples revealed that *Aeromonas* dominate the majority of the samples while other genera make more of an appearance possibly based on environmental circumstances such as antibiotic treatment. For example, *Cetobacterium* was present in day 1 control (2.15%) and DSS fish

(10.88%),and increased abundance in the antibiotic-treated fish (22.28%). Similarly, *Pseudomonas* was in markedly low abundances (0.7-8%) in samples with the exception of the Day 3 antibiotic treated fish (46.40%) and the Day 7 samples (8-26%). This is suggestive of antibiotic treatment resulting in profound changes within the community, similar to studies done in murine and mammalian models. It is not clear whether inflammation could have a similar influence on the community within the guts of the *Gambusia* as MPO levels in the experimental fish were unreliable.

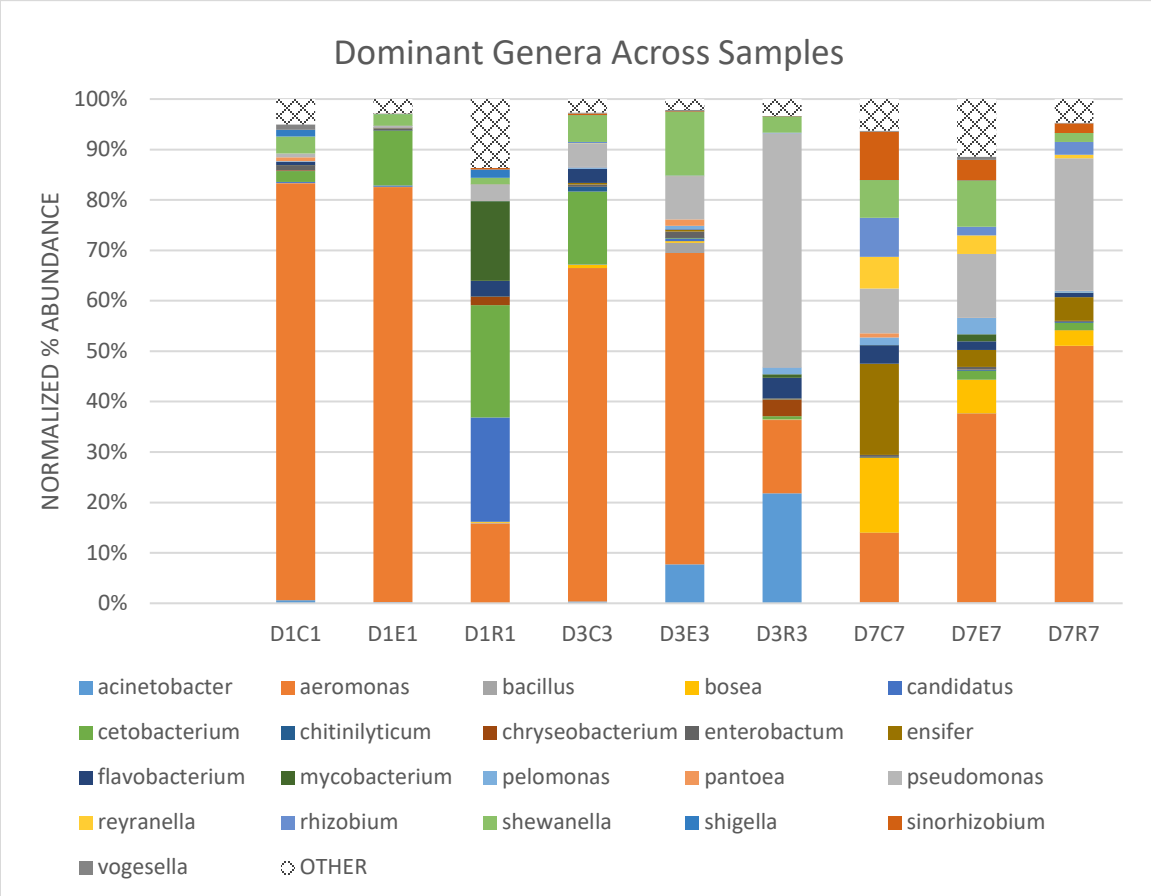


Figure 16. Genera present at 1% or greater in at least one sample on days 1, 3, and 7 denoted as D1, D3, and D7, respectively. Here, "C," "E," and "R" represent the control, dextran sulfate sodium-treated, and rifampicin-treated fish, respectively. "Other" indicates genera not assigned a classification.

An average of the dominant genera within the DSS treated group revealed *Aeromonas* made up 60% of the dominant taxa while *Pseudomonas, Cetobacterium,* and *Shewanella* together constituted approximately 20% of the dominant genera (Figure 14). Additionally, within the antibiotic treated samples, *Aeromonas* makes up 26% of the community while *Pseudomonas* makes up approximately 25% of the community, thereby making up half

the dominant genera across those samples. Furthermore, *Cetobacterium, Candidatus Arthrimotus, Mycoplasma,* and *Acinetobacter* together make up approximately 27% of the dominant genera across those samples (Figure 15).

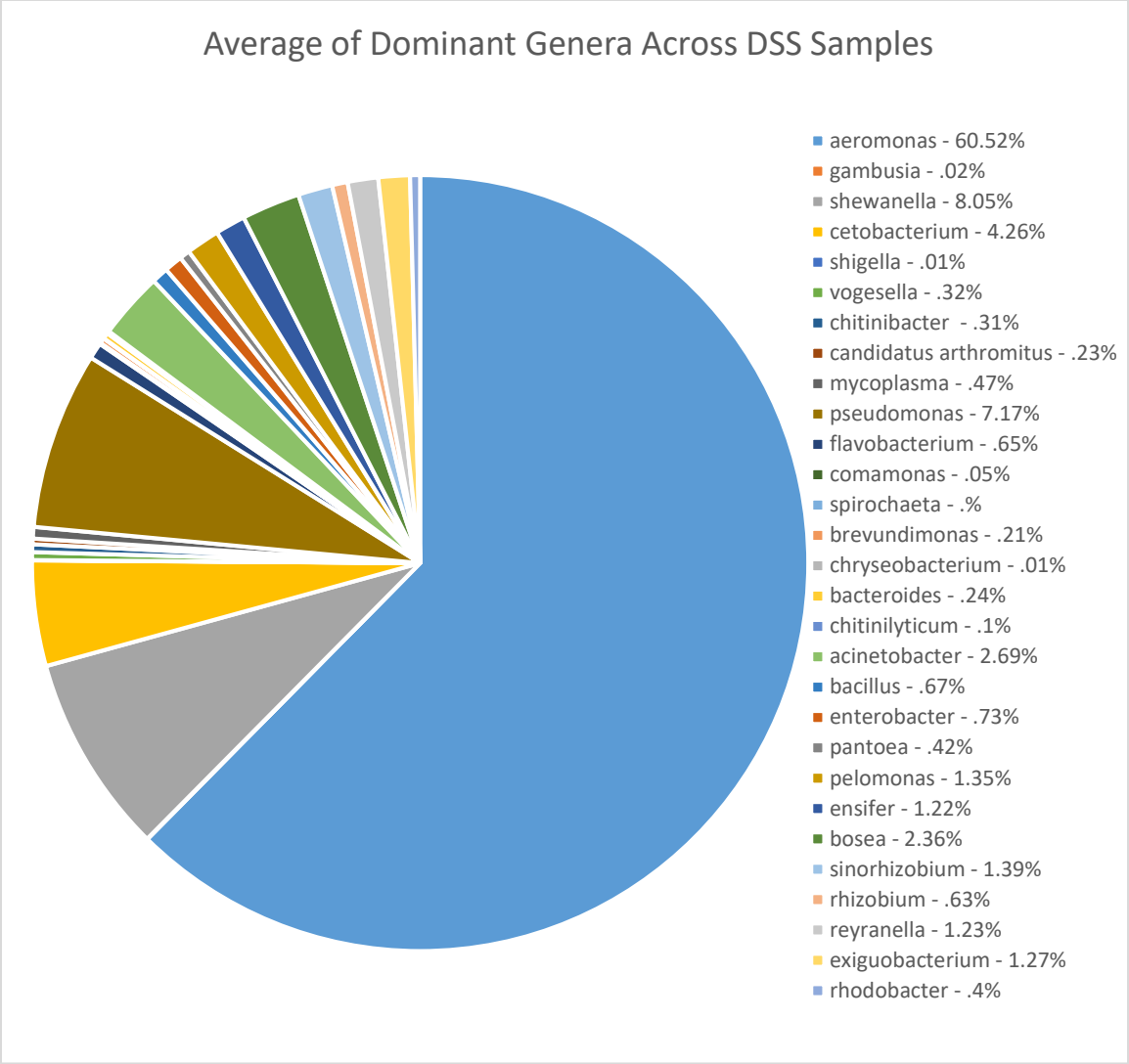


Figure 17. Pie chart of dominant genera of the gut microbiome across dextran sulfate sodium treated fish. Abundances shown are average percentage.

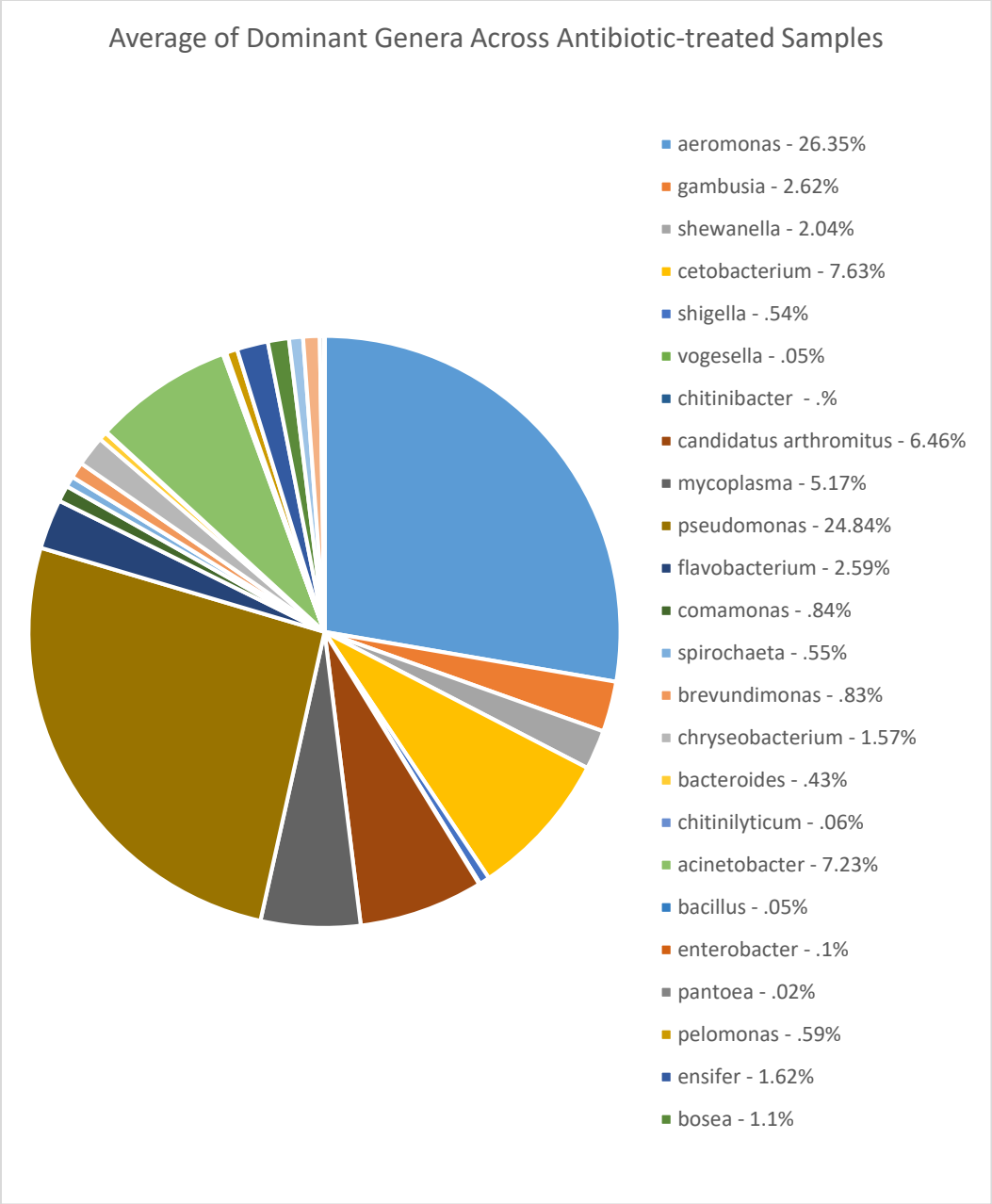
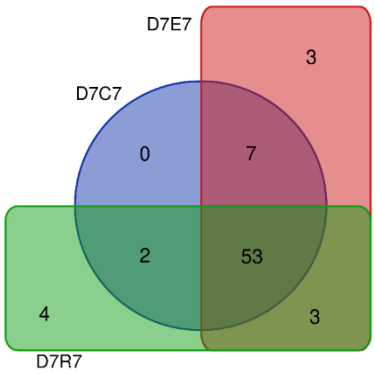
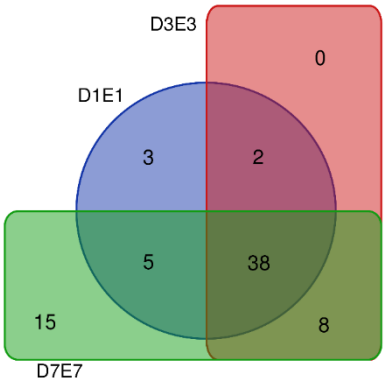


Figure 18. Pie chart of the dominant genera of the gut microbiome across Rifampicin treated fish. Abundances shown are average percentages.

Additionally, further analyses of genera across samples revealed that certain genera are unique to particular samples (Figure 7), suggestive of diversity across samples.





Genera unique to D7E7:
Planctomyces, Legionella, Aciditerrimonas
Genera unique to D7R7
Fusobacterium, Sphingomonas, Rheinheimera, Sphingobium

71 unique genera total (g diversity).
54% common to all 3 samples,
21% unique to d7 sample.

Figure 19. Venn diagram comparison of selected samples.

Sample Alpha Diversity

	D1C1	D1E1	D1R1	D3C3	D3E3	D3R3	D7C7	D7E7	D7R7
# Seq	54,868	44,374	45,741	52,781	53,693	73,790	66,250	66,289	60,690
# Genera	82	69	83	72	63	76	98	103	101
Dom Genera	5	3	13	6	7	7	10	14	8

Seq is number of sequences in that sample (each sample from one fish gut). 57,600 ± 9,999 mean sequences across all 9 samples. # Genera is total number of genera present. Dom Genera is genera present at 1% or higher abundance.

Figure 20. Alpha diversity of all nine samples.

Analysis of beta species diversity as represented by Whittaker dissimilarity indices revealed that some of the more similar samples were between D1E1 and D3C3,

D1E3; D3C3 and D3E3 and the D7 samples; and Day 7 samples as compared to each other while some of the more diverse samples were D1C1 and D1R1; D1E1 and D3R3; and D1R1 and D7E7. Unsurprisingly, a lot of these similarities and diversities are reflected in the changes in percentages as represented by the bar graph in Figure 4 and can also be observed among genera in Figure 7.

Table 15

Beta Species Diversity as Represented by Whittaker Dissimilarity Indices

	D1C1	D1E1	D1R1	D3C3	D3E3	D3R3	D7C7	D7E7	D7R7
D1C1	0	0.35094	0.48822	0.38849	0.40876	0.40411	0.41433	0.43202	0.37539
D1E1	0.35094	0	0.41429	0.2567	0.28405	0.46909	0.375	0.35669	0.34667
D1R1	0.48822	0.41429	0	0.45392	0.43253	0.4202	0.44048	0.45665	0.39157
D3C3	0.38849	0.2567	0.45392	0	0.25185	0.38889	0.27445	0.24771	0.28435
D3E3	0.40876	0.28405	0.43253	0.25185	0	0.40845	0.27796	0.31269	0.30744
D3R3	0.40411	0.46909	0.4202	0.38889	0.40845	0	0.41994	0.43695	0.36391
D7C7	0.41433	0.375	0.44048	0.27445	0.27796	0.41994	0	0.18919	0.24719
D7E7	0.43202	0.35669	0.45665	0.24771	0.31269	0.43695	0.18919	0	0.25683
D7R7	0.37539	0.34667	0.39157	0.28435	0.30744	0.36391	0.24719	0.25683	0

Note: Beta species diversity as represented by Whittaker dissimilarity indices where "0" indicates identical species and "1" indicates perfect diversity in which no species is shared between the samples.

Table 16

Beta Genus Diversity as Represented by Whittaker Dissimilarity Indices

	D1C1	D1E1	D1R1	D3C3	D3E3	D3R3	D7C7	D7E7	D7R7
D1C1	0	0.41818	0.48936	0.44706	0.46405	0.40782	0.48187	0.50754	0.39
D1E1	0.41818	0	0.4386	0.28105	0.26471	0.49383	0.39773	0.38462	0.333333
D1R1	0.48936	0.4386	0	0.53409	0.50943	0.37297	0.52764	0.53171	0.42718
D3C3	0.44706	0.28105	0.53409	0	0.26241	0.48503	0.28177	0.30481	0.34043
D3E3	0.46405	0.26471	0.50943	0.26241	0	0.52	0.35366	0.4	0.35673
D3R3	0.40782	0.49383	0.37297	0.48503	0.52	0	0.48421	0.52041	0.42132
D7C7	0.48187	0.39773	0.52764	0.28177	0.35366	0.48421	0	0.20952	0.31754
D7E7	0.50754	0.38462	0.53171	0.30481	0.4	0.52041	0.20952	0	0.33641
D7R7	0.39	0.33333	0.42718	0.34043	0.35673	0.42132	0.31754	0.33641	0

Note: Beta genus diversity as represented by Whittaker dissimilarity indices where "0" indicates identical species and "1" indicates perfect diversity in which no species is shared between the samples.

Species-level identification of the short sequences in our data is not very accurate. Genus-level identification is more confident. As a comparison, sequence data was also compared at a much higher level of taxonomy (Class level, which is three steps above genus; species, genus, family, order, then class). The most dominant class across all fish was Gamma Proteobacteria, not a surprise since this class include the genera *Aeromonas* and *Pseudomonas*. The second-most dominant class was Alpha Proteobacteria, which includes the genera *Bosea* and *Ensifer*. With the exception of the day1 rifampicin-treated fish, the *Proteobacteria* make up more than 75% of the gut microbiome of the fish. This is consistent with other studies of fish, where Proteobacteria dominate (14, 19, 23).

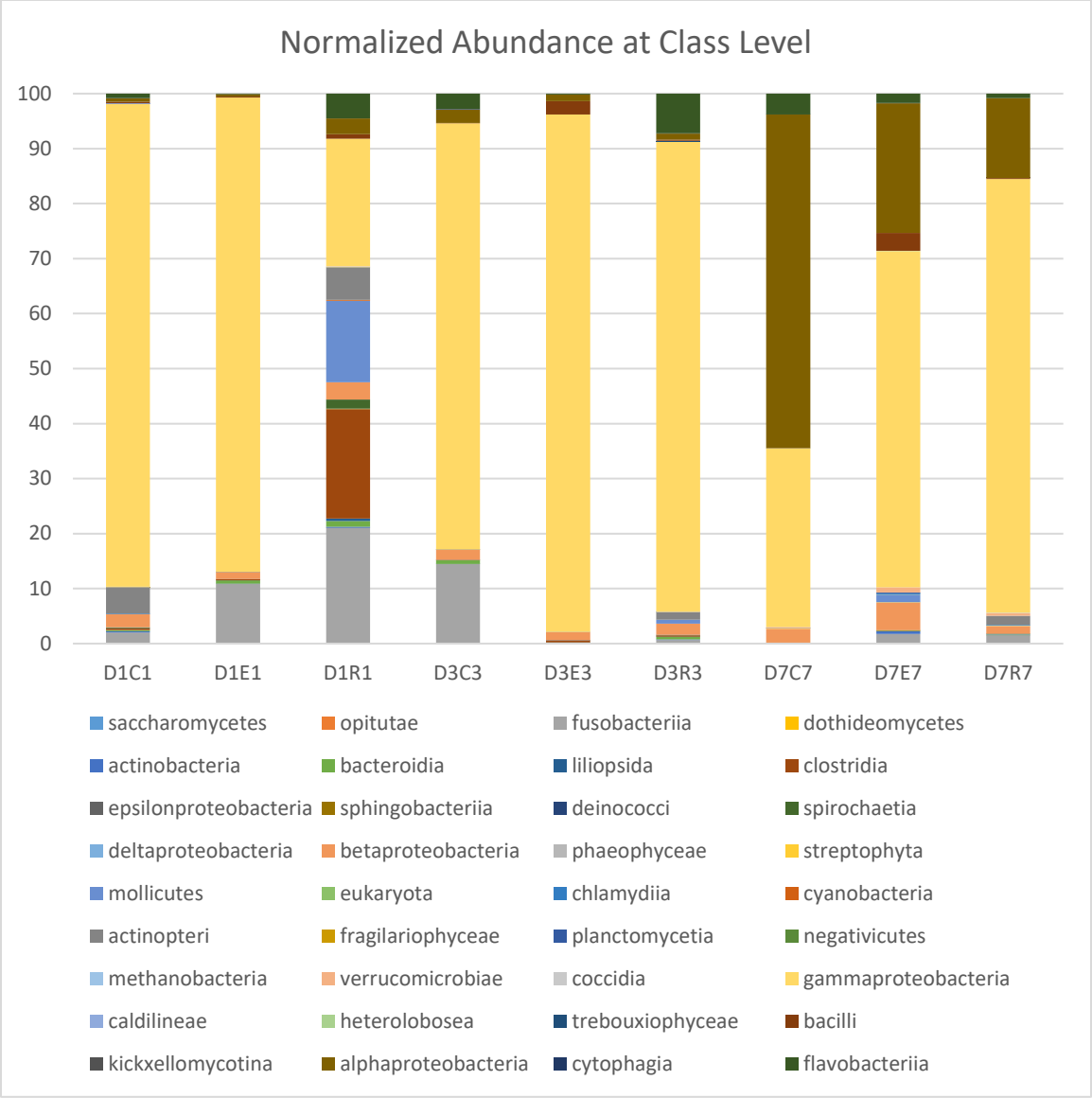


Figure 21. Normalized abundance at class level.

Non-metric multidimensional scaling (NMDS) shows differences in the day 1 antibiotic treated fish as well as in the Day 7 fish across samples as compared to the other samples while days 1 and 3 control and DSS treated fish cluster closer together.

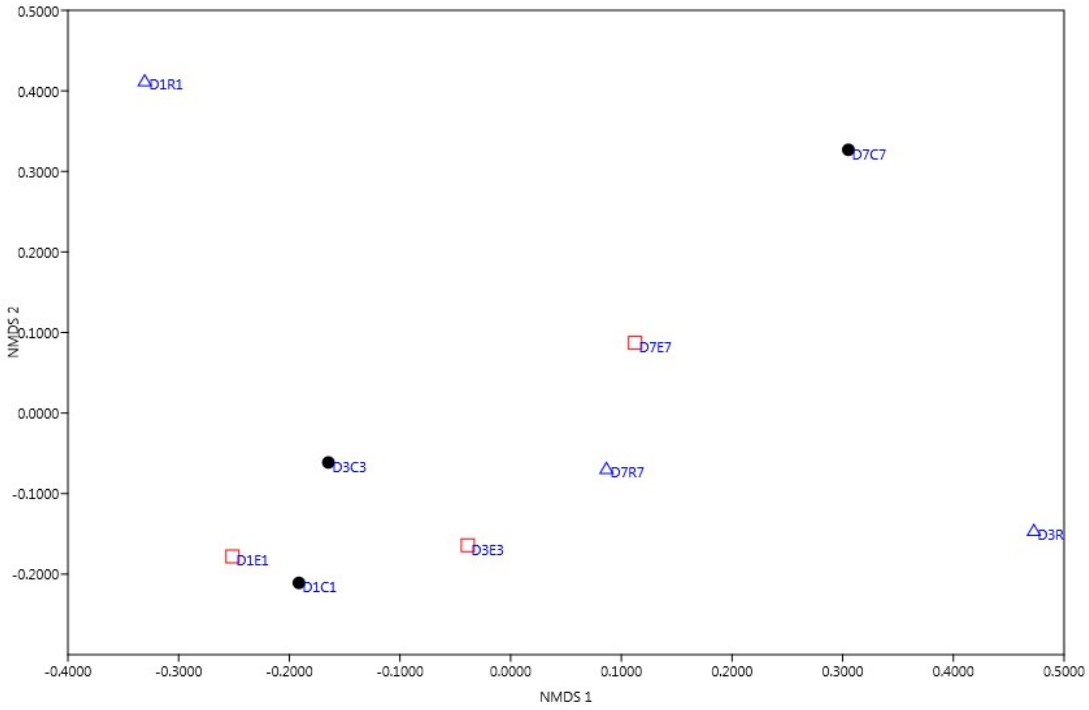


Figure 22. Non-metric multidimensional scaling (NMDS) to visualize differences in overall community (all genera 0.1% abundance or higher in at least one sample) composition of samples.

Histology.

The anatomy of the *Gambusia affinis* is similar to that of the zebrafish (Menke et al., 2011): intestinal folds lined by absorptive cells with the presence of goblet cells. A healthy intestinal tract is noted for its defined architecture, epithelial integrity, and not overly abundant presence of leukocytes at the base of the folds. Pictured below, Figure 20, is an example of a healthy, "normal" section of the intestinal tract. As can be observed, the architecture of the folds is well defined, the epithelium is intact, and while there is a presence of leukocytes, they do not overwhelm the presence of other cells:

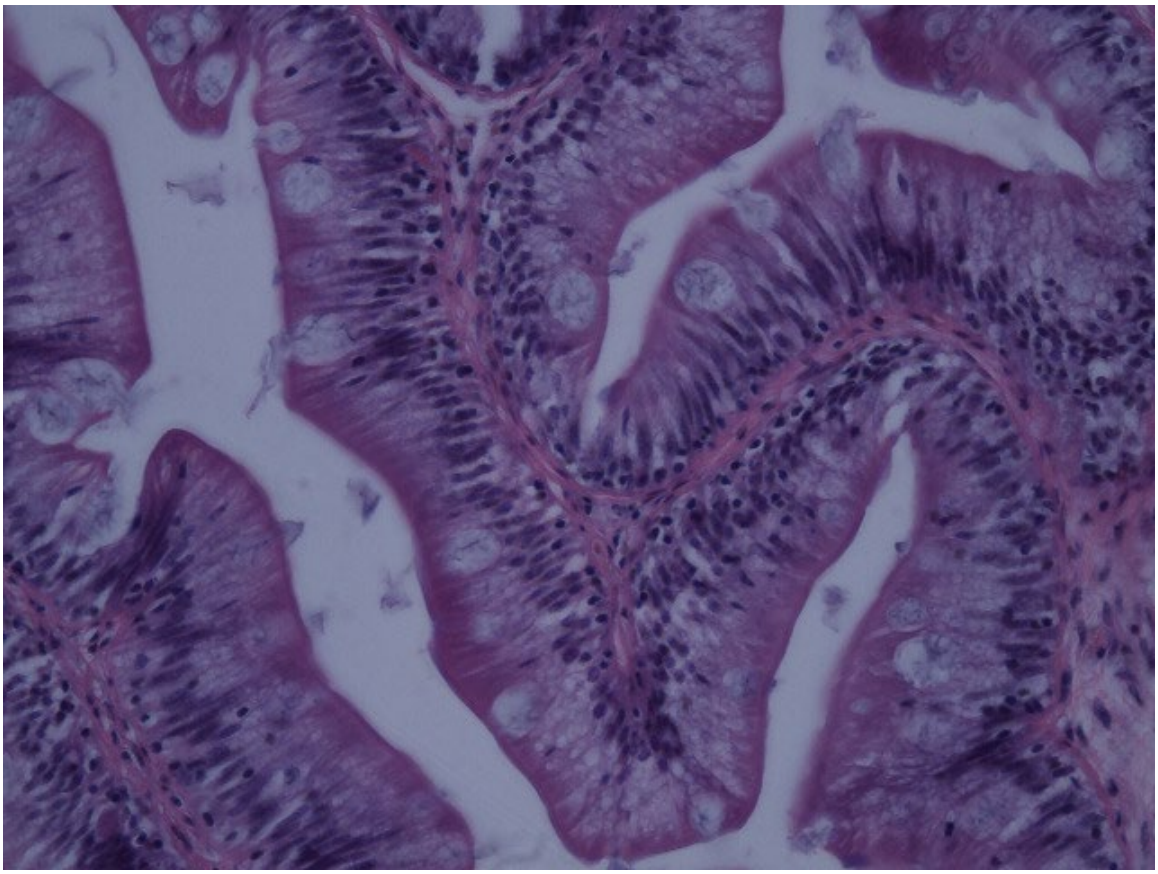


Figure 23. Histological image from gut sample of Day 7 rifampicin-treated fish.

In Figure 27-28, on the other hand, a complete disruption of the architecture as well as the epithelial integrity can be observed. There is also a significant infiltration of leukocytes, suggestive of acute inflammation in this region.

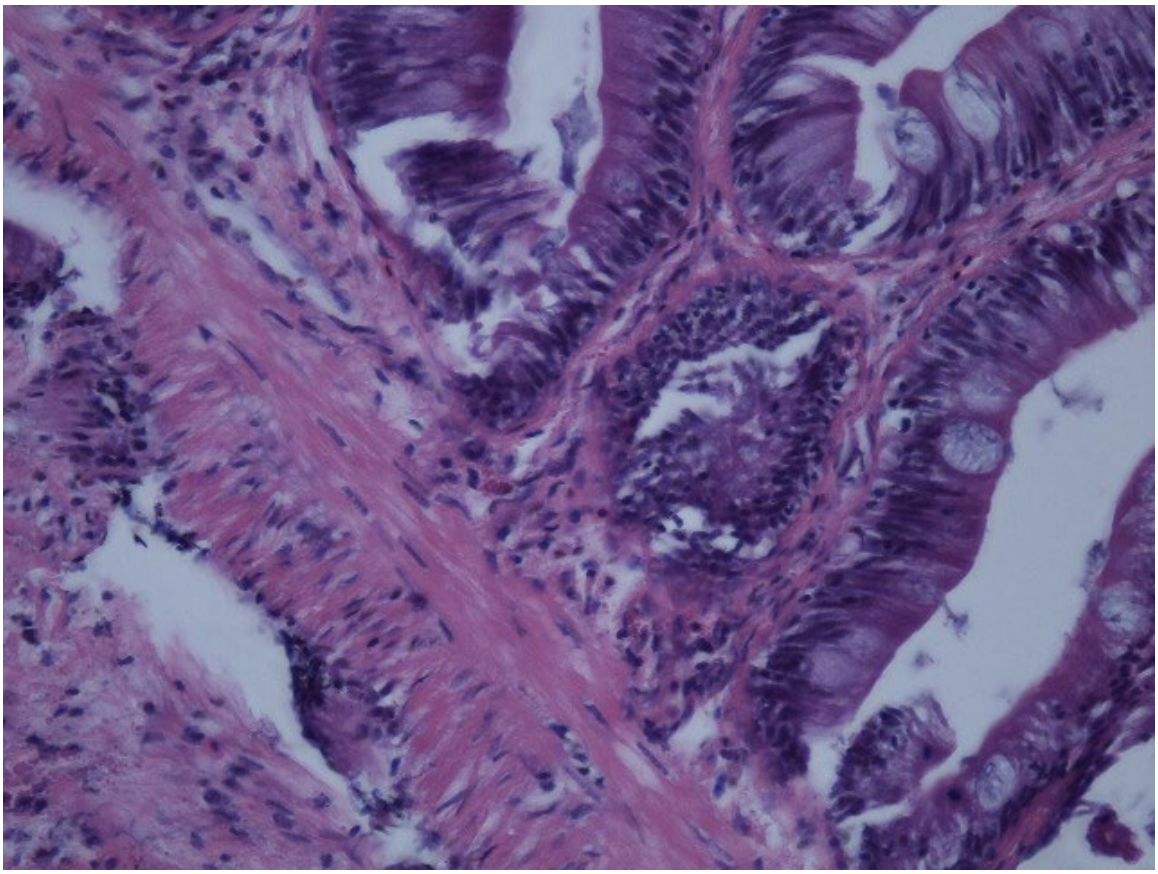


Figure 24. Histological image from gut sample of Day 7 DSS-treated fish.

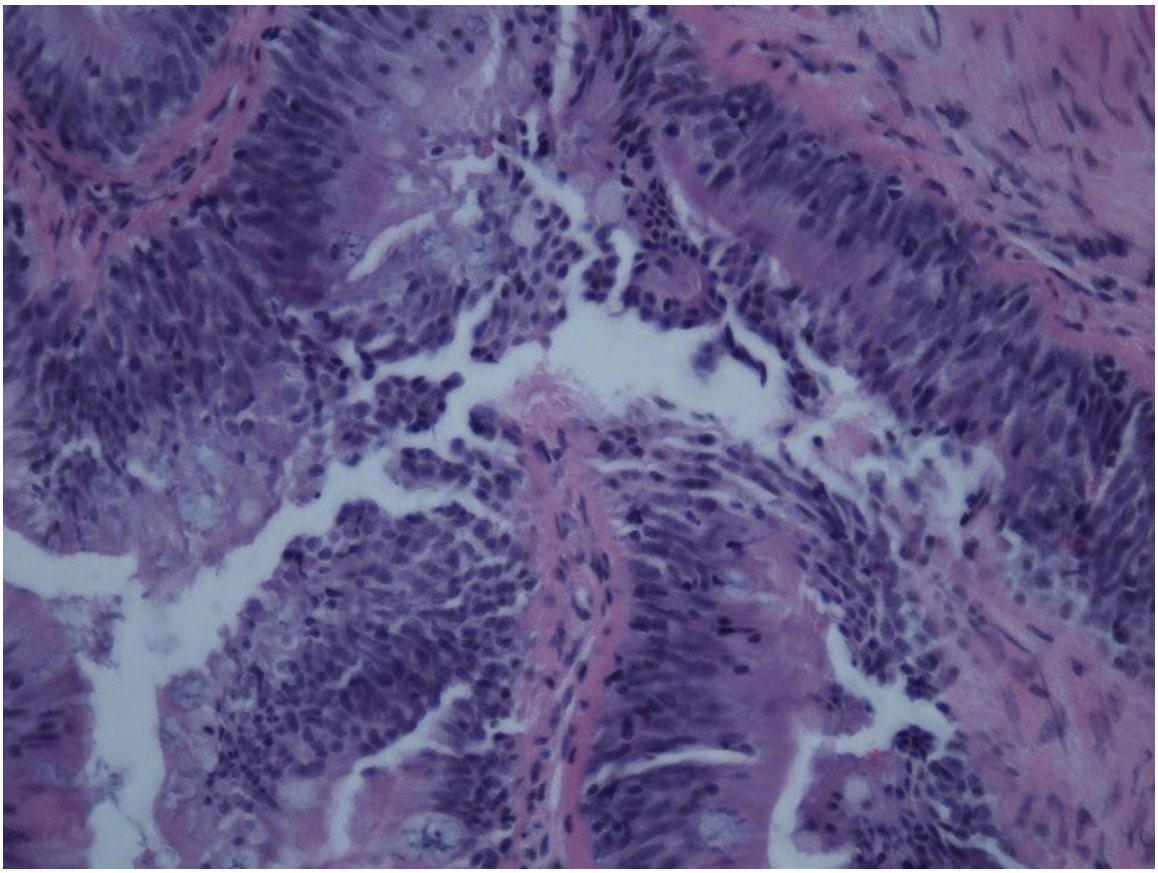


Figure 25. Histological image from gut sample of Day 3 untreated fish.

CFU Counts and Plating.

In order to further validate observations made based on sequencing data, an aerobic versus anaerobic plating experiment was conducted. Here, the experimental design was set up as previously outlined: fish were removed from the aquarium, weighed, and placed into individual Styrofoam cups. The control and antibiotic-treated fish were fed regular gelatin feed while the dextran sodium sulfate-treated fish were fed gelatin feed that contained the dextran sulfate sodium colitogen. As before, fish intestines were excised on days 1, 3, and 7, cut into small pieces, placed in 1.5 ml tubes containing PBST, and vortexed for 1 minute on 01-29-2019 and for 2 minutes after resting in PBST for 5 minutes on days 3 and 7. Afterwards, standard dilutions were made and 100 µl of the 1X and the dilutions were plated onto both nitrate and nutrient agar plates and placed into a 25°C incubator under both aerobic and anaerobic conditions (plates denoted for anaerobic conditions are placed into anaerobic chambers). After approximately 72 hours, plates were noted for growth and CFU counts were documented.

**It was noted that the first gut excision did not produce substantial results. For this reason, the method was modified.*

Table 17

Calculated CFUs Under Aerobic Conditions

Sample	Nutrient Agar	Nitrate Agar
C1	TFTC	TFTC
E1	TFTC	TFTC
R1	TFTC	TFTC
C3	1.36×10^5	2.8×10^5
E3	3.12×10^5	9.4×10^4
R3	3.1×10^2	TFTC
C7	TFTC	TFTC
E7	3.0×10^5	TMTC
R7	TFTC	TFTC

Table 18

Calculated CFUs Under Anaerobic Conditions

Sample	Nutrient Agar	Nitrate Agar
C1	no growth	TFTC
E1	no growth	no growth
R1	no growth	no growth
C3	2.7×10^5	1.0×10^4
E3	2.3×10^5	2.52×10^5
R3	no growth	no growth
C7	no growth	no growth
E7	1.3×10^5	9.7×10^4
R7	no growth	no growth

Note: The tables above denote the calculated colony forming units (CFUs) for each respective plate (nitrate or nutrient agar) under either aerobic or anaerobic conditions. The dilutions are listed under the respective group of fish (C=control, E=DSS-treated, and R=rifampicin-treated). “TFTC” is “too few to count” meaning that the colonies were under 20.

Anaerobic to Aerobic Plating. In all cases, when colonies were taken from anaerobic conditions on either nitrate or nutrient agar and inoculated onto nutrient agar plates and stored at 25°C for approximately 48-72 hours, growth was observed. However, CFU counts were not possible due to the lack of individual colonies formed. Nevertheless, this is suggestive of facultative anaerobes present within the gut of the *Gambusia affinis*. On the other hand, when colonies were inoculated from aerobic conditions and stored at 25°C in anaerobic chambers for 48-72 hours, growth was not observed in all cases. For example, when colonies were taken from sample C3 (10^{-3} dilution), originally plated on 02.06.2019 (representative of day 7 samples) on nitrate agar and grown under aerobic conditions, no growth was observed. This is suggestive of bacteria present which are strict aerobes within the day 7 samples.

CHAPTER IV

DISCUSSION

Inflammation

While the MPO pre-trials were suggestive of inflammation induced by DSS both at 6 mg and at 20 mg doses, trial results were unclear. Additional experiments conducted in an attempt to isolate and purify the MPO enzyme also failed to illuminate the reliability of this method. It is possible that the lack of specificity of o-dianisidine towards MPO makes this method unreliable overall. As Pulli et al point out, o-dianisidine, along with TMB and guaiacol, are general peroxidase substrates and therefore do not interact with MPO alone[27]. Nevertheless, the MPO assay utilized in this experiment is one that is used widely in the literature in murine and zebrafish models. It is also possible that this particular approach cannot be used reliably in *Gambusia affinis*. Two major hurdles in this experiment were avoiding female fish who were not pregnant and fish without parasites: it is not clear the ways in which pregnancy affects *Gambusia affinis* specifically, so it not known what peroxidases are present or elevated as a result. Additionally, as these fish were caught in the wild and are not bred in a lab, parasitic infections were relatively common and although care was taken to omit those infected, it is possible that other infections (perhaps not immediately apparent) interfered with MPO measurements such that control fish may have had greater inflammation than the dextran sodium sulfate-treated fish.

Furthermore, it is also possible that the initial gut microbiota structure is too variable between the fish. Although the fish are homogenous in that they come from the same pond, they are nevertheless wild and may perhaps exhibit nuances within their

individual gut microbiomes that could interfere with results which are dependent on generalizations within a "core" microbiome. As is seen in the data from previous work by Carlson et al., significant variations in the control gut microbiome data are observed as compared to the control fish within this experimental design. See pie charts below:

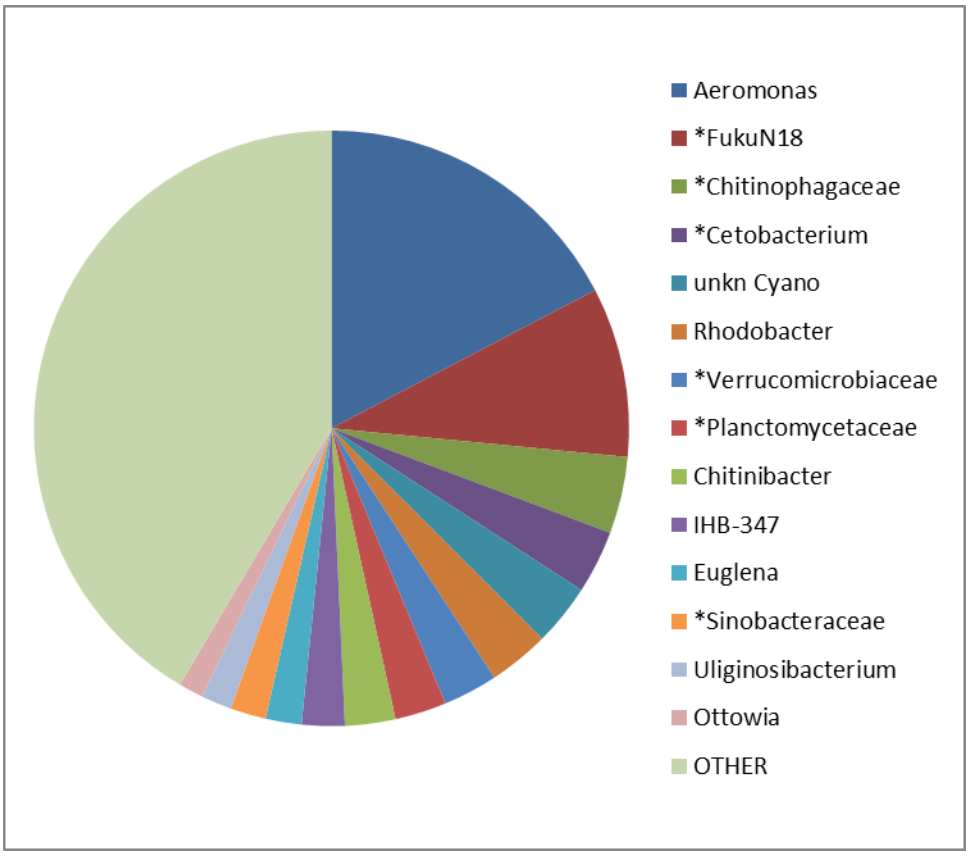


Figure 26. Chart taken from unpublished, raw data of control gut samples (n= 4) from Jeanette Carlson with her permission.

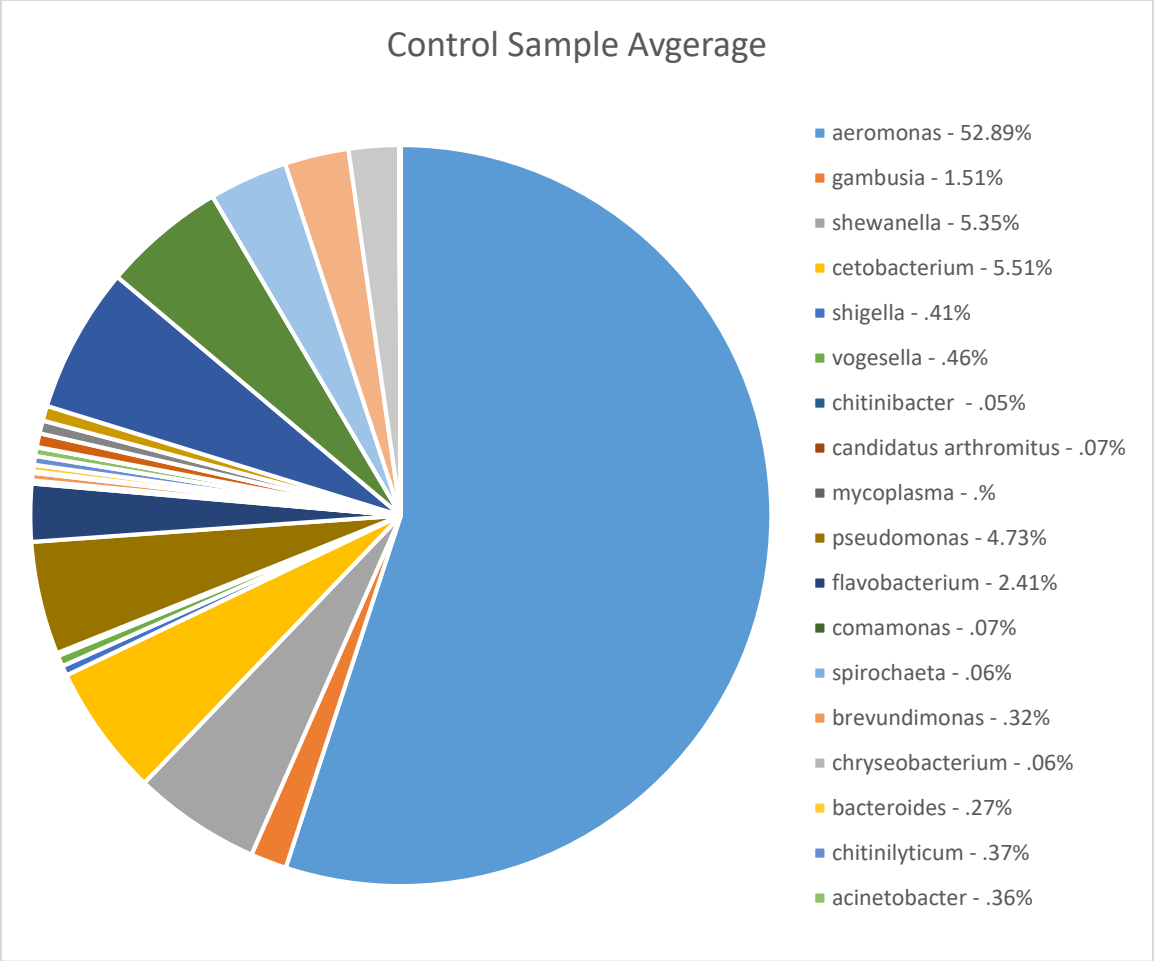


Figure 27. Average of control gut samples (n = 3), genus level.

For this reason, it may be necessary to conduct 16S rRNA sequencing on a large population of fish in order to assess what a "core" microbiome may look like within the *Gambusia*. This data would further illuminate whether the initial gut microbiome of the individual fish plays a role in the effectiveness of dextran sulfate sodium. As Li et al point out in a study conducted in mice, the initial structure of the gut microbiome affects the sensitivity to DSS-induced colitis (2017). For this reason, it is possible that whatever the initial gut microbiota was prior to treatment with DSS affected the results and may therefore explain the inconsistency of the assay on this organism.

The nitrate assay reveals slightly higher values of the nitrate anion in both the day 1 and day 7 DSS-treated fish. Interestingly, the day 3 antibiotic-treated fish reveals slightly elevated levels of nitrate anion as well. While the DSS-treated fish do appear to have higher levels across samples with an average of 0.04 micrograms of nitrate anion per gram of fish weight as compared to 0.01 and 0.03 in the control and antibiotic treated samples, respectively, these values do not necessarily explain the differences seen within the sequence data. For example, while the day 1 DSS treated sample has a higher value of nitrate anion at 0.05 than either of the day 1 control or antibiotic treated samples, its community composition is more similar to the control sample than the antibiotic treated sample which undergoes a dramatic shift in its community composition. Similarly, while the day 7 DSS-treated sample reveals higher levels of the nitrate anion, and while the community shifts to a higher abundance of aerobic and/or nitrate-fixing microbes, an increase in the nitrate anion alone cannot account for this shift as similar shifts are observed in the day 7 control and antibiotic treated samples which show decreased levels of the nitrate anion. In this way, potential inflammation as represented by nitrate anion values cannot adequately explain community shifts within the *Gambusia* samples. Instead, other factors need to be considered. Perhaps starvation plays a role in the ways in which the gut microbiome is affected. In a study conducted by Xia et al., sequencing analysis of the gut microbiome of the Asian seabass *Lates calcarifer* after eight days under starvation revealed dramatic differences in microbial community composition as compared to the regularly fed cohort (2014). In this way, it is possible that the day 7 fish that have not eaten since day 0 of the experiment are experiencing similar compositional shifts due to experiencing the same dietary restrictions. Additionally, Winters et al

conduct their research in mice which, like humans, are dominated by obligate anaerobes within the colon and it is for this reason that the nitrate anion offers a selective advantage for the facultative anaerobes as they can use the nitrate anion and perform nitrate respiration. It is not yet clear how this mechanism may be used by facultative anaerobes and how this may change the community composition of *Gambusia affinis* specifically. Human and murine intestinal communities are normally dominated by obligate anaerobes, while our fish is dominated by facultative anaerobes. So, the theory from Winters related to inflammation and nitrate does not apply. Furthermore, it should be noted that fish like zebrafish and salmonids like trout are dominated by aerobes or facultative aerobes (Roeselers et al., 2011) while large aquatic animals such as sperm whales are dominated by obligate anaerobes (Erwin high diversity and unique comp). In this way, the tendency toward aerobic and facultative aerobic microbial communities is not true of aquatic animals in general, as may be assumed, but instead appears to be true for non-mammalian aquatic animals. This observation brings into question the validity of using zebrafish for gut microbiome studies as they apply to human models. It is possible that the intricacies and complexities of the gut microbiome is too species specific, such that translating gut microbiome studies is currently limited to murine and mammalian studies.

16S rRNA Sequencing

Results of 16S rRNA sequencing revealed that the first and third days of rifampicin treatment disrupted the gut microbiome of the Gambusia fish profoundly. On the first day, the genus *Aeromonas* was in an abundance of approximately 15% as compared to approximately 82% in both the control and the dextran sodium sulfate-

treated fish. Additionally, the community shifts such that *Cetobacterium, Candidatus*, and *Mycobacterium* become pronounced at 22%, 20%, and 15%, respectively, whereas *Candidatus* and *Mycobacterium* are either rare or nonexistent across the other samples. On day 3 (two days post-antibiotic treatment), there is a marked abundance in *Acinetobacter* at approximately 22%, whereas it is rare (0.5% or less) across all other samples with the exception of the day 3 DSS treated fish (two days post-DSS treatment) in which it is at 7% abundance. Furthermore, there is a marked abundance of *Pseudomonas* at 46%. Interestingly, *Pseudomonas* emerges in the day 7 control, DSS treated, and antibiotic treated samples at 8%, 12%, and 26%, respectively. Of note, based on other gut samples (n=3) from previous work done in the lab, *Pseudomonas* was in low abundance in the control fish (prior to antibiotic treatment), at 0.8% and lower. In recovery samples, *Pseudomonas* remained low. It should be noted, however, that the fish underwent treatment and recovery over the span of a two weeks (one week for treatment, one week for recovery), thus the community was impacted and experienced shifts that cannot adequately and reliably be compared to this experimental design. Nevertheless, with so little known about the gut microbiome of the *Gambusia affinis*, comparisons such as these are helpful in gaining insight into the possible core microbiome of this potential model and the ways in which various environmental factors and chemicals may affect that microbiome.

Additionally, *Bosea* and *Ensifer* emerge across day 7 samples, both of which are obligate aerobes as opposed to the facultatively anaerobic *Aeromonas*. In fact, a number of aerobes are in day 7 samples which are in very low abundance across the other samples. As is seen in Figure 4, a pattern of emergence can be seen across day 7 samples.

For example, in the day 7 control sample*, Bosea sp*. is an obligate aerobe, *Ensifer adherens* is aerobic and *Ensifer meliloti,* and *Reyranella massiliensis* are both microaerophilic. The day 7 DSS-treated fish exhibits *Bosea, Pseudomonas straminae,* and *Pseudomonas putida*, all of which are aerobes or obligate aerobes. The day 7 antibiotic treated fish, similar to the DSS-treated fish, also exhibits an increase in *Pseudomonas straminea* and *Pseudomonas putida* along with a re-emergence of *Aeromonas* species such as *Aermonas salmonicida* and *Aeromonas veronii.* The theory of Winters is that in the mammalian gut, which is normally dominated by obligate anaerobes), release of nitrate during inflammation gives a metabolic advantage to facultative anaerobes (using the nitrate during anaerobic respiration). This does not apply in our fish, as they are normally dominated by facultative anaerobes. However, future work can examine a possibility suggested by this work, that inflammation in the fish gut causes an increase in abundance of obligate aerobes compared to facultative. Perhaps this results from oxidative radicals being released in the gut.

When comparing dextran sodium sulfate treated fish to antibiotic treated fish, patterns can be seen within the averages across samples. In Figure 5, *Aeromonas* comprises approximately 60% of the genera present while in antibiotic treated fish, *Aeromonas* comprises approximately 26% of the community while *Pseudomonas* comprises approximately 24%. In this way, the two groups are dissimilar to one another.

In reviewing the beta genus diversity as represented by Whittaker dissimilarity indices, again, these patterns in genera lost and gained and differences in community composition, as represented in Figures 3-8 (not set until nitrate done), can be observed. For example, a beta diversity index of 0.48 exists between D1C1 and D1R1. In this way,

D1C1 and D1R1 are more diverse than D7C7 and D7E7 which have a dissimilarity index of only 0.19.. When D7C7 is then compared to D7R7, for example, a beta diversity index of 0.25 is noted. Additionally, D7E7 compared to D7R7 resulted in a beta diversity index of 0.25. In this way, the day 7 DSS treated fish is more similar to the control fish than the antibiotic treated fishIn this way, antibiotic disruption may be more dramatic than DSS disruption as conducted in this experiment. Furthermore, when samples are compared among groups, the DSS treated groups remain relatively similar to one another, resulting in indices between 0.28 and 0.35 while the control and the antibiotic treated groups remain more dissimilar across their own groups, ranging between 0.27-0.41 and 0.36-0.42, respectively. These patterns can be observed in the NMDS as well. While these patterns are notable, they cannot be adequately explained by either MPO or the nitrate anion. This suggests that rifampicin is more disruptive to the gut microbiome than DSS, which is not a surprise. Alternatively, it may take longer for the DSS effects to manifest.

Histology

Histological samples were prepared at Texas Tech Health Sciences Center and analyzed by Derek Fleming, a post-doctoral research associate in Dr. Kendra Rumbaugh's lab. Dr. Rumbaugh also inspected the images and confirmed Dr. Fleming's conclusions. From all nine samples, it was concluded that the day 3 untreated and day 7 DSS-treated samples were most different from what is regarded as "healthy" or "normal" tissue, which is characterized by a well-defined tissue structure with intact crypts and epithelia. Additionally, there was not an overabundance of leukocytes/lymphocytes beyond what is expected in the colon, which itself houses a great deal of immune cells. In

this way, the first image in the histology section is from the day 7 Rifampicin-treated fish gut and represents normal tissue. Here, there is an observable and defined structure without a massive infiltration of immune cells. On the other hand, in the second and third images, there is significant architectural disruption, reduction of goblet cells, and a noticeable infiltration of immune cells. Of note, Dr. Fleming did not detect the appearance of neutrophils as he has characterized them in mammalian and murine gut samples but did note what could be a large amount of monocytes. Additionally, an in-depth paper on the intestinal histology of *Gambusia affinis* reveals what is seen in our study: folds covered by simple columnar epithelium with the presence of Goblet cells and macrophages. There is no mention of neutrophils, however (bullock). This raises the question of whether it is possible that *Gambusia* lack neutrophils. If they do, then the MPO data is not representative of neutrophil activity, but rather of monocyte/macrophage activity.

While Dr. Fleming did not provide official scores for the histological samples, samples are typically analyzed and severity of disease assessed in the following manner: bowel-wall thickening (0 normal, 1= slightly thickened, 2 = moderately thickened, 3 = severely thickened), intestinal-fold architecture disruption (0 = normal, 1 = slight disruption, 2 = moderate disruption [characterized by increased interfold distance and/or disruption of epithelial integrity], 3 = severe disruption), goblet cell appearance (0 = normal, 1 = decreased in number or size, 2 = complete depletion), and infiltration of leukocyte/ granular cells (0 = sporadic, 1 = some scattered cells, 2 = clusters of infiltrating cells at base of the folds, 3 = massive infiltrates, clusters of infiltrating cells at

the base and tips of the folds). Using this general scale, Dr. Fleming assessed the samples and provided me with his impressions. The samples were de-identified prior to analysis.

The results of the histological analysis suggest that the possible inflammation seen in the day 3 untreated fish and the day 7 DSS-treated fish is likely due to confounding factors and individual heterogeneity and not the result of treatment as both the control and the DSS-treated samples exhibit possible inflammation. The results thus also suggest that the DSS, at least as it is utilized in this study, is possibly not an effective colitogen in this model. This could be due to the fact that the amount of DSS is too low, that the bacterial communities of the fish combat its effects, or that the method of ingestion is ineffective. In a study by Oehelers, zebrafish larvae were exposed to DSS through immersion. In mice, however, DSS is administered through the drinking water.

Finally, the aerobic and anaerobic CFU counts on either nitrate or nutrient agar are inconclusive. Day 1 samples revealed nothing due to possibly vortexing the tissue samples too quickly after placing them in PBST, thereby not allowing the detergent to work properly. Day 3 samples revealed growth, both aerobically and anaerobically and on nitrate and nutrient agar, in the control and DSS-treated samples but not in the antibiotic treated samples. Day 7 samples revealed a significant amount of growth, both aerobically and anaerobically, in the DSS treated samples but not in the control or antibiotic-treated samples. Furthermore, when colonies were inoculated from anaerobic conditions onto nutrient agar and stored under aerobic conditions, growth was noted across samples. Of note, however, was that when day 7 samples were taken from the control fish and the DSS-treated fish, growth was not observed in the control but was observed in the DSS-treated sample.

Additional MPO statistics

An additional focus was placed on MPO activity. Using the same experimental design as before (with the exception that Rifampicin-treated fish were eliminated), a total of five fish were used per category per day. Thus, there were five untreated fish for days 1, 3, and 7 and five DSS-treated fish for days 1, 3, and 7 for a total of thirty fish. The MPO assay was run on each gut sample. For statistical analysis, graphs illustrating mean absorbances with standard deviation bars were created and a pairwise t-test was run on each group comparing untreated to DSS-treated samples:

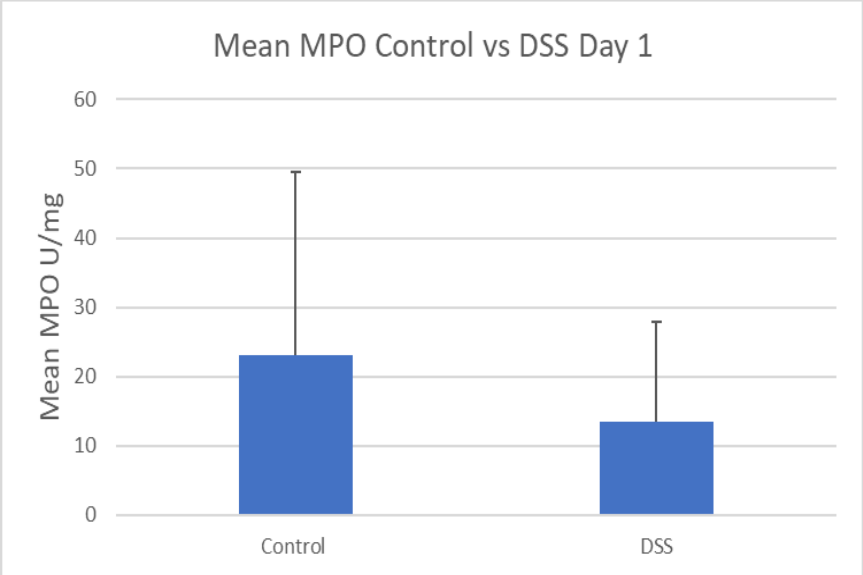


Figure 28. Mean MPO Control vs DSS Day 1

Table 19

T-test on additional MPO samples, n=5

	Control	*DSS*
Mean	23.02	*13.24*
Observations	5	5
Hypothesized mean difference	0	206.963

(continued)

Observations	5	5
t Stat	0.726681	
P(T<=t) two-tail	0.494784	
t Critical two-tail	2.446912	

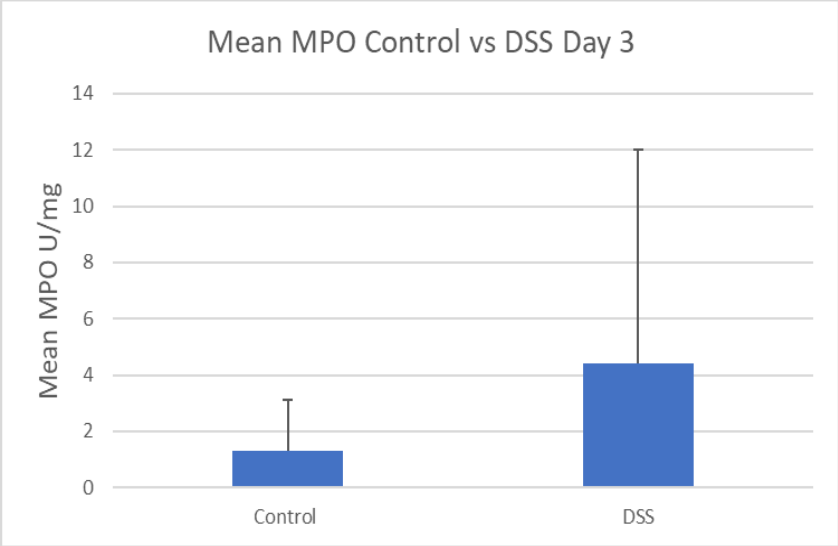


Figure 29. Mean MPO Control vs DSS Day 3

Table 20

T-test on additional MPO samples, n=5

	Control	*DSS*
Mean	1.3	4.364
Observations	5	5
Hypothesized Mean Difference	0	
t Stat	0.8717	
P(T<=t) two-tail	0.432567	
t Critical two-tail	2.776445	

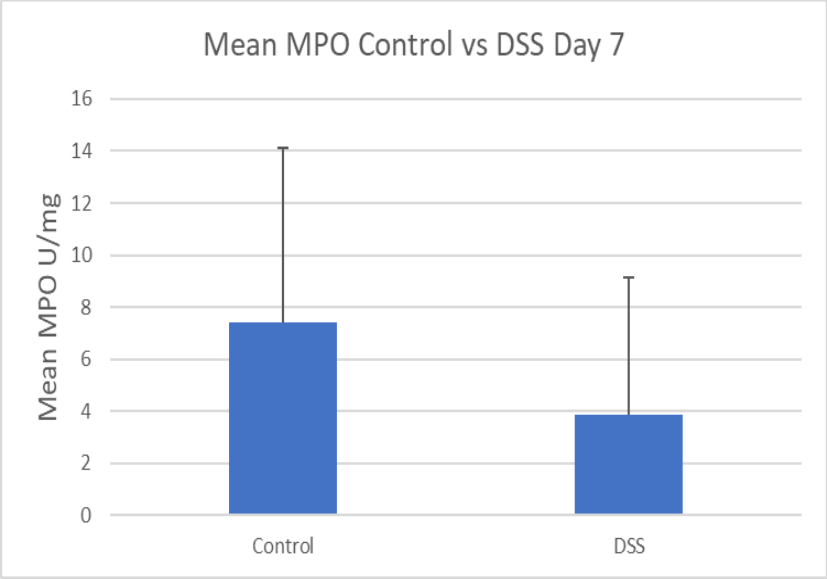


Figure 30. Mean MPO Control vs DSS Day 7

Table 21

T-test on additional MPO samples, n=5

	Control	*DSS*
Mean	7.4	3.86
Observations	5	5
Hypothesized Mean Difference	0	
t Stat	0.922094	
P(T<=t) two-tail	0.383446	
t Critical two-tail	2.306004	

The results of this statistical analysis indicate that there is not a significant difference between the untreated fish versus DSS-treated fish as measured by the MPO assay. Based on the analyses of the data and results of the first set of nine fish, this is not altogether surprising. As previously discussed, it is possible that DSS, at least in the amount utilized in this study, is not sufficient to consistently induce inflammation. It is also possible that the microbiota of the *Gambusia* is such that any inflammation induced by administration of DSS is attenuated, or some other as yet undetermined confounding factor (such as pregnancy or genetic predisposition) may be behind the lack of consistency. Further

studies need to be conducted before conclusions can be drawn. *Gambusia* may be a good gut microbiome model to explore fish-related topics, such as probiotics or antibiotics or pathogens in commercial fish farms. However, this work suggests it is not a good model for mammalian gut inflammatory diseases, due to two primary factors that were uncovered in this study. First, while the healthy mammalian gut is dominated by obligate anaerobes, and a shift to more facultative anaerobes is a disease marker, instead the fish gut is already normally dominated by facultative taxa. Secondly, while a shift to more Proteobacteria is a potential marker of dysbiosis and inflammation in the mammalian gut (4), the fish gut is normally dominated by Proteobacteria. So, while the "oxygen hypothesis" of Winter and colleagues that oxidative radicals released during gut inflammation metabolically drive a shift in the mammalian gut microbiome is very promising for human health, it may not apply to fish. This deserves future study, since fish are the largest group of vertebrates on Earth.

Future Work

While this study contributes to things not previously known about *Gambusia affinis*, it is nevertheless preliminary and exploratory. With such a small sample size, it is impossible to draw conclusions or make predictions with confidence. As such, future work should include a large sample size on which larger comparisons can be made and statistical analyses conducted. Additionally, it would be beneficial to conduct a sequence-based study on a large population of *Gambusia* in order to assess a "core" microbiome that can then be compared to experimentally treated microbiomes. Furthermore, as 20 mg of DSS administered through the gelatin fish food ultimately did not yield consistent and reliable results, future work should include separating fish into

individual Styrofoam cups and conducting a toxicity experiment in which the levels amounts of DSS are increased until there is a mortality. It is possible that 20 mg of DSS simply is not enough to induce colitis in *Gambusia affinis*, which are known for being rather resilient fish.Lastly, it is possible that wild caught *Gambusia* have too many inherent confounding factors to be used reliably in gut microbiome research. Should this study be explored further, it may be beneficial to order *Gambusia* bred in a lab where confounding factors such parasites and pregnancies can be avoided. To explore the potential relationship between nitrate during inflammation and DSS, an additional control group should be used in future experiments. This group of fish would have nitrate added to the food, and effects compared to the DSS-treated group.

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APPENDIX

Permission to use image

Carlson, Jeanette
Tue 11/5/2019 11:22 PM

Yes, of course.

-Jett

Jeanette M. Carlson

Master's student in Dr. Todd Primm's Microbiology Laboratory
Assistant Pre-JAMP camp Coordinator
Department of Biological Sciences
Sam Houston State University

Stetco, Eliana
Tue 11/5/2019 2:57 PM
Sent Items

To: Carlson, Jeanette;

Hello, Jeanette.

Hope you are well!

I am emailing you to ask permission to use an unpublished image (the pie graph of control gut microbiome) in my thesis.

Thank you!

Eliana

VITA

ELIANA STETCO

EDUCATION

2017-2019	M.S. in Biology, *Sam Houston State University*
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2010	B.A. in English, *University of Louisiana at Lafayette*

PROFESSIONAL EXPERIENCE

2019-present	Research Assistant, Houston Methodist Research Institute
2017-present	Graduate Research Assistant, Sam Houston State University
2017-present	Graduate Teaching Assistant, Honors Introduction to Cellular Biology, Sam Houston State University
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2012- 2014	Patient Screening Services Volunteer, Lafayette Community Health Care Clinic
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2009-2010	Tutor, UL Lafayette English Department's Writing Center

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2017-2019	Graduate Research Assistant to Dr. Todd Primm who uses the western mosquito fish, *Gambusia Affinis*, to study the impact antibiotic therapies have on mucosal microbiomes, Sam Houston State University
2013-2014	Undergraduate Research Assistant to Dr. Karen Muller Smith who is recognized for her research in the roles genes play in mammalian brain development and neuropsychiatric disorders, UL Lafayette, Department of Biology
2013	Participant in an intensive two-week course in Neurobiology and Electrophysiology held at the Louisiana Universities Marine Consortium. This program is headed and funded by Dr. Benjamin Hall, Tulane University, Neuroscience Program

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2017-present	Member, Texas Branch of the American Society for Microbiology
2017-present	Member, Biological Sciences Graduate Student Organization
2013-2014	Member, Phi Beta Delta, Honor Society for International Scholars
2013	Invited to participate in the Neurobiology Program at the Louisiana Universities Marine Consortium (LUMCON)
2012- 2013	Volunteer Coordinator, Pre-Professional Society, UL Lafayette
2012-2013	Member, Student Affiliates of American Chemical Society (SAACS), UL Lafayette
2010	Member, Pre-Professional Society, UL Lafayette
2009-2011	Member, Phi Kappa Phi Honor Society
2009-2011	Member, Sigma Tau Delta (English Honor Society
2009, 2010	Judge Social Science Fair

2009-2010	Vice President, Phi Kappa Phi
2009	Member, Popular Culture Association
2008	Recipient of the Doris Meriwether Endowed English Scholarship
2006	Honorable Mention, English Freshman Essay
2005-2006	Member, Biology Club