IMPACT OF NITRITE ON GOLDFISH (*CARASSIUS AURATUS*) MICROBIOMES AND PROBIOTIC DESIGN

by

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TABLE OF CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

I. INTRODUCTION

Gut and Skin Microbiomes

The microbiome is representative of all microbiota, including bacteria, fungi, and viruses, that reside on and within an organism (McFarland, 2000). The microbiome plays an important role in physiological functions throughout the tissues of an organism, from digestion to stimulation of the immune system (Bienenstock et al., 2018). These functions require a specific community of bacteria in balance with each other. Therefore, changes in the composition of the microbiome could compromise the host (Kostic et al., 2019). Dysbiosis is an imbalance of the microbiome that can result in stress or increase susceptibility to an infection (Hooks and O'Malley, 2017). Changes in the microbiome occur naturally throughout the developmental stages and can be influenced by factors including diet, stress, and environmental conditions (Bomar et al., 2018). Moreover, the bacterial composition of the microbiome is differentiated between tissues based on their function (Carthey et al., 2018).

For example, the bacteria that populate the gut microbiome are usually specialized to help the host breakdown food and nutrients. The host would not be able to digest these foods without them, and, in return, the bacteria receive a steady source of nutrients and a relatively stable habitat (Colston and Jackson, 2016). In mammals, dysbiosis of the gut microbiome has been linked to inflammation, intestinal and extra-intestinal disease, and some cancers (Carding et al., 2015). The gut microbiome is compartmentalized into communities that allow the host to control their interactions and prevent pathogens from colonizing the gut. Because of this, the gut microbiome is relatively stable (Coyte et al., 2015). Other tissue microbiomes are not as stable because they are more susceptible to

environmental conditions.

The skin microbiome, for example, has a relatively low bacterial biomass that is continuously exposed to environmental bacteria (Kong et al., 2017). The composition of the skin microbiome also varies depending on what part of the skin the bacteria are colonizing. This means that there is no all-encompassing skin microbiome, but smaller regions that are adapted to the area's specific skin conditions (Sanford and Gallo, 2013). In humans, the stability of the skin microbiome is heavily dependent on the site and can be highly variable between individuals (Grice et al., 2009). The skin microbiome provides cutaneous immunity by out competing potential pathogens. Dysbiosis of the skin microbiome usually occurs in tandem with other chronic conditions and can result in the emergence of opportunistic pathogens that cause ulcers and inflammation (Sanford and Gallo, 2013).

The skin microbiome in fish has also been found to vary between individuals along with species specific skin microbiota. The host is subjected to large amounts of environmental bacteria, but the mucus layer prevents over colonization and helps to maintain a healthy skin microbiome. The skin microbiome helps inhibit the growth of fungal pathogens and serves a similar role as in terrestrial vertebrates. (Larsen et al., 2013; Lowrey et al., 2015)

Nasal Microbiomes

Research tends to focus on the importance of the gut microbiome because of its clear importance in digestion, gut health, and its broad impact on overall immune health (Colston and Jackson, 2016). As a result, the microbiome of the nasal mucosa is poorly

understood, but in mammals it has been linked to changes in odorant detection, appetite, overall immune health, and susceptibility to specific infections (Sepahi and Salinas, 2016; Bomar et al., 2018; Koskinen et al., 2018; Ahmed et al., 2019).

In humans, the nasal mucosa and microbiome is usually studied in the context of human respiratory disease (Ahmed et al., 2019). One function of the nasal microbiome is as a barrier to pathogens because the nose is a portal for disease. The olfactory system is directly connected to the central nervous system which allows it to bypass the blood-brain barrier (Bell et al., 2019). This direct connection, aided by sinus drainage, may allow microbes and their waste and byproducts direct access to the brain through the olfactory bulb (Lafay et al., 1991). These byproducts and small microbes, like viruses, could cause infection, inflammation, or buildup of dangerous proteins resulting in neurodegeneration (Lafay et al., 1991; Bell et al., 2019). Neurodegeneration from protein buildup has been implicated in severe diseases including Parkinson's, Alzheimer's, and Huntington's disease (Bell et al., 2019). Additionally, the nasal microbiome supports the immune system by preventing infection by pathogens (Debertin et al., 2003; Tacchi et al., 2014). Thus, the genera that dominate the nasal microbiome can be used to infer overall respiratory health and can indicate susceptibility to infection, asthma, or even neurological disease (Mika et al., 2015; Bell et al., 2019).

In addition to supporting the immune system, bacteria can secrete metabolites that can help or hinder olfaction by modulating odor threshold, discrimination, and identification (Koskinen et al., 2018). The nasal microbiome may be able to influence olfactory function through the production of secondary metabolites, organic molecules not directly involved in growth or reproduction of the microorganism (Francois et al.,

2016). These metabolites can act as a way for the host and bacteria to communicate and for the microbiome to influence the physiology of the host (Holmes et al., 2011; Koskinen et al., 2018). Additionally, the presence of metabolites can interact with the host to change their physiology directly or, in the case of olfactory function, overwhelm the sensory neurons with a strong odor (Holmes et al., 2011; Koskinen et al., 2018). Studies have found differences in microbiome composition when comparing the microbiome between individuals with normal olfactory function and those with decreased olfactory function (Koskinen et al., 2018). Individuals with lower odor discrimination scores had a greater abundance of bacterial species that are producers of butyric acid, which is characterized by a strong, unpleasant odor (Holmes et al., 2011). Consequentially, prolonged exposure to this odor could decrease olfactory function and is an example of how secondary metabolites indirectly affect olfactory function (Holmes et al., 2011). Although modulation of olfactory sensitivity is not related with acute disease, decreased olfactory function in humans has been tied to bad mood, lessened enjoyment of food, poor hygiene, and diminished social interaction (Koskinen et al., 2018).

In humans, dysbiosis of the nasal microbiome is most relevant with regards to immune function as humans are less dependent on their olfactory sense than other species (Sela and Sobel, 2010). In contrast, many nonhuman mammals with higher dependence on olfaction may be significantly impacted by changes in their olfactory sensitivity or secondary metabolite cues. Nursing age animals depend on olfaction to reliably find their mother, initiate nursing, and identify home areas. Olfaction also influences sexual maturity in some species, and exposure to odors from mature individuals will induce maturation in immature individuals. Many animals also use odor to determine the health

of potential mates and conspecifics. Certain smells may indicate if an animal is sick, they can also mark territory boundaries and roosting sites (Cheal, 1975; Carthey et al., 2018; Maraci et al., 2018). Loss of olfaction would make it difficult for social animals to interact with each other and their environment.

The human nasal microbiome develops in the first year and is shaped by diet, age, and climate (Mika et al., 2015). In other mammal species the timeframe for the development of a unique nasal microbiome is on the same relative timeline (Mika et al., 2015; McDaneld et al., 2019). For example, beef calves experience fluctuations in their microbiome from when they are born leading up until weaning. The changes in microbial composition are unique between individual calves, but healthy individuals show similar patterns in predominant genera (McDaneld et al., 2019). Another mammalian study, with slaughter-age pigs, shows that there are similarities between the structure of the nasal microbiome of humans and pigs. The nasal microbiomes in both species show high diversity with low evenness, meaning only a few genera dominate the microbiome (Weese et al., 2014). The similarities in development and structure of the nasal microbiome are consistent across mammalian species, likely because these bacterial communities have coevolved as a part of the immune system and the olfactory organs (Sepahi and Salinas, 2016).

The limited available literature indicates the nasal microbiome contributes heavily to a functional immune system (Colombo et al., 2015; Colston and Jackson, 2016; Maraci et al., 2018; Weitzman et al., 2018). In addition, the similarities between the structure of the nasal-associated lymphatic tissue (NALT) across vertebrates suggests that the nasal mucosa and immune system evolved to perform similar functions across multiple

vertebrate groups, including fish (Debertin et al., 2003; Tacchi et al., 2014).

Tissue Microbiomes in Fish

Fish are an important group to study for various reasons. They are a major source of protein, especially in developing countries (Llewellyn et al., 2014), which contribute to over 95% of global aquaculture production. In 2017, fish species production was valued at US$ 139.7 billion; as a result, fish production in aquaculture is a major part of the global economy and this demand is only growing as the world population increases (Tacon, 2020).

Fish species appeared over 500 million years ago and are thought to be the first vertebrates on the planet. This paraphyletic group evolved numerous species creating the most diverse and numerous extant vertebrate group, allowing researchers to study the evolution of vertebrates. The diversity of functional adaptations to a wide range of environments means that fish are a unique group that can provide model organisms for various functions ranging from genomics to endocrine systems to behavioral toxicology (Ulloa et al., 2011; Blanco et al., 2018; Hong and Zha, 2019).

Many studies have investigated the gut microbiomes in fish and determined that they are important in preventing disease and aiding in important physiological functions, such as digestion and stimulation of the immune system (Ramirez and Dixon, 2003; Gomez and Balcazar, 2008; Colston and Jackson, 2016). As fish age, their gut microbiome differentiates more from the aquatic environment and plays increasing roles in digestion and immune health (Kelly and Salinas, 2017). Most of the analysis of the gut microbiome focuses on understanding the composition. It has been found that there are

similar phyla despite fish taxonomy or geographic location which indicates that the microbiome plays a similar role in nutrient absorption, digestion, and the immune response. Despite this, diet and environment can impact the composition of the gut microbiome along with the host. The degree to which the environment impacts the composition of the microbiome is unclear, as is how pollutants change the gut microbiome (Talwar et al., 2018). Pollutants, like nitrite, may drastically change the composition of the gut microbiome which would negatively affect the growth, development, and health of the fish. The gut, skin, gill, and nasal microbiomes all play a key role in the mucosal barrier by competing with pathogens and the production of antimicrobial peptides.

Fish are a crucial model organism for studying olfaction. This is because they rely on olfaction for feeding, avoiding predators, territory control, migration, and numerous other behavioral and physiological functions (Kasumyan, 2004). Fish can detect 5 types of chemical odorants, amino acids, steroids, bile acids, nucleotides, and prostaglandins, which are detected by separate receptor mechanisms (Laberge and Hara, 2001). The olfactory organ of most teleost fish is a peripheral epithelial rosette which allows for an interaction between the odorant and the olfactory sensory neuron (OSN) (Hara, 1975). Odorants like amino acids are typically associated with food and can be detected at low concentrations in water. Alternatively, conspecifics release molecules that can serve as a lingering warning if they have been attacked or injured (Olivares and Schmachtenberg, 2019). Unlike some vertebrates, fish depend on a well-functioning olfactory system to mediate both their behavior and physiology. Since fish are aquatic, they must be able to detect water soluble molecules. This makes the olfactory organs particularly vulnerable to

damage from water contaminants (Tierney et al., 2010) and a portal to the brain for pathogens.

Unfortunately, disease is a major problem in aquaculture, where fish are cultured at high densities and water contaminants and pathogens are more likely to occur. Disease is common within the farmed fish stock and is known to spread into wild populations (Lai et al., 2018). Investigating new ways to prevent the spread of disease in these populations could make aquaculture more efficient and protect wild populations. Despite their dependence on olfaction for survival, the nasal microbiome of fish is severely understudied (Tierney et al., 2010; Colombo et al., 2015).

The limited literature shows that fish nasal microbiomes are dominated by Proteobacteria with high variability between individuals and large numbers of unidentified taxa. The microbiome regulates many genes associated with odorant receptors including maintaining healthy olfactory receptors, vomeronasal receptors, and the pseudostratification of the olfactory epithelium. As a result, the microbiota affects the ability of fish to detect and discriminate odors in their environment. These results mirror what are found in other vertebrates, suggesting the nasal microbiome in fish impacts olfaction similarly to other vertebrates (Tacchi et al., 2014).

Additionally, given the similarities between the functions of the gut and skin microbiomes in fish to terrestrial vertebrates, it is possible that the nasal microbiome also shares similar functions with terrestrial vertebrates. If the nasal microbiome has an impact on immune health or olfactory function in fish, then characterizing it would be valuable to conservation efforts or increasing efficiency in aquaculture (Lai et al., 2018). Dysbiosis is known to increase the chance for disease, so knowing what aspects of the

microbiome are important to a healthy organism could serve as an indicator to monitor the health of fish populations (Hooks and O'Malley, 2017).

The importance of olfaction to fish and the unique nature of their olfactory system makes olfaction a key area in researching fish. Despite this, the nasal microbiome is severely understudied. Investigating the microbiome of the nose would fill an important gap in the current knowledge.

The nose in fish is not a part of the respiratory system, which happens in the gills instead. This unique process means both the nasal and gill microbiomes are unique from other vertebrates and the gill microbiome must be studied to understand how the microbiome effects the respiratory system. Despite this, the gill microbiome has not been studied extensively. The gills are a difficult environment for bacteria to colonize due to the continuous water flow. This may limit a stable microbiome to the lamellae and pharyngeal arches, which are more protected. The gill microbiome has some overlap with the skin microbiome although it is less diverse. Both microbiomes are impacted by stress, water quality, nutrition, and can fluctuate seasonally (Merrifield and Rodiles, 2015). The gill and skin microbiome's role has not been well studied outside of its association with the mucosal barrier.

Fish model

Goldfish (*Carassius auratus*) are an important model organism and research on them is generally applied to other fish species (Hansen et al., 1999). They thrive in laboratory settings, being able to tolerate a wide range of water quality and recover relatively quickly from handling and sampling (Blanco et al., 2018). Goldfish have been

well characterized and are a significantly better model organism than most commercial species because they are well understood physiologically, genetically, and behaviorally (Blanco et al., 2018).

Fish use pheromones to school, mark territory, identify conspecifics and mates, and in parent interactions. Alarm pheromones are released and cause other individuals to avoid the area due to predators or injury (Liley, 1982). In goldfish, the response of males to mates is dictated almost entirely through olfaction by pheromones (Partridge et al., 1976). They also use a complex multicomponent pheromone to identify conspecifics at all life stages, and this pheromone may also indicate sexual maturity (Levesque et al., 2011).

Despite extensive goldfish research, information on the goldfish microbiome is limited to the composition of the gut microbiome. The gut microbiome in goldfish is predominantly *Aeromonas* and all contained *Shewanella* species. The composition and diversity of the gut microbiome is affected by diet, where plant-based diets decreased the bacterial diversity (Silva et al., 2011). While the composition of the gill and skin microbiomes have been studied to some extent in other fish species, there is no information specific to these microbiomes or their role.

The microbiomes associated with the gut, gills, skin, and nose are all understudied in goldfish, which means their role and the effect of pollution and disease is unknown. Characterizing the nasal microbiome of goldfish will fill a gap in knowledge about a vital aquatic model organism, and fish in general. This information could be used to understand the interaction between the nasal microbiome and olfaction, which relates to feeding, communication, and immune health which are important parts of conservation,

aquaculture, and general research.

Nitrite Pollution

Pollution is a global issue affecting all parts of the environment. Water pollution is one of the most consequential types of pollution, affecting human health, food production, and the environment in severe ways (Moss, 2008). In addition, up to 80% of oceanic pollution originates on land with agricultural runoff being a top source of contamination (NOAA, 2018). Agricultural runoff contains fertilizer, resulting in localized spikes of nitrogen and phosphorus, which can trigger massive algal blooms and be devastating to the surrounding aquatic life (Moss, 2008). Nitrogen is a dangerous agricultural pollutant because it undergoes nitrification as a part of the nitrogen cycle producing toxic nitrogenous compounds, such as ammonia, nitrate, and nitrite (Fig. 1) (Eddy and Williams, 1986). Nitrite is a normal part of the aquatic ecosystem in minute amounts because it is utilized by prokaryotes and aquatic plants. However, given the inverse relationship between dissolved nitrites and dissolved oxygen in the water, excess amounts can have negative effects (Eddy and Williams, 1986; Kroupova et al., 2005).

Ammonia is oxidized first to nitrite and then to nitrate by nitrifying bacteria in the environment (Figure 1) (Lai et al., 2018). The distribution of nitrifying bacteria is uneven across phyla, however Actinobacteria, Cyanobacteria, Firmicutes, and Proteobacteria are phyla that contain diazotrophs, bacteria that can fix atmospheric nitrogen (Dos Santos et al., 2012). The nitrogen cycle is characterized by very low levels of nitrite in the environment as it is typically oxidized by bacteria faster than it accumulates (Eddy and Williams, 1986). However, accumulation of nitrite occurs when ammonia is not

completely oxidized or excess nitrites are added to a system from outside sources, like pollution (Moss, 2008). The limiting factor in these cases is the presence of ammonia-oxidizing bacteria and archaea (Shiozaki et al., 2016). Additional nitrogenous compounds added to the environment may have a significant impact on the bacteria present in the water column. As a result, these changes could shift the microbiome to species better suited for utilizing nitrogenous compounds (Pramod K. Pandey, 2014).

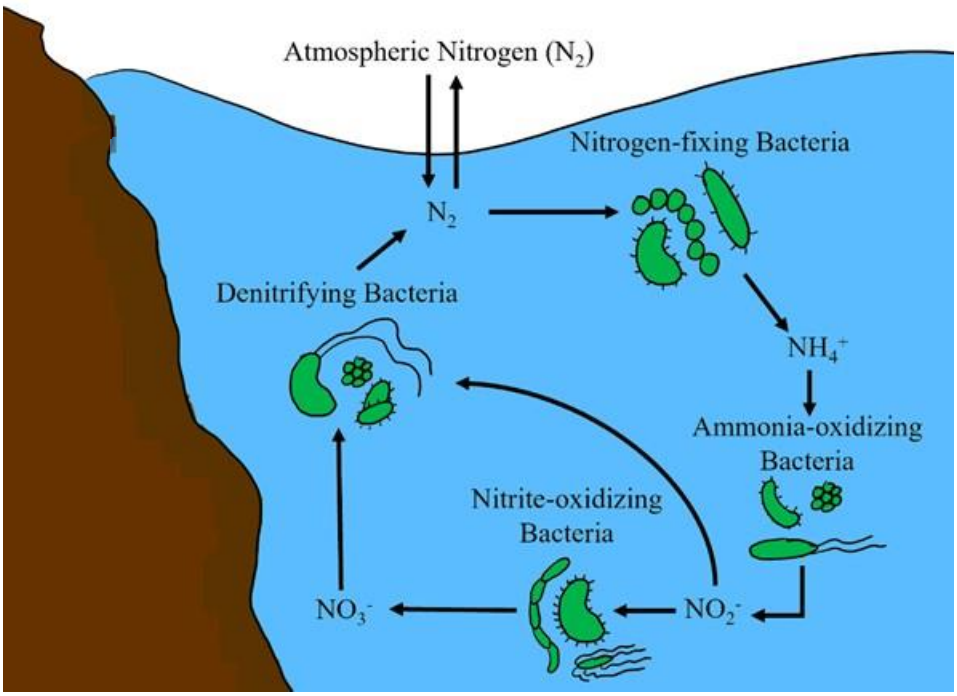


Figure 1. Nitrogen cycle. Nitrification and denitrification steps of the nitrogen cycle. These steps are important aspects of the natural conversion of nitrogen throughout the environment. Nitrification is the conversion of ammonium into nitrite, and then nitrate, and denitrification is the conversion of oxidized nitrogen into nitrogen gas.

Nitrite can accumulate in natural water systems, but it is a particularly big problem in intensive, recirculatory aquaculture systems. These systems use excessive

amounts of proteinaceous feed, nitrogenous fertilizers, and have high fish stock densities which cause a buildup of toxic metabolites, including nitrite. The main sources of nitrogenous compounds in aquaculture are fish waste, dead organisms, uneaten feed and feces, and atmospheric nitrogen. The nitrite levels are usually mediated by ammonia-oxidizing and nitrite-oxidizing bacteria, but an imbalance can exacerbate already elevated levels. Some intensive systems have poor or insufficient filtration systems or use systems which use ultraviolet irradiation to disinfect water which converts nitrate to nitrite (Ciji and Akhtar, 2020). There is a focus on keeping nitrite below toxic concentrations, but it is unknown how sublethal levels impact fish microbiomes.

The negative effects of nitrite on fish are well documented and it is toxic in relatively low concentrations (Hanson and Grizzle, 1985). A main culprit in nitrite exposure is its ability to oxidize hemoglobin to methemoglobin which is unable to effectively bind oxygen (Tomasso, 1986; Williams and Eddy, 1986; Jensen et al., 1987; Kroupova et al., 2008; Kroupova et al., 2018). However, nitrite exposure impacts almost every physiological system in fish and can devastate the regulatory systems fish have. Nitrite can enter the fish through the skin or gut epithelium but is primarily absorbed through the gills. This is done by competing with chloride ions in the chloride exchanger at chloride cells. As a result, nitrite has been linked to elevated levels of plasma potassium, decreased levels of plasma chloride, and reduced production of hydrogen ions (Gisbert et al., 2004; Evans et al., 2005). Active uptake by the chloride cells means that freshwater fish are far more susceptible to environmental nitrite than their marine counterparts (Wright et al., 1989). Nitrite exposure has been shown to induce clubbing, fusion of the secondary lamella, disruption of the mucus and chloride cells, and damage

to the gill epithelium (Tomasso, 1986; Kroupova et al., 2018; Martinez and Huertas, 2019).

The nose may be more sensitive to nitrite exposure than other associated lymphatic tissues, because of its structural anatomy and physiology. In teleost fish, the water comes into direct contact with the olfactory receptor neurons because they need to detect soluble molecules in the water. Additionally, the water travels into the anterior nares, through the nasal cavity, and out the posterior nares, meaning that the nasal cavity is continuously saturated with environmental water. Other tissues are likely better at regulating nitrogenous exchange with water. The gills, for example, play an important role in osmotic regulation and have various systems in place that allow them to remove ions and contaminants from the tissue (Blanco et al., 2018). Internal tissues, like the gut, are also less sensitive to water contamination because the tissue is not in continuous contact with the environmental water and both the gills and gut can actively filter nitrite from the tissue.

Nitrite pollution is a major problem in many water systems and especially in areas with farmed fish, aquaculture, or agricultural runoff (Moss, 2008). Ascertaining the effects of nitrite exposure at sub-lethal concentrations may indicate problems at levels that would normally be considered safe. This information might inform conservation efforts, legislation involving acceptable nitrite levels, and aquaculture.

Fish Probiotics

Studying the changes to the microbiome caused by sub-lethal nitrite exposure could reveal changes that make fish more susceptible to infection. This increased

susceptibility could be devastating in aquacultural settings. In fact, fisheries tend to have higher concentrations of nitrite than is naturally found in the environment (Ciji and Akhtar, 2020). This means that any damage caused by nitrite should be more apparent in these fish populations. If nitrite changes the nasal microbiome significantly, then fish in sublethal and nontoxic concentrations of nitrite, could still be negatively affected. It may not always be possible to remove the nitrite from the environment. In this case it could be possible to use a probiotic to support the bacteria found in a healthy microbiome. Probiotics are live cultures of microorganisms that provide health benefits, and are typically used to promote gut health by introducing bacteria that compete with pathogenic bacteria and help metabolize acids (Mombelli and Gismondo, 2000).

Probiotics serve several functions depending on the bacteria they are composed of and the system they are supporting. They can boost immune health by activating the immune system and competing with pathogens for resources like space and nutrients (Kelly and Salinas, 2017). Alternatively, if the probiotic is a bacterium that is normally associated with the microbiome but has been lost, the probiotic can help replace the lost bacteria and recolonize. Thus, preventing opportunistic pathogens from emerging and filling the niche of the lost bacteria.

Probiotics have been successfully used in aquaculture to improve immune health, growth, and feed conversion (Williams and Eddy, 1986). The aim of a probiotic during nitrite exposure would be to support the nasal microbiome of the fish. As mentioned, fish rely heavily on olfaction and the physiology of the nose may make it sensitive to damage from pollutants. If the nasal microbiome helps with olfaction, like in other vertebrates, a nasal probiotic may help recover any damage caused to the tissue or recuperate their

sense of olfaction. A probiotic derived from the healthy nasal microbiome would hypothetically serve these functions with little risk to the fish. There is evidence that probiotics administered orally can support the health of the nasal microbiome in other vertebrates (Dimitri-Pinheiro et al., 2020). Assisting the nasal microbiome would not necessarily decrease the physiological damage in tissues done by the nitrite but could combat the negative effects of a disrupted microbiome. Additionally, given the association between stronger olfactory ability and a healthy microbiome, it could help return olfactory function to fish.

Aims

I **hypothesize that sublethal concentrations of nitrite in the water will cause dysbiosis of fish microbiomes.** As a result, fish exposed to nitrite will experience changes in the nose, gill, gut, and skin microbiomes that could make them more vulnerable to disease. The dynamics of microbial changes during nitrite exposure will inform possible probiotic formulation. These specific bacterial groups will be more resilient against pathogens in a wide range of nitrite concentrations. The probiotic candidates identified in this study would hypothetically be used to correct the vulnerable state generated by dysbiosis.

To test our hypothesis, we devised two aims for the study.

Aim 1: To characterize the nasal microbiome of goldfish and identify the changes to various tissue microbiomes when subjected to sublethal concentrations of nitrite.

Aim 2: To identify potential probiotics that could be used to mitigate the effects

of nitrite exposure.

II. IMPACT OF NITRITE ON GOLDFISH MICROBIOMES

Introduction

Despite the prevalence of nitrite in aquaculture, the effect of nitrite on tissue microbiomes is unstudied. Each of these tissues plays an important role in fish health which could be disrupted if nitrite causes dysbiosis to the tissue microbiomes. Considering the importance of aquaculture as a food source, reducing pressure on wild stock and reducing the negative impacts of farming on the environment, it is necessary to understand all aspects of disease prevention and immunity, (Llewellyn et al., 2014). The tissues examined in this study all have important physiological functions and are key members of the immune system as mucosal-associated lymphoid tissues (MALTs) (Merrifield and Rodiles, 2015; Parra et al., 2015; Das and Salinas, 2020). The MALTs interact with the microbiome of each tissue to shape the immune response, and disruption of the microbiome can disrupt the immune response in these tissues (Kelly and Salinas, 2017). Therefore, the goal of this study was to determine how the microbiome changes because of sublethal concentrations of environmental nitrite. If sublethal nitrite disrupts the microbiome, then levels that are currently considered healthy, may be harmful.

The nasal microbiome, in particular, is significantly understudied across the animal kingdom, but especially in many aquatic species who use olfaction as their primary sense (Colston and Jackson, 2016). There is limited information on the normal nasal microbiome in fish, though it has been linked to maintenance of both the olfactory neurons and the epithelial structure (Casadei et al., 2019). It has been completely unstudied in goldfish, which is limiting to the goldfish as a model species. The nasal microbiome needs to be characterized before studying how it can be manipulated by

pollutants like nitrite.

Materials and Methods

1. *Nitrite Exposure and Sample Collection*

Goldfish were acquired from a certified local hatchery and allowed to acclimate in a recirculating living stream with aerated water at 25°C for 1 week. Fish were fed TetraFin Goldfish Vitamin C Enriched flake food once a day for the duration of the experiment. After acclimation, they were transferred and allowed to acclimate for 1 week in a continuous flow water aquarium system. This system had a 200 L tote that served as a water reservoir for each of the four treatments and was feeding water by gravity to four aquariums. Each tote was refilled every 2 days. The water continuously flowed from the tote to the aquariums and out of the aquariums to allow for a consistent concentration of nitrite while otherwise maintaining proper water quality by allowing a complete replacement of water in the aquarium twice a day (Figure 2). Each treatment had 4 replicate tanks with 10 fish per tank.

Figure 2. Continuous flow water setup. Depicted is the setup for two of the nitrite treatments. Water was added into the tote every 2 days and flowed down into the aquariums using gravity at a rate of 10-15 mL/ second. The water then flowed out of the aquariums and into a drainage system. This setup was designed to prevent the accumulation of nitrate and ammonia and keep the nitrite concentration at the desired level.

After the acclimation period, the fish were exposed to one of four nitrite treatments (0.0 mM, 0.01 mM, 0.1 mM, or 1.0 mM) for 2 months. During this time, water samples were collected and nitrite, nitrate, ammonium, and pH levels were monitored. Any goldfish that died were immediately recorded and removed.

After 2 months, the fish were anesthetized with Tricaine mesylate (MS-222) at a concentration of 0.8 g/ L before being sacrificed. The nose was dissected out of the head and collected in its entirety. The tail was clipped to serve as a skin sample. Gut samples were collected by removing the remaining fecal material from the intestines. The gills were also dissected out in one piece to preserve any bacteria that might be associated between the gill lamellae. A subsample of 2 L of water were collected from each tank to determine if the tissue microbiomes were unique from the water microbiome. All tools and surfaces were disinfected with 70% ethanol between each fish and tissue to minimize contamination. Each tissue was placed in a sterile microcentrifuge tube and stored at -20°C until processing. The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Texas State University (IACUC # 7074).

2. *Analysis of nitrite.*

The water that was collected during exposure was analyzed using the Invitrogen fluorometric Measure-iT High Sensitivity Nitrite Assay for the 0.0 mM and 0.01 mM treatments and the Sigma-Aldrich Nitrite/Nitrate colorimetric Assay Kit for the 0.1 mM and 1.0 mM nitrite treatments. To do the colorimetric assay, standards were made to create a standard curve. 100 µL of the standards and the samples were added to a 96-well plate and then 100 µL of Griess solution was added to each well. The plate was shaken and allowed to sit for 5 minutes before being read at 570 nm. Standards were also made to conduct the Measure-iT High Sensitivity Nitrite Assay, and the assay was conducted according to the assay protocols. The fluorescence was measured with an excitation/emission of 365/450 nm. The results for both assays were then analyzed using Prism 9 (GraphPad).

3. Microbiome Analysis of Tissue Samples

DNA was extracted from tissue samples from 2 fish from each of the 4 tanks from each treatment, giving a total of 8 tissue samples per treatment. The DNA was extracted using the QIAamp BiOstic Bacteremia DNA Kit and the procedure dictated by the kit. The water samples were filtered using Durapore 0.22 µm PVDF membranes and the DNA was extracted again using the Qiagen QIAamp BiOstic Bacteremia DNA Kit. The 16s rRNA gene was then amplified using KAPA Taq and primers 515F (5'-GTGCCAGCMGCCGCGGTAA) and 806R (5'-GGACTACHVHHHTWTCTAAT). The PCR products were tested for amplification using gel electrophoresis with a 1.5% agarose gel for 20 minutes at 90 V. Any samples that showed amplification underwent a second PCR to add barcode primers that would allow the identification of each sample after sequencing. The second PCR products were purified using the Applied Biosystems ExoSap- IT PCR Product Cleanup kit and each sample was quantified using the Invitrogen Qubit dsDNA BR assay kit. The samples were diluted to 10 ng/µL, combined to form a library, and stored at -20°C until sequencing.

The DNA was sequenced using Illumina MiSeq sequencing and was analyzed using R Studio. The samples were trimmed using a minimum quality score of 30 and filtered with a maximum of 5 ambiguous bases. The minimum overlap was determined for each tissue to maximize the number of reads. All samples except the gut samples were then decontaminated using the 0.5 filter of R decontam. There appeared to be some contamination in the negative control that was affecting how the gill and gut samples were being decontaminated. It was determined that the gut samples could not be

decontaminated without removing amplicon sequence variants (ASVs) normally associated with the gut, so these samples were not decontaminated.

The resulting ASVs were then analyzed using microbiomeanalyst.ca Marker Data Profiling. The minimum count of the low count filter was changed to 2 and the data transformation was changed to centered log ratio, while all other settings were left as the default.

4. Statistical analysis

The nitrite water concentration was analyzed using Prism 9 (GraphPad co) to graph the nitrite concentrations with mean ± SEM to show the variation between the four treatment replicates.

The resulting ASVs were then analyzed using microbiomeanalyst.ca Marker Data Profiling (McGill University)(Dhariwal et al., 2017). The minimum count of the low count filter was changed to 2 and the data transformation was changed to centered log ratio, while all other settings were left as the default. The relative abundance of each of the microbiomes was visualized using the Stacked bar/area plot option with the desired taxonomy level. The stacked bar plot used percentage abundance showing the top 10 taxa based on the total number of taxa. Significance was determined using a DESeq2 (Bioconductor) differential abundance analysis method with an adjusted P-value cutoff of 0.05.

The significant difference between overall microbial communities was determined using Past3 (PAlentological STatistics). This was determined using a one-way PERMANOVA multivariate test to create a pairwaise Bray-Curtis comparison with Bonferroni-corrected P-values.

Results

Water Quality Analysis

The control treatment did have some accumulation of nitrite which ranged between 0.0 mM and 0.02 mM. The 0.01 mM treatment was slightly more consistent, a little under 0.01 mM. Two days, 2/18/2020 and 3/7/2020, had large spikes in the nitrite concentration that lasted one day. The 0.1 mM treatment ranged between 0.025 mM and 0.05 mM and the 1.0 mM treatment ranged between 0.2 mM and 0.6 mM (Figure 3). When the water was tested using LifeGard all-purpose 5-way test strips, there were differences in the nitrite and nitrate concentrations (Figure 4).

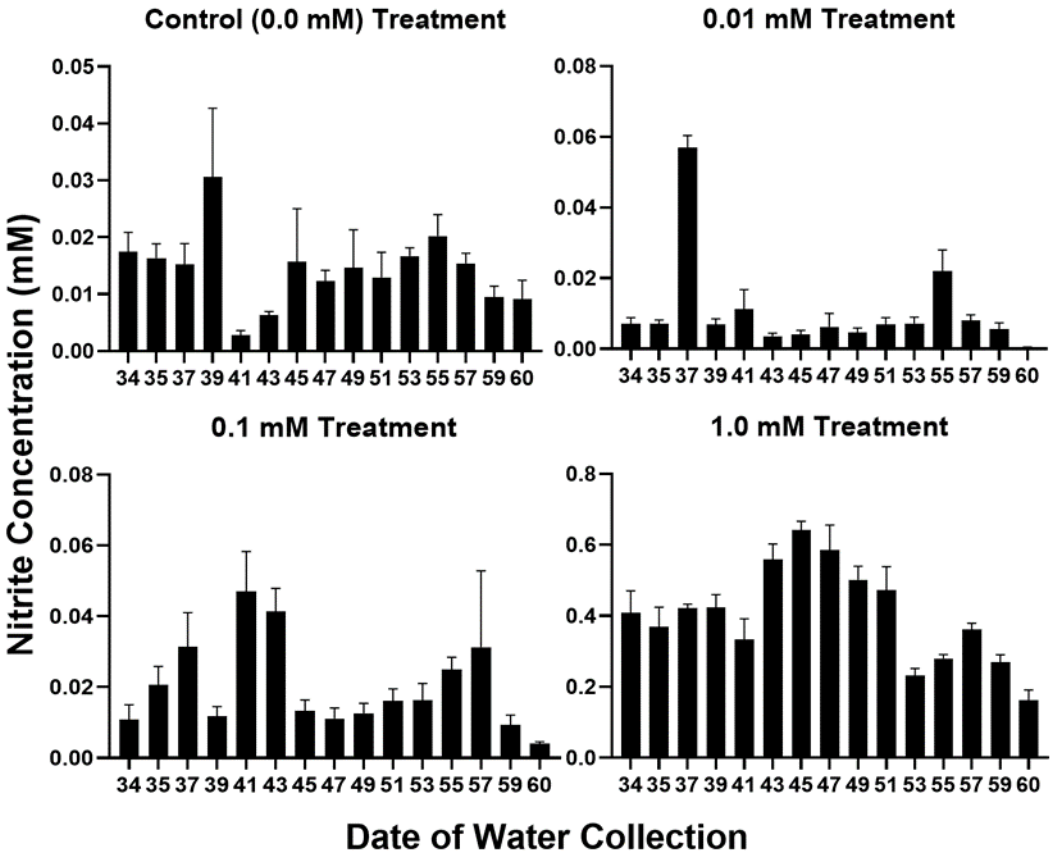


Figure 3. Nitrite concentration analysis. Nitrite water concentration for the different treatments during the nitrite exposure. The 0.0 mM and 0.01 mM treatments were not significantly different from each other, but the 0.1 mM and 1.0 mM treatments were both significantly different. The concentration of the 0.01 mM, 0.1 mM, and 1.0 mM treatments were all lower than the target concentration. Significance was determined using a one-way ANOVA, $p<0.05$.

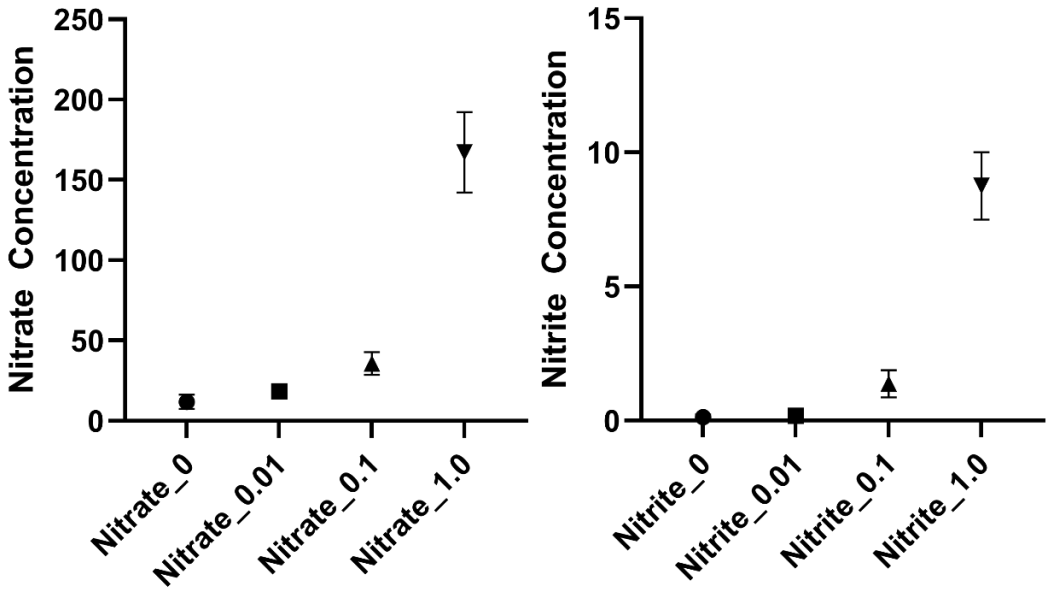


Figure 4. Nitrate/Nitrite concentration during exposure. Concentration of nitrate and nitrite taken during the nitrite exposure with water test strips. Nitrate increased as the nitrite concentration increased in the treatment.

Water microbiome

The water microbiomes were not significantly impacted by nitrite exposure. There was a decrease in Rhizobiales in the nitrite treatments and an increase in Azospirillaceae

in the 0.01 mM and 0.1 mM treatments (Figure 5). In total, there were 17 ASVs, 8 genera, 3 classes, and 1 phyla determined to be significantly different between at least two of the treatments (Table 7). Notably, none of the treatments were significantly different from each other despite some changes in the relative abundance of the classes.

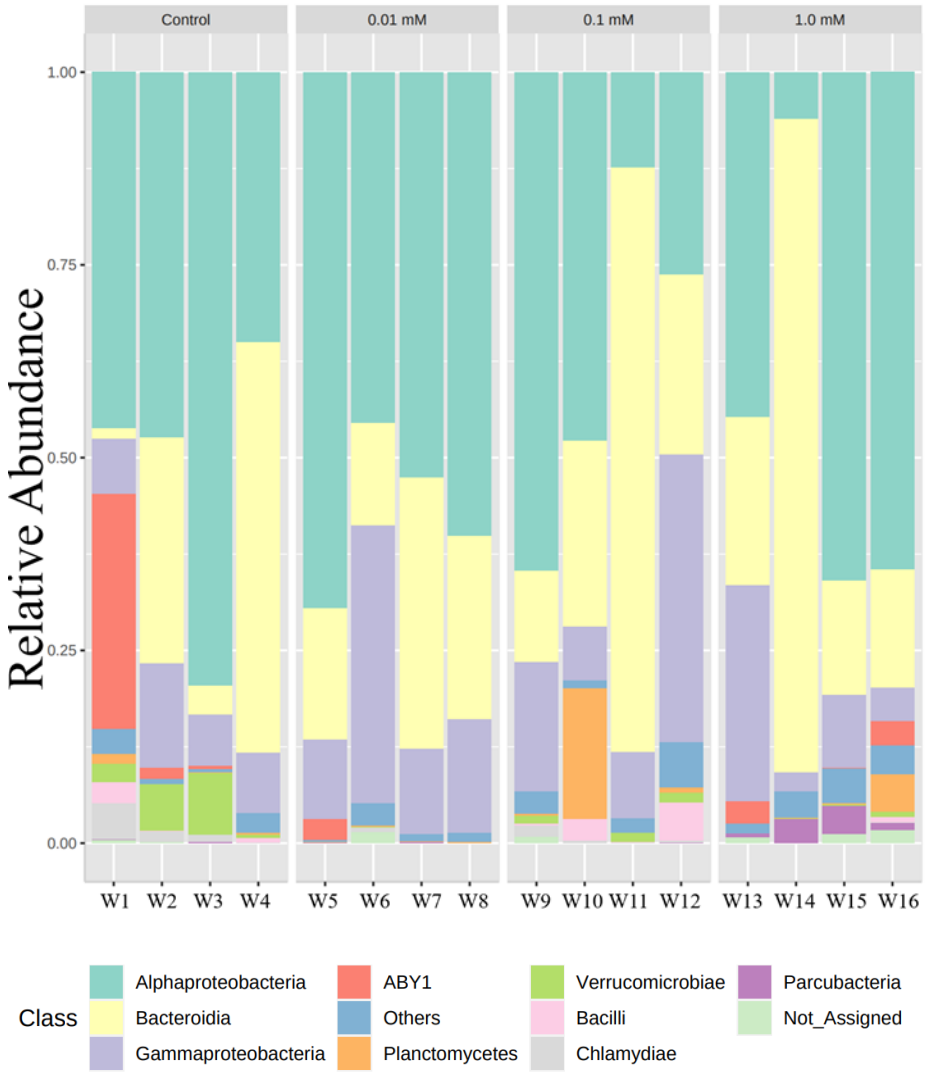


Figure 5. Relative abundance of water microbiome classes. Relative abundance of the top ten most abundant classes represented in the water microbiome across various nitrite treatments. There was an increase in Bacteroidia and Gammaproteobacteria. The 1.0 mM

treatment had Parcubacteria, which did not appear in the other treatments.

Microbiome in tissues within nitrite treatments

All the tissues and water showed several bacteria that changed significantly in the presence of nitrite, but most of them only changed in their respective microbiome. There was little overlap in the genera that changed between the tissues, but *Nitrobacter* increased in the gut, gills, and water and *Azospirillum* increased in the gills and water microbiomes (Figures 6 & 7).

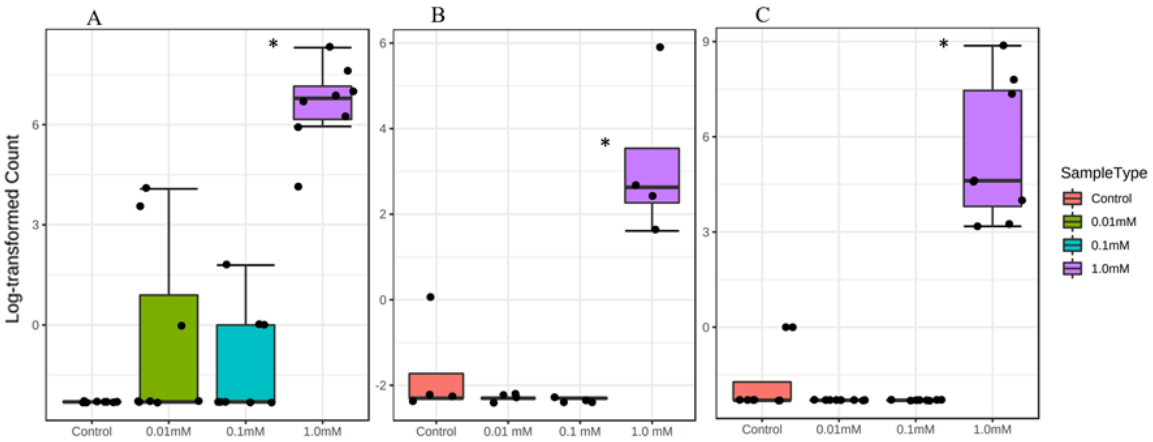


Figure 6. Log-transformed counts of *Nitrobacter.* Box plot of log transformed abundance of *Nitrobacter* counts among the nitrite treatments the gills (A), gut (B), and water (C). Asterisk denotes a significant difference (differential expression analysis (DESeq 2), $P < 0.05$).

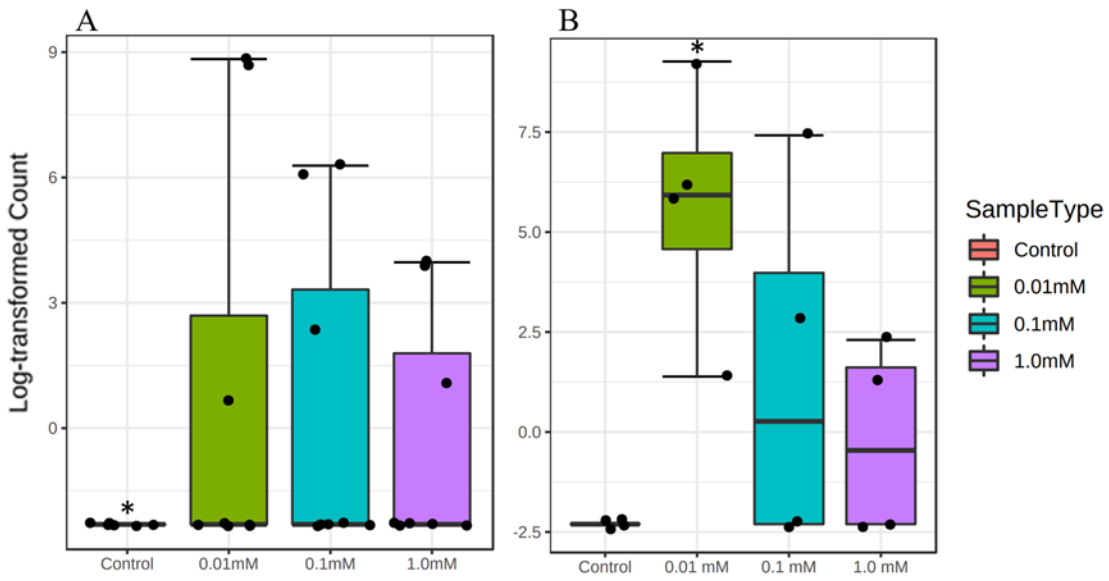


Figure 7. Log-transformed counts of *Azospirillum*. Box plot of log transformed abundance of *Azospirillum* counts among the nitrite treatments in the gills (A) and water (B). Asterisk denotes a significant difference (differential expression analysis (DESeq 2), $P < 0.05$).

The untreated nasal microbiome was first characterized to better inform how nitrite exposure influenced the microbiome. The nasal microbiome is dominated by Proteobacteria with one outlier (N2) which is 50% Dependentiae and 50% Proteobacteria. At the class level, the Proteobacteria is further classified as Gammaproteobacteria. Finally, at the genus level the microbiome is broken down into 25-50% *Pseudomonas*, 25% *Yersinia*, and smaller amounts of *Shewanella*, *Branchiomonas*, and an others group. N2, the outlier, was composed 50% of Babeliae, which was unclassified at the genus level (Figure 8).

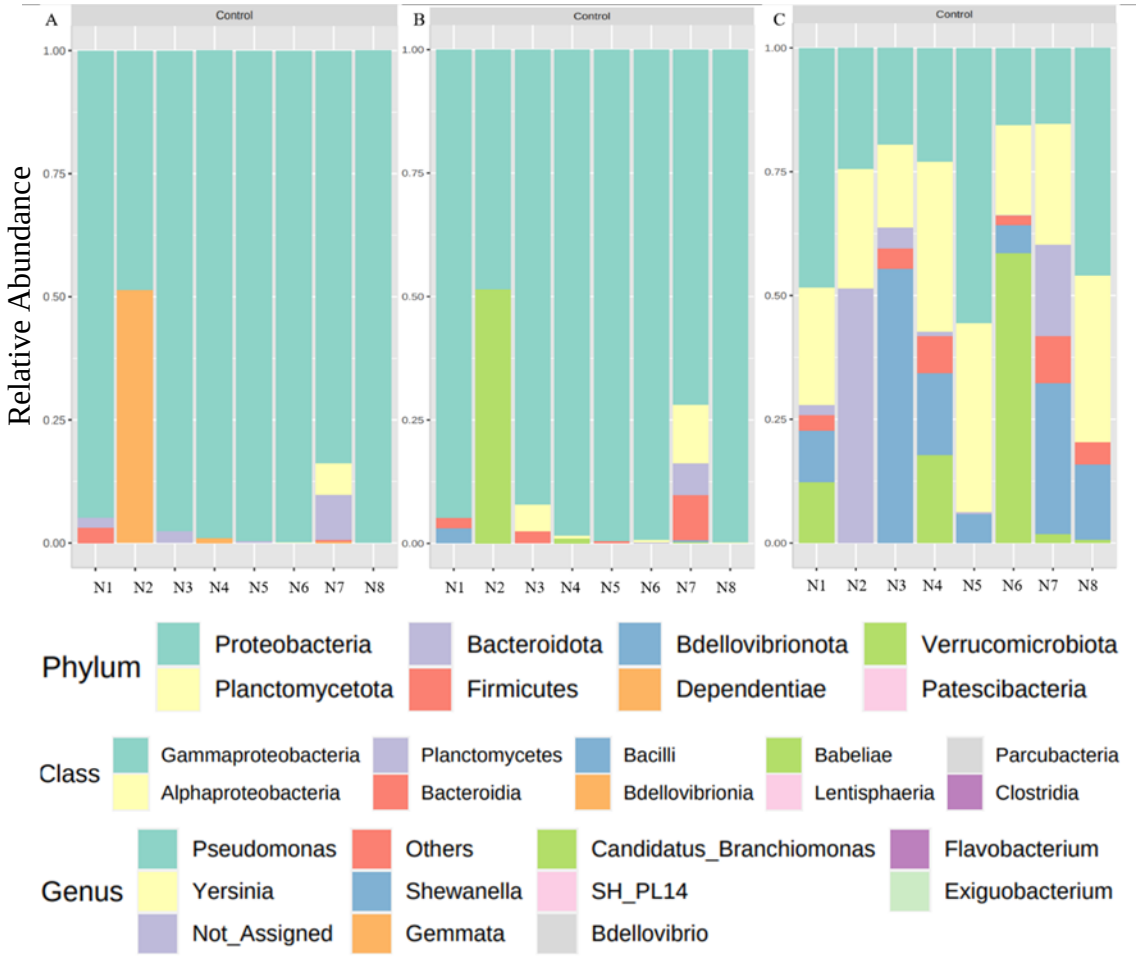


Figure 8. Relative abundance of untreated nasal microbiome. Nasal bacterial community in unexposed goldfish at the phyla (A), class (B), and genus (C) classifications. The samples are labeled N1-N8 which indicates that these are the nasal microbiomes of eight different fish. All fish labeled with the same number are the same fish. The nasal microbiome is mainly composed of Gammaproteobacteria with between 25-50% *Pseudomonas* and 25% *Yersinia*.

The untreated nasal microbiome was predominantly composed of Gammaproteobacteria. The nitrite treatments showed an increase in the number of classes present in the microbiome and a decrease in the amount of Gammaproteobacteria. These

changes were not consistent between individuals and there was no pattern of bacteria that were more prominent, only the disappearance of Gammaproteobacteria (Figure 9). Using a one-way PERMANOVA, only the 0.1 mM and 1.0 mM treatments were determined to be significantly different than the control with the 1.0 mM treatment also being significantly different from the 0.01 mM treatment (Table 1). In total, 4 ASVs, 3 genera, 3 classes, and 1 phyla was determined to be signifnicantly different in at least two treatments (Table 7).

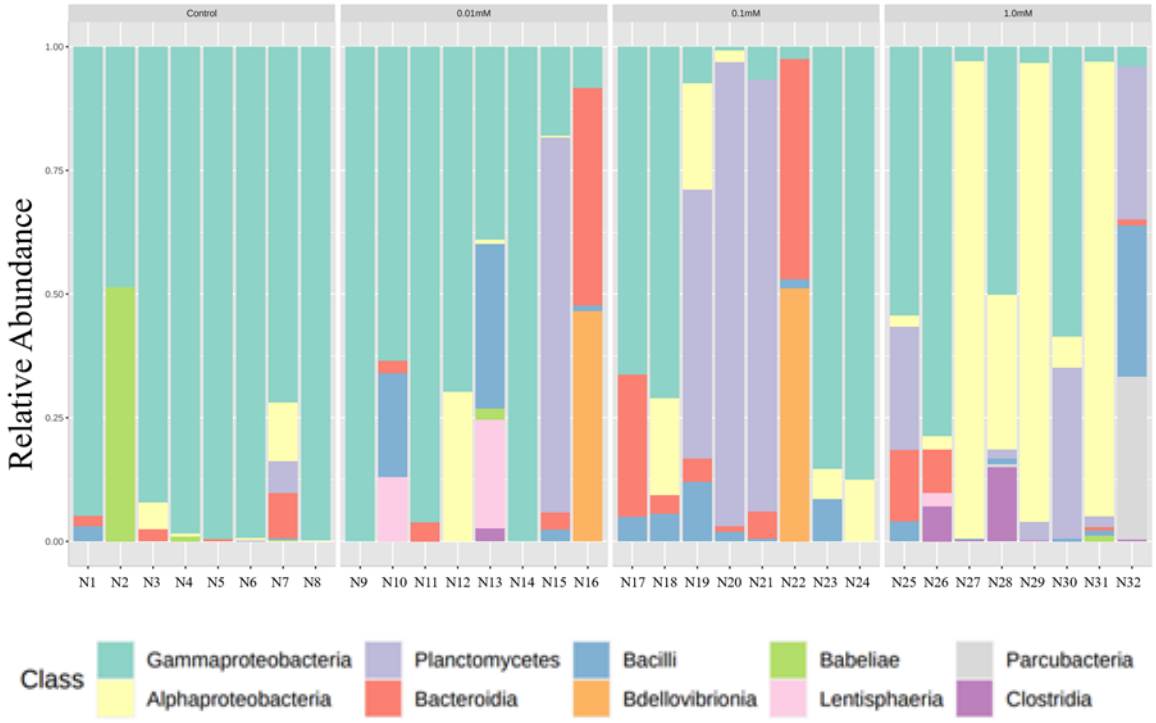


Figure 9. Relative abundance of nasal microbiome classes. The top ten classes represented in the nasal microbiome were examined across various nitrite treatments, (0.0 mM, 0.01 mM, 0.1 mM, and 1.0 mM). As the concentration of nitrite increased more classes of bacteria were incorporated into the microbiome.

Table 1. One-way PERMANOVA of nasal microbiomes. Significance was determined using Bray-Curtis and with p-values corrected using Bonferroni-corrected values ($P < 0.05$). These microbiomes were compared across the nitrite treatments. Only the 0.1 mM and 1.0 mM treatments were significantly different from the control and the 1.0 mM treatment was also significantly different from the 0.01 mM treatment.

	CONTROL	**0.01 mM**	**0.1 mM**	**1.0 mM**
CONTROL		1	0.0024*	0.0024*
0.01 mM	1		0.9684	0.021*
0.1 mM	0.0024*	0.9684		0.0738
1.0 mM	0.0024*	0.021*	0.0738	

In the gut, the 0.01 mM treatment had a different composition than the control and other nitrite treatments. This treatment had an increase in Fusobacteria, Bacteroida, Gammaproteobacteria, Bacilli, and unassigned classes while having less Alphaproteobacteria and Actinobacteria. The 0.1 mM and 1.0 mM treatments were more like the control, although there was still a decrease in Alphaproteobacteria and an increase in Fusobacteriia (Figure 10). In total, 18 ASVs, 6 genera, and 1 class were determined to be significnalty different in at least two treatments, no phyla changed significantly (Table 7). Similarly, to the nasal microbiome, the 0.01 mM and 0.1 mM treatments were significantly different from the control and the 0.1 mM treatment was different than all other treatments (Table 2).

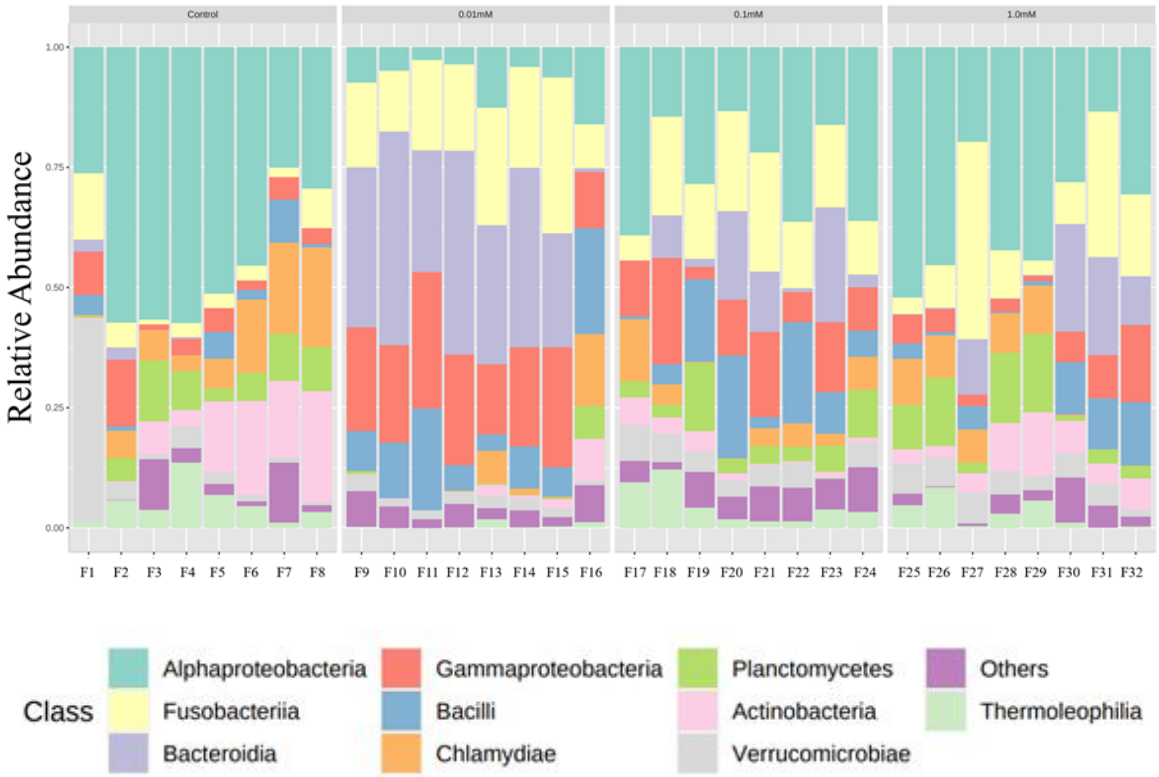


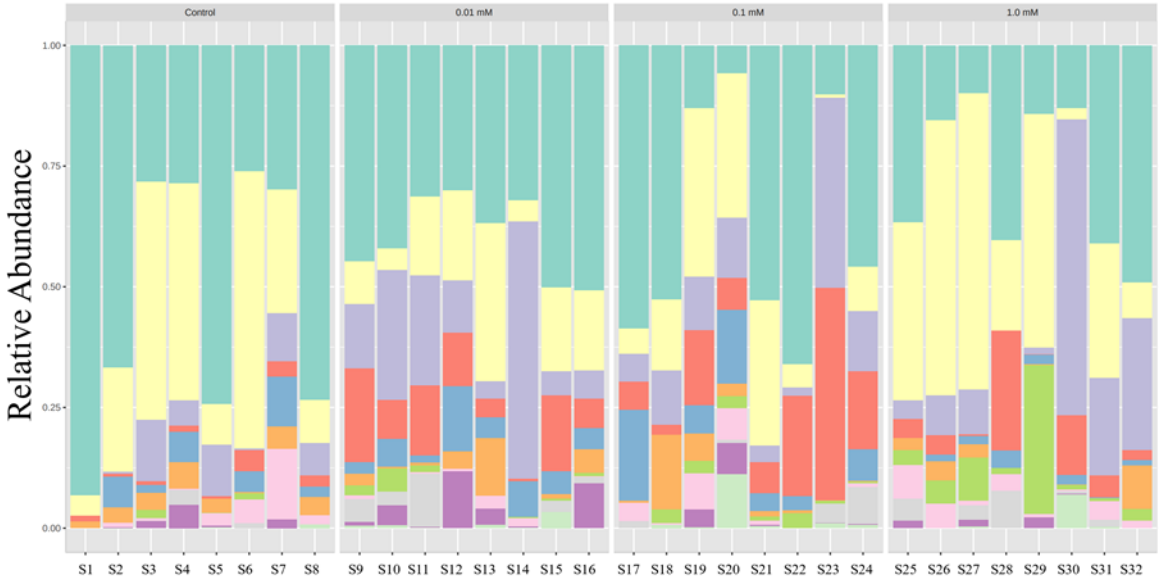
Figure 10. Relative abundance of gut microbiome classes. The top ten most prominent classes represented in the gut microbiome were examined across various nitrite treatments, (0.0 mM, 0.01 mM, 0.1 mM, and 1.0 mM). The 0.01 mM treatment had a different overall composition than both the control and other nitrite treatments. The 0.1 mM and 1.0 mM treatments had a composition more similar to the control, but there was a decrease in the amount of Alphaproteobacteria and an increase in Fusobacteria and Bacilli.

Table 2. One-way PERMANOVA of gut microbiomes. Significance was determined using Bray-Curtis and with p-values corrected using Bonferroni-corrected values ($P < 0.05$). These microbiomes were compared across the nitrite treatments. All the treatments were significantly different from each other except the 0.1 mM and 1.0 mM treatments

were not significantly different from each other.

	CONTROL	0.01 mM	0.1 mM	1.0 mM
CONTROL		0.0024*	0.0018*	0.087
0.01 mM	0.0024*		0.006*	0.0072*
0.1 mM	0.0018*	0.006*		0.1458
1.0 mM	0.087	0.0072*	0.1458	

The relative abundance of bacterial classes in the skin microbiome showed variation within and between treatments. There were some samples that were composed mainly of unassigned classes while some other samples had no unassigned classes. There was a clear increase in Bacterodia and Fusobacteria in the 0.1 mM and 1.0 mM treatments (Figure 11). In total, 4 ASVs and 2 genera were determined to be significantly different in at least two treatments, no classes or phyla were significantly changed (Table 7). The 1.0 mM treatment was significantly different from both the control and 0.01 mM treatments (Table 3).



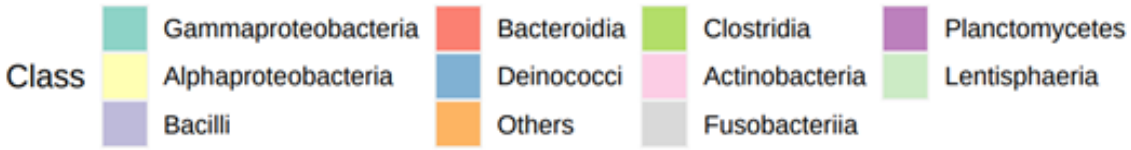


Figure 11. Relative abundance of skin microbiome classes. The top ten most abundant classes represented in the skin microbiome were examined across various nitrite treatments. The skin microbiome is variable both within and between treatments.

Table 3. One-way PERMANOVA of the skin microbiomes. Significance was determined using Bray-Curtis and with P-values corrected using Bonferroni-corrected values ($P < 0.05$). These microbiomes were compared across the nitrite treatments. The control and 0.01 mM treatments are significantly different from the 1.0 mM treatment.

	CONTROL	**0.01 mM**	**0.1 mM**	**1.0 mM**
CONTROL		0.1332	0.9288	0.0174*
0.01 mM	0.1332		1	0.0108*
0.1 mM	0.9288	1		1
1.0 mM	0.0174*	0.0108*	1	

In the gills, there was an increase of Planctomycetes and Gammaproteobacteria. The 0.1 mM and 1.0 mM treatments also had higher levels of these groups than the control, but less than the 0.01 mM treatment (Figure 12). In total, 42 ASVs, 20 genera, 2 classes, and 1 phyla were found to be significantly different in at least two treatments (Table 7). The 0.01 mM and 1.0 mM treatments were significantly different than the control treatment, with the 0.01 mM treatment being different than all the other treatments (Table 4).

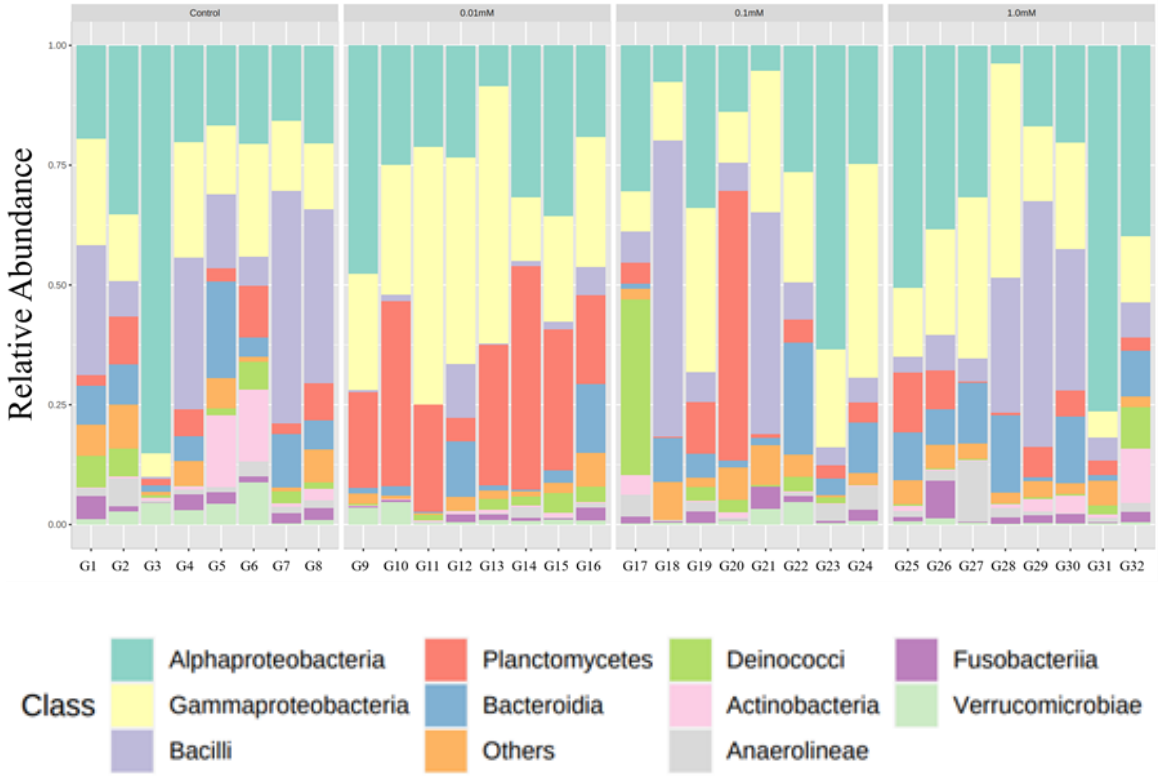


Figure 12. Relative abundance of gills microbiome classes The top ten classes represented in the gill microbiome were examined across various nitrite treatments, (0.0 mM, 0.01 mM, 0.1 mM, and 1.0 mM.) There appears to be an increase of Planctomycetes in the 0.01 mM treatment.

Table 4. One-way PERMANOVA of the gill microbiomes. Significance was determined using Bray-Curtis and with p-values corrected using Bonferroni-corrected values ($P < 0.05$). These microbiomes were compared across the nitrite treatments. All the treatments had significantly different compositions except the control and 0.1 mM treatments.

	CONTROL	**0.01 mM**	**0.1 mM**	**1.0 mM**
CONTROL		0.0012*	0.3948	0.0084*
0.01 mM	0.0012*		0.0042*	0.0018*
0.1 mM	0.3948	0.0042*		0.2244
1.0 mM	0.0084*	0.0018*	0.2244	

Discussion

The concentration of nitrite in the water was determined to be lower than the theoretical concentration. A possible explanation is that the nitrite was being converted into nitrate by the bacteria present in the water. The water flow rate was the same for all the treatments, but the concentration of nitrate increased along with the concentration of nitrite indicating that nitrification was occurring in the system. If this is true, nitrite would have been converted into nitrate, decreasing the concentration that could be detected. The tissue microbiomes were also shown to be significantly different between the 0.0 mM and 0.01 mM treatments in the gut and gills which implies there was a difference in the nitrite concentration they were exposed to.

The water microbiome, however, was the only microbiome that did not significantly change when exposed to nitrite. There is not much literature on how increased levels of nitrite change the water microbiome, but increased concentrations have been linked to increased amounts of methanotrophs (Shen et al., 2020). The water microbiome is predominantly Alphaproteobacteria and Bacteroidia, both of these classes contain numerous nitrifying bacteria (Boyd and Peters, 2013). The water microbiome might have already had a large population of bacteria capable of fixing nitrite, so there would have been less selective pressure on the overall microbial composition.

The conversion of nitrite to nitrate increased the concentration of nitrate in the treatments, so this elevated nitrate concentration could have also influenced the microbiomes by adding additional selective pressure. Like with nitrite, there is no literature on how nitrate would impact the tissue microbiomes, but it would likely select

for nitrate-reducing bacteria. Elevated nitrate levels have been shown to cause dysbiosis of the gut microbiome in some fish species and both nitrite and nitrate are positively correlated with increased potential pathogens, although the concentration of nitrite and nitrate was not specified (Sun et al., 2019). In a real-world setting, excess nitrite is converted into nitrate, so the changes to the microbiome observed reflect what would happen in a realistic setting. The results are therefore still informative about how increased nitrite changes the microbiome both directly and indirectly when it is converted to nitrate.

The normal nasal microbiome in fish is not well studied, however, the predominance of Gammaproteobacteria in the nasal microbiome has also been found in zebrafish and rainbow trout (Tacchi et al., 2014; Casadei et al., 2019). This indicates that the results from the untreated nasal microbiome are in line with the limited available literature. At the genus level, the microbiome is mainly composed of *Pseudomonas* and *Yersinia*. *Yersinia* species are usually pathogenic to fish, however, considering it composed about 25% of the microbiome and the fish were healthy, this may not always be the case. The nasal microbiome had a limited number of genus represented which indicates these have likely been selected to play a specific role in the tissue. This could be a role supporting the immune system, but it could also be playing a role in mediating the cells associated with the nasal epithelium (Tacchi et al., 2014). Bacteria have also been shown to directly influence behavior by releasing metabolites that are smelled by the host, however, this has not been confirmed in fish (Cheal, 1975; Maraci et al., 2018). Now that the basic structure of the goldfish microbiome has been identified, future works are needed to determine the role and importance of these bacteria in fish health and

olfaction.

With regards to nitrite exposure, our results confirm the hypothesis that environmental nitrite pressures fish microbiomes. The composition of the nasal microbiome changed significantly with increasing nitrite concentration in the water. As a result, the 0.1 mM and 1.0 mM treatments were both significantly different than the control. As the concentration of nitrite increased, the microbiome shifted from mainly Gammaproteobacteria to having a larger composition of other classes. Dysbiosis is characterized by a decrease in diversity of species associated with the microbiome and an increase in other groups, usually opportunistic pathogens (Infante-Villamil et al.). The increase in classes in our experiment may indicate that the microbiome is being disrupted and allowing other bacteria to colonize the tissue which are more likely to be pathogenic.

Notably, the gill and gut microbiomes showed a more drastic shift in the microbial composition in the 0.01 mM treatment than the higher treatments. This pattern was not reflected in the nose, skin, or water microbiomes. It is possible that the gill and gut tissues have a route of metabolism or nitrite transport that causes the nitrite to accumulate at low concentrations. The gut, on the other hand, actively takes up nitrite from the environment via sodium, potassium, and chloride transporters (Williams and Eddy, 1986). They also can produce nitrite when bacteria in the gut convert nitrate to nitrite (Eddy and Williams, 1986). At this low concentration, nitrite uptake may not be down regulated, causing a buildup of nitrite in the tissue.

The gut microbiomes in the 0.01 mM and 0.1 mM treatments were significantly different than the control. Interestingly, the 1.0 mM treatment was not different to the control. There was a drastic change in the 0.01 mM treatment that stabilized to something

similar to the control as the concentration increased. The intestinal epithelium is capable of nitrite uptake, up to two-thirds of the total nitrite uptake is through the gut (Jensen, 2003). It is possible that as the nitrite concentration increases in the external environment, the gut stops taking in nitrite or the excess nitrite is moved into the plasma and out of the tissue. The 0.01 mM treatment showed an increase in Fusobacteria, Bacteroidia, Gammaproteobacteria, Bacilli and a decrease in Alphaproteobacteria, Actinobacteria, Planctomycetes, and a smaller others group. The relative abundance of almost every class shifted between the control and the 0.01 mM treatment. There does not appear to be a correlation between ability to metabolize nitrite in these classes (Dos Santos et al., 2012; Boyd and Peters, 2013). It is possible that bacteria that metabolize nitrite began to outcompete the bacteria associated with the microbiome which allowed for the colonization by bacteria that would normally be pushed out by the microbiome. In this way, some of the new bacteria may simply be taking advantage of a weakened microbiome instead of metabolizing nitrite themselves.

In the gills, nitrite competes with chloride in the chloride exchanger. Chloride is preferred over nitrite, so small amounts will likely accumulate more because it is not able to outcompete chloride which leads to accumulation of nitrite in the external microenvironment of the tissues (Williams and Eddy, 1986). The gill microbiome showed a similar pattern to the gut where the 0.01 mM treatment was more different from the control than the higher treatments. In this case, the 0.1 mM treatment was not significantly different from the control. In the 0.01 mM treatment, there was an increase in the Gammaproteobacteria and Planctomycetes and a decrease in Bacilli. These changes are not the same as what was seen in the gut although the pattern is similar.

Gammaproteobacteria and Planctomycetes both have several species capable of fixing nitrite, however, so does Bacilli (Dos Santos et al., 2012; Boyd and Peters, 2013). Since the gills actively uptake nitrite, there may have been similar changes to the epithelium that changed the microbiome without selecting specifically for bacteria capable of nitrogen fixation (Jensen, 2003).

Overall, the gills had far more genera change during nitrite exposure than any of the other tissues. This implies that the bacteria in the gills are more susceptible to nitrite exposure than those in the other tissues, with the next highest being 8 genera in the water. The gills create a unique water environment to facilitate the movement of ions which is likely being changed with the addition of nitrite (Evans et al., 2005). Since the gills are filtering nitrite, they are likely experiencing higher concentrations than the other tissues, causing more selective pressure on the microbiome.

The pattern in how nitrite impacted the skin microbiome is more similar to the nose than the gut or gill microbiomes. This makes sense as the skin does not have the same ion channels found in the gut or gills which could remove excess nitrite. Therefore, for the same water nitrite concentration, the skin microbiome is exposed to higher concentration of nitrite that other tissues.

For the skin microbiome, the 1.0 mM treatment was significantly different from both the control and 0.01 mM treatment. There was a lot of diversity between individuals of the same treatment, which is common with skin microbiomes although the skin microbiome in fish has not been studied extensively (Larsen et al., 2013; Sanford and Gallo, 2013; Kong et al., 2017). Since the skin is in direct contact with the water and the external environment cannot be well regulated by the fish, changes to the microbiome are

more likely to increase as the external nitrite concentration increases.

The gut, gills, and water microbiome all saw the appearance of *Nitrobacter*, which reduces nitrite to nitrate, in the 1.0 mM treatment (DiSpirito and Hooper, 1986; Ciji and Akhtar, 2020; Liang et al., 2021). The gills and water also saw, to a lesser degree, the appearance of *Azospirillum* in all the nitrite treatments. This genus is also associated with denitrification, although it is part of the terrestrial nitrogen cycle and is found near plant roots and is not usually aquatic (Bothe et al., 1981; Han and New, 1997; Kloos et al., 2001; Fukami et al., 2018). Both species were likely selected for by increased levels of nitrogen.

Exposure to nitrite caused dysbiosis of each of the tissue microbiomes, although the changes vary depending on the physiology of the tissue. Significant changes of the microbiome can lead to dysbiosis which leads to an increased risk of disease and death in fish. Given that there are high mortality rates and high concentrations of nitrite in aquaculture, part of the problem may be significant dysbiosis of the tissue microbiomes which leads to disease and eventually death (Ciji and Akhtar, 2020). Moreover, most aquaculture systems are maintained at sublethal nitrite concentrations (0.1 mM to 3 mM). Our results show that even at these lower concentrations dysbiosis can occur and could lead to chronic stress and disease. Gaining a better understanding about how nitrite impacts the tissue microbiomes can help with nitrite management strategies in settings where high levels of nitrite are unavoidable. For instance, a probiotic can be used to stabilize a microbiome under dysbiosis, but none have been developed specifically to stabilize fish microbiomes exposed to nitrite. There is therefore a need to investigate and develop probiotics designed to mitigate the effects of nitrite on fish micrbiomes.

III. IDENTIFICATION OF PROBIOTIC CANDIDATES

Introduction

Nitrite has been shown here to influence the overall composition of the tissue microbiomes in fish and damages the tissues themselves (Jensen, 2003; Duan et al., 2018; Ciji and Akhtar, 2020). Nitrite is a harmful toxin to fish but is impossible to completely eliminate in aquaculture. Since exposure to moderate levels of nitrite is unavoidable, other measures must be taken to reduce the stress and increased risk of disease associated with nitrite exposure. Probiotics are microbes that support the health of a tissue or system. They are usually ingested to help with digestive and immune health (Mombelli and Gismondo, 2000). Probiotics may be able to stabilize fish microbiome and strengthen the immune system which could mitigate the toxic effects of nitrite.

Probiotics have long been used in aquaculture to promote growth, immune health, feed utilization, and decrease stress response. Gut probiotics are the most common type of probiotic used and are easy to administer orally through feed (Llewellyn et al., 2014; Kelly and Salinas, 2017; Wanka et al., 2018). We choose to develop a nasal probiotic because nitrite exposure decreases olfaction in fish, and a probiotic could potentially mitigate the damage by boosting the immune system or replenishing the bacteria lost due to nitrite exposure (Martinez and Huertas, 2019).

To develop a nasal probiotic for use in an aquacultural setting, suitable bacteria must be selected, shown safe to fish, and help recover olfaction, boost the immune system, or otherwise promote fish health in the presence of nitrite. Probiotics support the immune system by stimulating the immune system and directly competing with pathogens (Gomez and Balcazar, 2008). Probiotics should be non-pathogenic and non-toxic, so those used in aquaculture are usually isolated from the microbiome of aquatic

animals (Lara-Flores, 2011). For this reason, the probiotic candidates were isolated from the tissues of healthy fish and tested for antimicrobial activity to identify a candidate that was part of the healthy microbiome and had the capacity to compete directly with pathogens.

The pathogens used to determine antimicrobial activity are *Edwardsiella ictaluri*, *Aeromonas hydrophilia*, *Yersinia ruckeri*, and *Vibrio harveyi*. Each of these pathogens is associated with a prevalent and deadly disease in fish. *E. ictaluri* is primarily connected to enteric septicemia in catfish, although it also causes disease in non-catfish families. This infection causes mass die-offs that disrupt wild fish populations as well as farmed catfish (Gaafar et al., 2021). *A. hydrophilia* is an opportunistic pathogen that only causes disease in stressed fish making it a biomarker for polluted water. When it does cause disease, it does not discriminate between species or habitat making it a lethal pathogen, especially in aquaculture (Harikrishnan and Balasundaram, 2005). *Y. ruckeri* causes enteric red mouth disease in fish, typically in rainbow trout, but is known to infect other fish. Like the other pathogens selected, it can be devastating to aquaculture and reared fish (Terzi et al., 2021). *V. harveyi* is a major pathogen for both fish and invertebrates, causing vasculitis, gastro-enteritis, and eye lesions in fish. It is mainly found to infect marine fish but can also infect freshwater fish. All the pathogens were selected because they cause severe disease that can lead to death, infect a wide range of fish, and are problems to aquaculture.

We hypothesize that a probiotic candidate isolated from a healthy host that can inhibit any of these pathogens would be a viable probiotic candidate. If a candidate met these criteria, then it is unlikely to be pathogenic and would reduce the risk of infection

by these or other pathogens.

Materials and Methods

1. *Identifying Probiotic Candidates*

Nasal, gill, and skin swabs samples were taken from untreated goldfish using hand-made sterile swabs to sample the microbiome of these tissues. The bacteria were then cultured on Reasoner's (Oxoid) (R2A) or ½ Brain Heart Infusion agar (Oxoid) (BHI) for up to 7 days at 25°C. Individual colonies with unique morphology were isolated by streaking and made into long-term stocks. These were prepared using 400 mL 50% Glycerol and 1.2 mL broth culture of either R2A or ½ BHI, depending on which media they were cultured on, and stored at -80°C. Colony PCR was then used to extract the DNA to be used for sequencing. A sterile toothpick was used to pick up a small amount of the isolated colony and placed in the PCR mix and amplified using universal primers 27F (AGAGTTTGATCMTGGCTCAG) and proK1492R (GGWTACCTTGTTACGACTT). After the PCR, they were run on a 1.5% agarose gel at 90V for between 20-30 minutes. The bands were then removed from the gel and the DNA was extracted and purified using the GFX PCR DNA and Gel Band Purification Kit (Illustra). The purified DNA was then prepared for sanger sequencing. The PCR products were sequenced on the ABI 3500xL platform and the quality of the sequences were checked using UGene (Unipro). The bacteria were then identified by comparison of the 16s rRNA gene against the Ribosomal Database Project database with the RDP classifier tool, with an identity threshold ≥80% (Wang et al., 2007).

These isolates were then tested for antibacterial activity against *Yersinia ruckeri*,

Edwardsiella ictaluri (ATCC 33830), *Vibrio harveyi* (ATCC 35084), and *Aeromonas hydrophilia* (ATCC 7966) to identify potential fish probiotics. These probiotic candidates were tested through a direct competition assay and an agar plug assay. In the direct competition assay, the pathogens were plated to create a lawn on ½ BHI and then 5µL of the probiotic candidate was added to the plate. This was done in triplicate for each pathogen. They were then incubated at 26°C for 24 hours and observed for inhibition of the pathogen growth. The probiotic candidate was determined to be inhibitory if it inhibited pathogen growth on at least 2 of the 3 plates.

For the agar plug assay, the probiotic candidates were plated on ½ BHI at 26°C for 3 days. A plug was then taken using a sterile core borer and placed on a ½ BHI plate where one of the pathogens had been plated to create a lawn. These plates were also done in triplicate for each candidate-pathogen pair. The plates were incubated at 26°C for 24 hours and a zone of inhibition was measured to determine the degree of inhibition. These zones were averaged across the 3 plates to determine the degree of inhibitory power each candidate had.

From the results of these 2 assays, 9 probiotic candidates were selected for further testing. The direct competition assay and agar plug assays were repeated on 0.0 mM, 0.01 mM, and 0.3 mM nitrite plates to determine the effect of nitrite on the inhibitory power of the candidates. The ½ BHI agar was autoclaved and then combined with a 2X concentration of the desired nitrite to create agar plates with the desired concentration. The assays were then repeated using the same procedures as detailed above. The incubation time for the direct assay was increased to 3 days, the incubation time for the candidates in the agar plug was increased to 5 days, and the plug-pathogen incubation

was increased to 5 days. This time increase was due to slower and less dense bacterial growth on the nitrite plates. The analysis criteria was the same as in the initial tests, however, not all plates showed growth and could not be used to determine antibacterial activity. From the results of the initial tests and these nitrite tests, 2 probiotic candidates were selected as showing the strongest antibacterial activity while representing the least threat to the fish host.

Results

A preliminary group of 22 probiotic candidates were tested for antimicrobial activity using an agar plug assay and a direct competition assay. These candidates were tested against *Yersinea ruckeri*, *Vibrio harveyi*, *Edwardsiella ictaluri*, and *Aeromonas hydrophilia*. *A. hydrophilia* was unable to be inhibited by any of the candidates in either the competition or agar plug assay. *E. ictaluri* was inhibited by the most candidates, and this trend was reflected in all control experiments.

In the direct competition assay, 11 candidates were able to inhibit *E. ictaluri*, 8 candidates inhibited *Y. ruckeri*, and 3 candidates inhibited *V. harveyi*. There were 6 candidates that inhibited multiple pathogens, indicating they may have a more generalized antimicrobial than the other candidates. The agar plug assay showed that 7 candidates were able to inhibit *E. ictaluri* only. The zone of inhibition showed some variation but was similar for each of the candidates that had any inhibitory ability. *E. ictaluri* showed less dense lawn growth than the other pathogens which may be part of the reason it was more susceptible.

The candidates from these preliminary experiments that showed antimicrobial

abilities were identified using 16S rRNA gene sequencing (Table 5). This group of 9 candidates was tested again using the agar plug assay and direct competition assay, but these tests were run with 0.0 mM, 0.01 mM, and 0.3 mM nitrite concentrations to determine the impact of nitrite on their ability to inhibit the selected pathogens. The 0.3 mM concentration was chosen as a midpoint between the 0.1 mM and 1.0 mM treatments the fish were exposed to. The pathogens did not grow well on the plates with nitrite. They took twice as long to form a lawn, and the lawn was less dense than on 0.01 mM control plates. Only *E. ictaluri* was consistently inhibited by any of the probiotic candidates despite *V. harveyi* and *Y. ruckeri* being inhibited G1, N1, N2, N3, and N6 during the initial competition assay. Only N4 was able to inhibit *V. harveyi*, and that only occurred on the 0.3 mM nitrite plate. Overall, the candidates showed more inhibition on the plates without nitrite in the direct competition assay (Figure 13). In the agar plug assay, none of the pathogens plated with nitrite were inhibited (Table 6). The pathogens did not grow as consistently on these plates so, in some cases, it was difficult to determine if the nitrite or candidate was inhibiting the pathogen's growth.

Table 5. Summary of probiotic candidates. Candidates were derived from the skin, gills, and nose of healthy goldfish. A direct competition assay and an agar plug assay were conducted to determine if the candidates had any antimicrobial activity against pathogens, *E. ictaluri* (EI), *V. harveyi* (VH), *Y. ruckeri* (YR), and *A. hydrophilia* (AH). EI was inhibited by all of the selected candidates while AH was never inhibited by the candidates.

Code	Strain Code	Source	Genus	EI	VH	YR	AH

S1	S00367	Skin	*Aeromonas*	Yes	No	No	No
G1	S00359	Gill	*Cellulomonas*	Yes	Yes	No	No
G2	S00368	Gill	*Aeromonas*	Yes	No	No	No
N1	S00553	Nose	*Chitinilyticum*	Yes	Yes	No	No
N2	S00551	Nose	*Aeromonas*	Yes	No	Yes	No
N3	S00557	Nose	*Pseudoxanthomonas*	Yes	Yes	Yes	No
N4	S00541	Nose	Contaminated	Yes	No	No	No
N5	S00544	Nose	*Pseudomonas*	Yes	No	No	No
N6	S00542	Nose	*Pseudomonas*	Yes	No	Yes	No

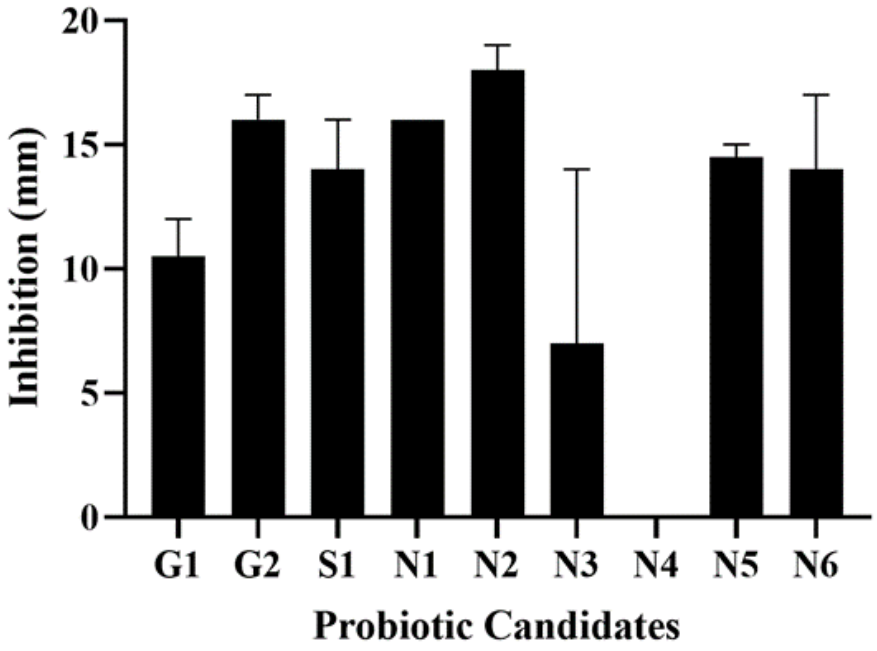


Figure 13. Probiotic agar plug assay. Inhibition of plated on ½ BHI by probiotic candidates using an agar plug assay (mean ± SEM). The rest of the pathogens were not inhibited by any probiotic candidate (graphs not shown). The zones of inhibition shown are against *E. ictaluri* when with no added nitrite.

Table 6. Probiotic compeititon assay. Group candidate's inhibition power among nitrite concentration. The nitrite concentrations tested were 0.0 mM, 0.01 mM, and 1.0 mM. The pathogens are the same from the preliminary experiment, however only *V. harveyi* (VH)

and *E. ictaluri* (EI) were able to be inhibited by the candidates. Check marks indicate the candidate inhibited the pathogen while the "X" indicated no inhibition.

PATHOGEN	S1	G1	G2	N1	N2	N3	N4	N5	N6
EI 0.0 mM	✓	✓	✓	✓	✓	✓	✓	✓	✓
EI 0.01 mM	✓	✓	✓	✓	✓	✓	✓	✓	✓
EI 0.3 mM	X	X	X	X	X	X	X	X	X
VH 0.0 mM	X	X	X	X	X	X	X	X	X
VH 0.01 mM	X	X	X	X	X	X	X	X	X
VH 0.3 mM	X	X	X	X	X	X	✓	X	X
YR 0.0 mM	X	X	X	X	X	X	X	X	X
YR 0.01 mM	X	X	X	X	X	X	X	X	X
YR 0.3 mM	X	X	X	X	X	X	X	X	X
AH 0.0 mM	X	X	X	X	X	X	X	X	X
AH 0.01 mM	X	X	X	X	X	X	X	X	X
AH 0.3 mM	X	X	X	X	X	X	X	X	X

Examining the results of these experiments and the characteristics of the genus of each candidate was used to select the best probiotic for further experiments. N3 and N5 are the best potential probiotics out of the candidates tested. They were able to inhibit the growth of *E. ictaluri* in both the competitive assay and the agar plug assay. N3 is *Pseudoxanthomonas* which is not heavily associated with fish pathogens and was able to inhibit *E. ictaluri* and *V. harveyi*. N5 is *Pseudomonas* which can be associated with fish pathogens but was able to inhibit the pathogen's growth the most.

Discussion

We confirmed our hypothesis that bacterial groups identified in goldfish microbiomes can protect from pathogen infection, making them adequate candidates for probiotic treatments. *E. ictaluri* was the most susceptible pathogen to probiotic treatments, whereas *A. hydrophilia* was not inhibited by any of the candidates tested. Of the candidates tested, most of them showed inhibitory activity against at least one of the pathogens. This makes sense if part of their role in the microbiome is to compete with pathogens. This also means that there are plenty of bacteria that likely possess antimicrobial activity that can still be cultured from the various tissue microbiomes.

A clear understanding of how the probiotic candidates inhibited the pathogens when exposed to nitrite was not possible. This is because the growth of the candidates was mildly inhibited by the nitrite, but the pathogens were almost entirely inhibited by the presence of nitrite. None of the pathogens can metabolize nitrite, but it is unclear how the tissues environments would change with increased concentration of nitrite. It may be that tissue conditions with high concentrations of nitrite are optimal for these pathogens when it is not *in vitro*. These pathogens are known to cause disease when fish are stressed, including during nitrite induced stress, particularly *Y. ruckeri* causes disease in catfish when exposed to poor water quality (Terzi et al., 2021). These pathogens colonize the fish and take advantage of the shelter and nutrients available which means that they may not need to tolerate or metabolize nitrite to thrive as a fish pathogen. The probiotic candidates that were isolated from the fish microbiomes were more tolerant of nitrite exposure, which indicate they are adequate candidates for probiotic treatments in nitrite-

abundant aquaculture systems. These putative probiotics were isolated from goldfish that had not been exposed to nitrite, so withstanding low levels of nitrite may be a more valuable ability to bacteria associated with the microbiome than to pathogens.

The candidates chosen were a *Pseudoxanthomonas*, N3, and a *Pseudomonas*, N5, species. *Pseudoxanthomonas* species are not common fish pathogens so this probiotic candidate is less likely to be dangerous to the fish as a live probiotic. There is no published literature about *Pseudoxanthomonas* or its potential as a probiotic with antimicrobial properties. *Pseudoxanthomonas* species are commonly isolated from soil samples or waste sites. Members of this genus can reduce nitrate but not nitrite. (Weon et al., 2006; Young et al., 2007; Klankeo et al., 2009).

Some *Pseudomonas* species are pathogenic, but there are several species which are currently being used as probiotics in fish to combat infections by *A. hydrophilia*, *Flavobacterium*, *Streptococcus*, and other *Pseudomonas* species (Korkea-Aho et al., 2011; Giri et al., 2012; Eissa et al., 2014). *Pseudoxanthomonas* is a relatively safe choice, in that it is unlikely to cause disease in fish, but some *Pseudomonas* species have been shown to be effective probiotics. There is some evidence, in humans, that probiotics targeting the nasal microbiome can be effectively administered orally, but this research is very limited (Dimitri-Pinheiro et al., 2020; Bourdillon and Edwards, 2021). An *in vivo* study would need to be performed to confirm that the candidates are not pathogenic and provide a benefit to the fish host.

These fish have the potential to be probiotics capable of supporting the nasal microbiome during nitrite exposure. They were isolated from the healthy nasal microbiome of goldfish and both showed antimicrobial activity against *E. ictaluri* while

the *Pseudoxanthomonas* inhibited *E. ictaluri*, *Y. ruckeri*, and *V. harveyi* in at least one of the experiments. These bacteria were chosen as the best candidates out of the bacteria tested, however, many of the tested bacteria showed some level of antimicrobial activity against the pathogens. Not only are these candidates good potential probiotics, there is reason to believe that more bacteria in the nasal microbiome could possess antimicrobial activity and have potential as a probiotic. This could be investigated by working to isolate the bacteria under culture conditions more similar to those found in the nose and working to test the antimicrobial ability of the candidates when exposed to nitrite.

Since nitrite is disruptive to the nasal microbiome, introducing these bacteria could help to restore a healthy nasal microbiome by recolonizing the epithelium with bacteria present under normal conditions, but also by preventing the colonization of pathogens like *E. ictaluri*. Each of the pathogens tested is common in the stressful, high nitrite conditions found when rearing fish and a nasal probiotic could go a long way to minimize stress and death by disease.

We hypothesized that nitrite would disrupt the tissue microbiomes causing dysbiosis, and that is what was observed. Although the nitrite impacted each tissue differently, it is clear that nitrite pollution is systemically impacting the tissue microbiomes. The additional stress caused by dysbiosis of the microbiomes makes fish at higher risk for disease and disorders associated with chronic stress. In aquaculture, where high concentrations of nitrite are common, dysbiosis is likely causing health problems in fish that are otherwise not showing symptoms of nitrite toxicity, reducing yield and raising costs. Future works should focus on better characterizing the tissue microbiomes and determining the role that the bacteria diminished with nitrite exposure play in the

microbiome. This insight would better allow for the treatment and management of fish who experience chronic elevated nitrite levels.

Additionally, we hypothesized that bacteria from a healthy fish could show antimicrobial activity, which we found. These bacteria, and others present in the microbiome, could serve as a probiotic to mitigate the effects of nitrite exposure. They show antimicrobial activity and should be safe for the fish, making them good candidates. Future work should investigate their effectiveness *in vivo* to determine how they colonize the tissue, deal with nitrite exposure, and their ability to combat colonization by pathogens. An effective probiotic would support the overall health of fish reared in poor water quality, which is a huge benefit in aquaculture.

IV. CONCLUSION

The toxicity of nitrite on fish has been studied extensively, but research into how nitrite impacts the microbiome has been neglected. Nitrite significantly changed the composition of each of the tissue microbiomes. The change appears to be dependent of the function of both the tissue and its microbiome. The concentrations of nitrite examined here are not uncommon in closed intensive culture systems and the stress caused by dysbiosis may be occurring without outward signs of nitrite toxicity. This stress is likely causing many unnecessary losses in fish farms which drive up costs and decreases the efficiency of aquaculture.

Probiotics like the candidates identified could prevent fish deaths associated with nitrite stress and disease which would heavily benefit aquaculture. Further work needs to be done on the efficacy of a probiotic to combat nitrite toxicity and its ability to protect the nasal microbiome specifically. Understanding the importance of the microbiome both on regular functions, like olfaction, and its role in disease and exposure to toxins is vital information. Without fully understanding the role the microbiome plays in these areas, it is impossible to find the best solution for dealing with disease and physiology.

APPENDIX SECTION

Table 7. Number of bacteria groups that were significantly different at the amplicon sequence varient (ASV), genus, class, and phylum classifications. These were determined using DESeq2 with an adjusted p-value cutoff of 0.05.

Tissue	ASV	Genus	Class	Phylum
Nose	4	3	3	1
Gills	42	20	2	1
Gut	18	6	1	0
Skin	4	2	0	0
Water	17	8	3	1

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