Studies of the host-microbe relationship in aquaculture-raised animals

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Abstract

Aquatic animals, such as fish and shellfish, provide important economic and nutritional benefits for human society. Due to overexploitation of natural fish sources through traditional wild-caught fisheries, aquaculture (generally described as fish farming or culturing) has grown into an economically important industry. A major focus area for the aquaculture field is related to sustainability by ensuring the health and welfare of the aquatic animals. Communities of microorganisms inhabiting the various niches of a given host comprise its microbiome and provide several key health benefits. The microbiome impacts nutrient acquisition, gut homeostasis, protection against pathogens, and immune system modulation. Therefore, much attention has been placed on studying how various culturing conditions and host factors impact the microbiomes of aquatic animals.

Here, multiple studies were conducted to elucidate the impacts of various parameters on the microbiomes of rainbow trout, steelhead trout, and Nile tilapia, including dietary supplementation, administration of probiotics and animal age. Though there is a significant correlation between the diet fed to fish and their microbiome communities, small dietary changes such as the inclusion of a dried and lysed yeast product, acting as a protein source alternative to unsustainable fishmeal did not significantly alter the intestinal adherent microbiome of rainbow trout. Moreover, an optimal percentage of yeast replacement that did not negatively impact weight gain for the aquaculture-raised fish was identified, suggesting its

efficacy for the industry. Similarly, the intestinal adherent microbiomes of steelhead trout were not significantly altered by diet supplementation with a *Bacillus subtilis* probiotic. The total microbiome of steelhead trout (mucosa combined with digesta) was instead significantly changed when they were only fed the probiotic additive at an early stage of intestinal development. This change in the microbiome of steelhead trout correlated with a significant increase in weight gain compared to fish only fed the probiotic during later stages of intestinal development. These findings also corroborate previous observations wherein the intestinal microbiome of fish varies during their developmental stages but then stabilizes over time.

Determining the core set of bacteria present in fish microbiomes, independent of treatment variables, is another important factor when considering attempts to manipulate the microbiome. To that end, a literature review was conducted in which the phyla Firmicutes, Proteobacteria and, to a lesser extent, Actinobacteria, Bacteroides, and Tenericutes were identified as likely members of the rainbow trout core microbiome. Bacterial families identified as part of the core phyla included *Lactobacilliaceae* that are commonly used as probiotics and *Mycoplasmataceae* that lack cell walls. Preventing dysbiosis of the rainbow trout microbiomes will be crucial to ensuring the health of the fish hosts and increasing longevity and profitability of the aquaculture industry.

Another important aquaculture-raised species is the Eastern oyster. This animal is critical for the ecological health of the Chesapeake Bay, and it is also an important source of revenue. A significant portion of the revenue flow is the harvest and sale of live oysters for consumption. Unfortunately, consumption of raw or undercooked oysters is the most common route of infection by the human pathogen *Vibrio parahaemolyticus* (VP) as oysters are a natural

reservoir for VP. This bacterium is responsible for a debilitating acute gastroenteritis with potential to cause fatal septicemia. Despite efforts to mitigate infection by this CDC-reportable pathogen, cases continue to increase. The understudied host-microbe relationship between the Eastern oyster and VP has been implicated as a path to research for potential future therapeutics. A novel culturing system for oysters was created using fermentation jars within a BSL-2 ready biosafety cabinet. Using this system, the effect of harvest season was tested against the inoculation efficiency of VP. It was found that higher native *Vibrio* levels within the oysters were present during the summer compared to the winter. Moreover, addition of the bacteriostatic antibiotic chloramphenicol (Cm) enabled a higher inoculation efficiency by VP during both the summer and winter compared to oysters not exposed to the antibiotic. During the winter, exposure to Cm led to the highest inoculation efficiency (~100%). These findings confirm the importance of the existing microbial communities against exogenous inoculation. Therefore, a year-long study was conducted to investigate the microbiome of oysters during each season. This pan-microbiome study identified a significant impact of harvest season on the microbiome structure. An increased diversity, including higher levels of *Cyanobacteriaceae,* was observed during the summer. Whereas an increase in *Arcobacteriaceae* was observed during the winter. Bacteria that persisted throughout the year included *Mycoplamataceae* and *Spirochaeteacae*; these families may represent potential members of the Eastern oyster core microbiome.

Further work is needed to study the localization patterns of VP within oysters. Such work includes further optimization of immunohistochemistry (IHC) and intracellular colonization assay methods under development here. Collectively, studies of the oyster-

microbe interactions will help improve aquaculture methods and identify mitigation targets to reduce VP-related clinical infections.

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General audience abstract

Fish and shellfish provide important economic and nutritional benefits for human society across the globe. Unfortunately, over-fishing of traditional sources of fish and shellfish has led to a reduced supply for world markets, even as the human population increases. Aquaculture, or fish farming, has been around for centuries, but its role in society has significantly increased in the past 50 years. It currently provides about half of fish and other aquatic products on the market today. To better maintain and increase the sustainability and profitability of this industry, more focus is being placed on the health of the fish. The microbiome is the collection of communities of microorganisms, including bacteria, fungi, and archaea, that inhabit various environments including animal hosts. The majority of this dissertation focuses on the impact of factors like diet and age on the microbiomes of aquaculture-raised animals, especially fish. Dietary changes such as the addition of dried yeast-products had a significant impact on fish health but not on the microbiome communities. However, a common probiotic, *Bacillus subtilis*, did significantly increase not only the growth rate of trout but it also significantly altered the total intestinal microbiome found in the feces and the intestinal mucosal layer. Moreover, it was found that early exposure of the animals to the probiotic had enhanced benefits even though the microbiome appeared to stabilize over time as the fish developed. Maintaining or improving the microbiomes of fish, paying close attention to the microbes that exist as part of a core group of bacteria always present, is vital to

ensuring fish health and understanding vertebrate host-microbe relationships. Thus, an analysis of the core microbiome of trout was performed.

The final set of projects within this dissertation focused on the relationship between the Eastern oyster, a mollusk native to the Chesapeake Bay, and the bacterial human pathogen *Vibrio parahaemolyticus* (VP). VP is the leading cause of seafood-borne acute gastroenteritis worldwide, and efforts are needed to mitigate the increasing rate of human infections. Therefore, a simple system using fermentation jars within the laboratory biosafety cabinet was designed to enable safe culture of oysters that were exposed to VP under experimentally controlled conditions. Oysters harvested during the summer naturally harbored higher amounts of native *Vibrio* organisms in contrast to the winter oysters that harbored much lower levels. A separate microbiome analysis revealed large shifts in the oyster microbiome between summer and winter, although some microbes were continually present. The lower levels of existing *Vibrio* species detected in winter oysters may have allowed for the higher efficiency of inoculation of winter animals by VP. In fact, these winter animals had *Vibrio* microbiomes that were completely dominated by the inoculated strain which will enable future work to observe the pattern by which VP localizes, or colonizes, the oysters. Ultimately, these efforts may lead to the development of future disease mitigation strategies against VP.

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Chapter One

Literature review

The microbiome

Microbial inhabitants of a host organism, whether commensal, symbiotic, or pathogenic, dwell in various niches and comprise the microbiome (Hooper et al., 2001). The relationship between microbial inhabitants and their vertebrate host has been established as a crucial dynamic for the survival of both entities. The composition of the microbiome is important for vertebrate host development, function, and health (Kinross et al., 2011) (Figure 1.1). In regard to host development, both vertically and horizontally acquired resident microbes may play a role in shaping early life development. Specifically, some microbiome constituents are capable of influencing host gene expression via signal molecule production (McFall-Ngai et al., 2013). The microbiome also acts as an evolutionary partner with its host organism. For example, some microbes appear to co-speciate with their hosts, as has been observed during the diversification between herbivores and carnivores (Phillips et al., 2012), (Groussin et al., 2017). In addition, the microbiome, especially the intestinal component, is implicated in modulating host physiology and the innate immune system (Hooper et al., 2001; Levy et al., 2015; Spor et al., 2011). Other studies looking specifically into the effects of the intestinal microbiota on host health have shown that it can affect neuronal health by directly producing metabolites that act as neurotransmitters (Sampson and Mazmanian, 2015). The intestinal microbiome is also vital for host health through nutrient acquisition, and the host can experience detrimental effects when this structured composition is destabilized (Nicholson et al., 2012). Microbiome destabilization and other disruptions to the microbiota (i.e., dysbiosis) are related to altered disease states (Ott et al., 2004; Turnbaugh et al., 2007). Targeting the microbiome for

prophylactic, therapeutic, and other clinically relevant applications can have beneficial outcomes in the health of humans and animals.

Molecular-based methods to study the microbiome

Methods for estimating bacterial diversity in environmental samples has been traditionally done by culturing isolated microorganisms in nutrient media and plating. However, plate-based culturing techniques have vastly underestimated the true numbers of viable but not culturable species (Li et al., 2014). The 16S rRNA gene is highly conserved among all known bacterial species. Therefore, using this gene as a target for diversity studies has made it possible to overcome the challenges of traditional plating methods (Woese et al., 1976). Within the 16S rRNA gene, there are nine regions with a higher degree of variability allowing for the identification of specific species (Chakravorty et al., 2007; Stackebrandt and Goebel, 1994; Van de Peer et al., 1996). Using these regions to define microbial abundance, however, can lead to biases for species with higher copy numbers of the 16S rRNA gene (Ghanbari et al., 2015). Mitigation of this issue may be possible through the use of more complex modeling systems (Kembel et al., 2012).

Historically, the 16S rRNA gene was sequenced using the Sanger method with dideoxy chain-terminating nucleotides (Sanger et al., 1977). Low throughput, cost constraints, and technical challenges have rendered this type of sequencing virtually obsolete with the advent of Next Generation Sequencing (NGS). NGS has the ability to process a mixture of PCR-amplified 16S rRNA sequences independent of culturing in a high-throughput manner. The high-throughput characteristic is associated with a relatively inexpensive cost per sample and high accuracy by performing millions of parallel reactions in a single sequencing run (Ghanbari et al.,

2015). By amplifying a hypervariable region from 16S rRNA genes within a sample of interest and running these amplicons on a NGS platform, it is possible to better estimate the microbial diversity in an environmental or animal-based sample. A majority of microbiome work, especially true for animal studies such as fish, has involved the Illumina HiSeq and MiSeq sequencing platforms (Tarnecki et al., 2017). Introduced first, the HiSeq system is capable of generating a very large set of data at a low cost per base with high accuracy, whereas the MiSeq platform has the ability to generate a lower number of reads at longer lengths for a lower cost and quick turn-around time (Caporaso et al., 2012). Both systems use the Sequence by Synthesis (SBS) method on a single flow cell, wherein each cell allows for millions of fluorescence-producing reactions to generate reads matching the input nucleotide sequences (Illumina, 2010).

Aquaculture

The "blue revolution," similar to the increased investment in land-based agriculture seen during the second half of the twentieth century deemed the "green revolution" (Pingali, 2012), pertains to the global push towards aquaculture and away from large-scale wild-caught fisheries (Krause et al., 2015). Overexploitation of wild-caught fish and shellfish has led to stagnated production levels. Increased aquaculture output helps to compensate for the ever-increasing demand for fish products, despite fishery stagnation (Figure 1.2) (Mustafa and Shapawi, 2015; Naylor et al., 2009). In fact, aquaculture currently makes up about 46% of the total global fishery production (FAO, 2020; FAO and FIAS, 2013) and has tremendous growth potential. Coincidentally the aquaculture industry grew much faster in comparison to other sectors in food production such as beef, grains, and poultry, between 1990 and 2010 (Crépin et

al., 2014). Continuing to improve this agricultural sector is necessary since, according to a recent report by the Food and Agriculture Organization of the United Nations (FAO), 17% of animal protein and 7% of the total protein in the global diet is derived from fish (FAO, 2020).

The significant push towards fish farming is currently not a sufficiently sustainable resource on a global scale (Krause et al., 2015). Whereas some countries are implementing methods to increase profits by optimizing safer and more efficient growth conditions, others have yet to adopt sustainable methods designed to protect the health of the fish. For example, a major component of fish feeds is fishmeal (a commercial fish product used in animal feeds). Because fishmeal is most often produced from wild capture-sourced fish, this practice is unsustainable due to limited supply. Continued use of fish from wild-caught sources also increases the cost of fishmeal. However, methods designed to reduce the time it takes to culture market-ready fish can improve the economic sustainability of aquaculture. Therefore, continuing to improve the overall welfare of animals farmed in aquaculture is critical to this industry's sustainability. Understanding the relationship between the physiology of the animal hosts and the microbiomes of fish and shellfish, which are vital effectors of overall health, will contribute to improved host health and growth. Considering the influence of the microbiome on animal health and physiology, studies that focus on the fish microbiome can potentially make significant economic and environmental impacts for aquaculture.

Fish microbiomes

To date, the majority of vertebrate microbiome research that has been generated originates from mammalian studies, although this group comprises less than 10% of all vertebrates. (Llewellyn et al., 2014; Mueller et al., 2012). Though fish species account for

almost half of the vertebrate subphylum, there have been much fewer microbiome analyses on these animals compared to mammals (Nelson et al., 2006). Thus far, most studies have focused on manipulation of the intestinal microbiome in the most economically relevant fish species with regard to disease protection and increasing feed efficiency (Tarnecki et al., 2017).

Intestinal microbiome studies may consider the adherent bacteria found in fish epithelial tissues as well as nonadherent feces-associated bacteria. The majority of research has focused on the intestinal tracts with fecal material present or the two niches separately (i.e., the adherent epithelial microbiota and the nonadherent fecal microbiota) without comparing to two niches to one another (Tarnecki et al., 2017). Thus, the general understanding of the interplay between nonadherent and adherent bacteria within the gastrointestinal tract is limited. In addition, adherent bacteria inhabiting the mucosal external epithelial tissues, comprising the skin microbiome, are poorly characterized as well though they are known to be important for pathogen defense and homeostasis (Xu et al., 2013).

Oyster microbiomes

Similar to fish, oysters are in constant contact with their aquatic environment. Though understanding of the relationship of the fish microbiome is increasing, similar investigations of aquatic invertebrates such as oysters have not been as prevalent. Related oyster species appear to harbor a core microbiome, however, there is a limited amount of information on the core constituents of the Eastern oyster microbiome in the literature (Chauhan et al., 2014; Dubé et al., 2019; Green et al., 2018; King et al., 2012, 2020, 2019).

Core microbiomes

The core microbiome is the community of microbiota consistently present across varying environmental and host conditions. Correlating the core microbiome with the influences of host phylogeny, health, and growth will provide fundamental insight into the host-microbe relationship. Data from fish studies investigating the presence of a core microbiome is limited, however, strong correlations between host genetics and the gut microbiome structure have been implicated in mammalian studies (Benson et al., 2010; Ley et al., 2008). Some studies indicate the presence of a core microbiome in fish that is independent of the environmental conditions (Rudi et al., 2018), while other studies indicate that environmental conditions play a role in selection of a core microbiome (Liu et al., 2016). For example, at different host trophic levels under similar environmental conditions, a microbiome comprised of the phyla *Proteobacteria, Firmicutes*, *Bacteroidetes*, and *Acidobacteria*, was found to exist (Bakke et al., 2015). Wilson et al. (2008) actually posited that the environment may be have more of an influence on microbiome determination than host genetics. Thus, the current consensus on the core microbiome effectors is inconclusive. However, it is conceivable that parameters effecting the core microbiome such as host genetics and environment (see below) are not mutually exclusive. Current research to define the core microbiome of aquatic animals, though limited, suggests that there exist distinctive core microbiomes in different tissues of fish, both internally and externally (Dehler et al., 2017; Lokesh and Kiron, 2016). This would be analogous to the different core sets of the microbiome found in different tissues throughout mammalian hosts (Oh et al., 2016; Turnbaugh and Gordon, 2009).

Internal parameters influencing the microbiome of fish and shellfish

Animal physiology

Fish live in environments constantly surrounded by stressors that can have detrimental effects on their overall physiology. These stressors can include, but are not limited to stocking density, temperature, water pH, and aquatic contaminants. (Davis, 2004; Ellis et al., 2002; Zahangir et al., 2015). Generally, stressors induce a change in fish homeostasis that results in decreased growth rates, higher mortality, and increased susceptibility to infection by opportunistic pathogens such as *Vibrio spp.* (Barton and Iwama, 1991; Wendelaar Bonga, 1997). For example, one study in rainbow trout (*Oncorhynchus mykiss*) highlighted the effects of titanium oxide, an aquatic transferrable component common in paint and building materials, to severe injury of gill tissues and systemic oxidative stress (Federici et al., 2007).

Fish respond to their environment in different ways according to their inherent phylogeny. The traditionally North American, Pacific coast-dwelling rainbow trout have been cultivated for hundreds of years with an exponential rise in global production since the 1950s (Pacha and Kiehn, 1969). This species is opportunistically carnivorous, wherein although they prefer meat, they it will feed on any edible organisms present. When needed, it can also utilize glucose from various sources in its intestinal tissues (Polakof et al., 2010) despite the fact that there is typically a lower concentration of glucose compared to amino acid carbon sources present there (Ferraris and Ahearn, 1984). In contrast, Nile tilapia (*Oreochromis niloticus*), the second most farmed fish worldwide, are an omnivorous species (Wang and Lu, 2016). Their value for aquaculture production is due to some of their inherent traits, including the wide range of diets they are able to metabolize. Tilapia are also highly desired because they are able

to be cultivated in either high or low salinity environments, thus mimicking marine and freshwater environments, respectively (Likongwe et al., 1996). Tilapia have an intestinal tract longer than carnivorous fish, like trout, that consists of specialized digestive enzymatic sections with the enzymes being localized mostly to the brush border (Tengjaroenkul et al., 2000).

Host age

Developmental stages of an animal also may influence the composition of the microbiome. For example, the intestinal microbiome of larval-stage fish can be highly reliant on the environmental conditions present in the water column, though the opposite is true for other species (Bakke et al., 2015; Stephens et al., 2016; Stewart et al., 2016). The parental mucosal layer has also been implicated as the origin of early-stage intestinal microbiome colonization (Sylvain and Derome, 2017). As the fish grow, bacterial species better adapted to inhabit the intestinal tract out-colonize (i.e., out-compete) the earlier inhabitants. This better adapted microbiome leads to increased community diversity and specialization which helps protect the animal against pathogens via competition for nutrients and less variation between host microbiomes (Llewellyn et al., 2014; Stewart et al., 2016).

Host phylogeny and genetics

Vertebrate studies have shown that, regardless of habitat, the genetic background of the host has a significant role in the development of the microbiome (Moeller et al., 2013; Sullam et al., 2015). In aquaculture species, a similar conclusion is drawn by the existence of a consistent core microbiome structure between closely related organisms regardless of environmental habitat (Tzeng et al., 2015). The core microbiome structure in fish species has also been found to be independent of sexual dimorphism (Tarnecki et al., 2017). Core

microbiome constituents for fish species such as the zebrafish include the phylum *Proteobacteria* and genus *Fusobacteria* (Roeselers et al., 2011). Furthermore, *Proteobacteria* have been found to inhabit the intestinal tracts of many other species of fish, thus indicating a possible key role in the core intestinal microbiome across a variety of fish (Bakke et al., 2015; Liu et al., 2016).

External parameters influencing the microbiome of fish and shellfish

Environmental conditions

Environmental conditions can have a significant impact on the structuring of the gastrointestinal microbiome in fish. For example, exposure to varying environmental conditions has led to altered microbiome compositions in migratory fish (Llewellyn et al., 2016). Sampling fish from different locations demonstrates a correlation between altered environmental conditions and the composition of host-associated bacteria (Roeselers et al., 2011). Abiotic environmental conditions, such as water column salinity, can influence the microbiome structure (Sullam et al., 2012). For example, higher salinity environments will select for halophilic bacteria not typically found in lower salinity environments (Franchini et al., 2014). Similarly, microflora in environments with high concentrations of ammonium will allow for the proliferation of more nitrifying bacteria (Giatsis et al., 2016). The bacteria present within the surrounding water column can then become associated with the fish microbiome. Thus, the intestinal physiology and microbiome diversity significantly differ between freshwater and marine fish in relation to their respective environments (German and Bittong, 2009; Mountfort et al., 2002). However, other studies have also indicated a low correlation between microbes in the water column and microflora inhabiting the gut (Schmidt et al., 2015).

Temperature has also been implicated as a possible driver of microbiome structuring. Oysters, initially inhabited with a diverse microbiome, experienced a decrease in microbiota diversity following a gradual heat-shock (Wegner et al., 2013). Higher temperatures (above 20°C) with concurrent decreased microbiome diversity have also been correlated with increased susceptibility to microbial pathogenesis (Garnier et al., 2007; Wegner et al., 2013). Moreover, there is evidence that the effects of global warming on coastal waters has led to an increase of incidences wherein the human pathogen *Vibrio parahaemolyticus* associates with oysters (see below).

Antibiotic application

Antibiotics have been widely used therapeutically in medicine to mitigate the growth and proliferation of pathogenic bacteria. However, high enough doses of the antibiotic will also result in unintended loss of beneficial microbes (Ferrer et al., 2017; McFarland, 2014). Unfortunately, antibiotics have also been used as prophylactic treatments in agriculture and aquaculture with the goal of increasing animal production. This application of antibiotics has been a key contributing factor to the proliferation of antibiotic-resistant pathogens. In turn, this has resulted in unmitigated disease and substantial economic losses in many agricultural sectors including the aquaculture industry (Schmidt et al., 2017; Sun et al., 2015). For example, following antibiotic treatment in mosquitofish, the intestinal microbiome experiences a loss of diversity. These fish with lower microbiome diversities are more susceptible to pathogen challenge, osmotic stress, and stalled weight gain (Carlson et al., 2015). To avoid these antibiotic-associated issues, aquaculture is focusing on supporting beneficial bacterial

communities as a more efficient defense measure through dietary supplements including prebiotics and probiotics (Llewellyn et al., 2014).

Diet

The microbes that colonize the gastrointestinal tract are often correlated with differential feed consumption (Giatsis et al., 2016). Herbivorous marine fish require short chain fatty acids, byproducts of hindgut bacterial fermentation, more than carnivorous marine fish (Hao et al., 2017). Carnivorous marine fish, on the other hand, require long chain fatty acids in their diets that are currently fed to them in the form of fish oil, a product of making fishmeal (Rhodes et al., 2016). Production of fishmeal typically involves utilizing fish from overexploited wild-caught fisheries. Therefore, more sustainable practices such as application of alternative dietary supplements including plant-based products and probiotics (i.e., beneficial live microbes) instead of fishmeal is an active area of research (Benedito-Palos et al., 2007; Li and Gatlin, 2003; Rumsey et al., 1991; Turchini et al., 2009). Understanding the impact of alternative dietary supplements is critical to ensuring the maintenance of fish health. Effects of these alternative diets on the microbiome vary in that some, such as corn oil, significantly impact the microbiome structure whereas other, such as grains, do not (Rhodes et al., 2016; Wong et al., 2013). Unfortunately, some alternative diets can lead to a higher prevalence of pathogen colonization and disease. However, this is most commonly the case if the fish are not consuming a sufficient amount of necessary nutrients (Tarnecki et al., 2017). The use of prebiotics, carbohydrates indigestible by the host but metabolizable by beneficial intestinal bacteria, can aid in the inhibition of pathogenesis and regulation of bacterial metabolite production (Ringø et al., 2010). These molecules can also lead to the production of anti-

inflammatory mediators and reverse microbiome dysbiosis (Montalban-Arques et al., 2015). Prebiotics are also associated with a lower cost and less regulatory issues than other aquaculture disease prevention methods, such as probiotics (Ringø et al., 2010).

Probiotics

Traditionally, probiotics have been used in animal husbandry for increased weight gain and disease prevention; these results are desired in aquaculture as well (Dowarah et al., 2017; Mudgal and Baghel, 2010). Probiotic organisms such as yeasts and other common microbiome commensals have been implicated in reversing dysbiosis and pathogen infection in host gastrointestinal microbiomes. The various mechanisms by which these probiotics function are not yet fully understood (Schmidt et al., 2017). In fish, a more diverse spectrum of possible probiotic candidates is required due to differences in the physiology of the intestinal tract among different fish species (Lauzon et al., 2014). Some organisms such as *Bacillus subtilis* are desirable probiotics due to their ability to sporulate and be easily coated onto fish feeds. Further, *B. subtilis* cells, when sporulated, are capable of resisting the hostile stomach environment. The spores can germinate in the more neutral pH environment of the intestinal tract wherein nutrients are more readily available to the bacteria. Fish have insufficiently developed immune systems as larvae, therefore probiotics are especially effective at increasing survival in these juvenile animals (Hai, 2015). Long-term effects of probiotic use in larval-stage tilapia showed that, although there is little persistence post-cessation of the administered probiotic, the total microbial community structure remains in its probiotic-modified state for a longer time period (Giatsis et al., 2016).

Pathogens

Tissues directly exposed to the environment, including the gills, scales, and gastrointestinal tract epithelium are common entry points for fish pathogens (Iregui et al., 2016; Pridgeon and Klesius, 2011). In addition to species-specific antimicrobial compounds, the skin-associated microbiome is considered to be an important protective barrier against pathogenic organisms. For example, disruption of the skin microbiome of channel catfish can lead to a significant increase in disease and mortality (Mohammed and Arias, 2015). Furthermore, disruptions to the microbiome associated with gill tissue caused by respiratory stress has led to increased pathogenesis (Hess et al., 2015). Understanding the mechanisms by which nonpathogenic microbes protect the host from infection may be crucial to implementing measures that support the enhanced presence of beneficial probiotic bacteria within the microbiome.

Equally important is understanding the mechanisms that potential pathogens use to colonize their target host. *Vibrio parahaemolyticus* (VP) is a Gram-negative Proteobacterium indigenous to coastal and estuarine waters that can colonize aquatic animals (Joseph et al., 1982; Sarkar et al., 1985; Thompson and Vanderzant, 1976). In shrimp, some strains cause a lethal disease known as Early Mortality Syndrome (Pérez-Acosta et al., 2018) that is having devastating effects on shrimp aquaculture. Conversely, VP appears to act as a commensal in oysters. Even clinical strains of VP don't appear to cause disease in oysters. In humans, VP is most commonly associated with a debilitating acute gastroenteritis, but it can also spread systemically resulting in deadly bacteremia depending upon the strain and host immune status (Gomez-Jimenez et al., 2014; Kim et al., 1999). Clinical infections caused by VP are most often

attributed to human consumption of VP-laden raw or undercooked oysters such as the Eastern oyster (*Crassostrea virginica*) (Blake et al., 1980; Letchumanan et al., 2014). The CDC has reported VP as an emerging pathogen with few treatments available for the rising number of gastroenteritis cases on a global scale. A better understanding of the host-microbe relationship between oysters and VP has the potential to lead to new disease intervention strategies that will improve both animal and human health.

Research plan

A major goal of aquaculture farmers is to maximize profits and ensuring the health and welfare of the fish is an important consideration in this regard. An integral component of host health is the microbiome, especially for aquatic animals like fish that are constantly exposed to exogenous microbial organisms. Understanding the influences of this community of microorganisms on the health of fish and how factors such as diet, environmental conditions, host age, and host phylogeny can impact the structural make-up of the microbiome is vital to the aquaculture sector. Chapter Two proves the efficacy of replacing portions of an unsustainable dietary ingredient of fish feeds (i.e., fish meal) with a lyzed yeast product; illustrated by the lack of impact on the rainbow trout microbiome. Chapter Three tests the impact of feeding a *Bacillus subtilis* probiotic on physiological parameters of steelhead trout and the clear influence of host age on the structure of the fish microbiome. Encompassing results from several previous studies, Chapter Four reviews the relevant literature to define potential constituents of the rainbow trout core microbiome and discuss their possible roles. Collectively these studies using 16S rRNA gene-based microbiome analyses have helped define

the relationship between the fish microbiome and the parameters influencing its development (e.g., diet and age).

Other aquaculture-raised species such as Eastern oysters are important both economically and ecologically to the Chesapeake Bay watershed. This animal is also a common vector of the food-borne pathogen *Vibrio parahaemolyticus* (VP). A jar-based oyster inoculation system used to safely test various parameters influencing the association of VP with consumer-ready oysters was developed, as described in Chapter Five; the impacts of season (summer versus winter) and antibiotic treatment were examined. Using this novel inoculation system, it was determined that the existing microbiome provides protection against artificial VP inoculation. Therefore, Chapter Six investigated the microbiome of consumer-ready Eastern oysters over the course of a year in order to establish some of the key core constituents of the microbiome. Understanding the core constituents of the Eastern oyster microbiome can provide an idea of the "normal" or healthy microbiota that inhabit oysters as well as identifying potential probiotic species that may help to improve aquaculture practices.

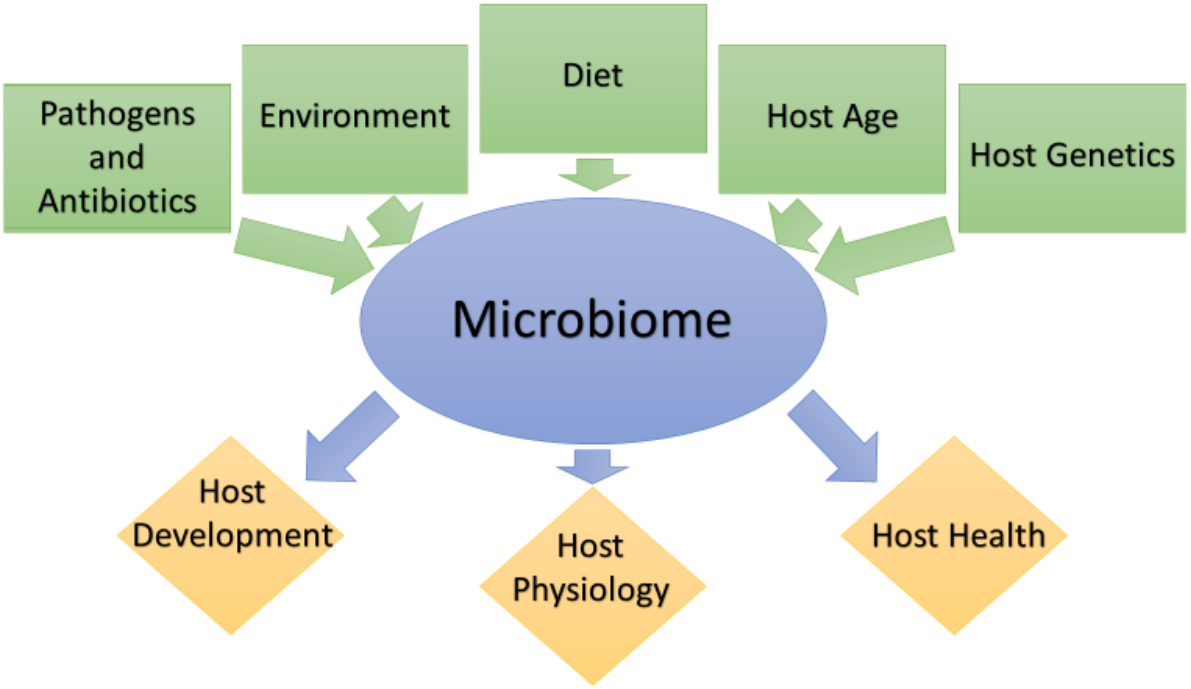

Figure 1.1. Influences on and by the host microbiome. External and internal factors that can impact the structure and function of the host microbiome are represented by green rectangles. The role the microbiome plays within the host is represented by yellow diamonds. See the text for additional details.

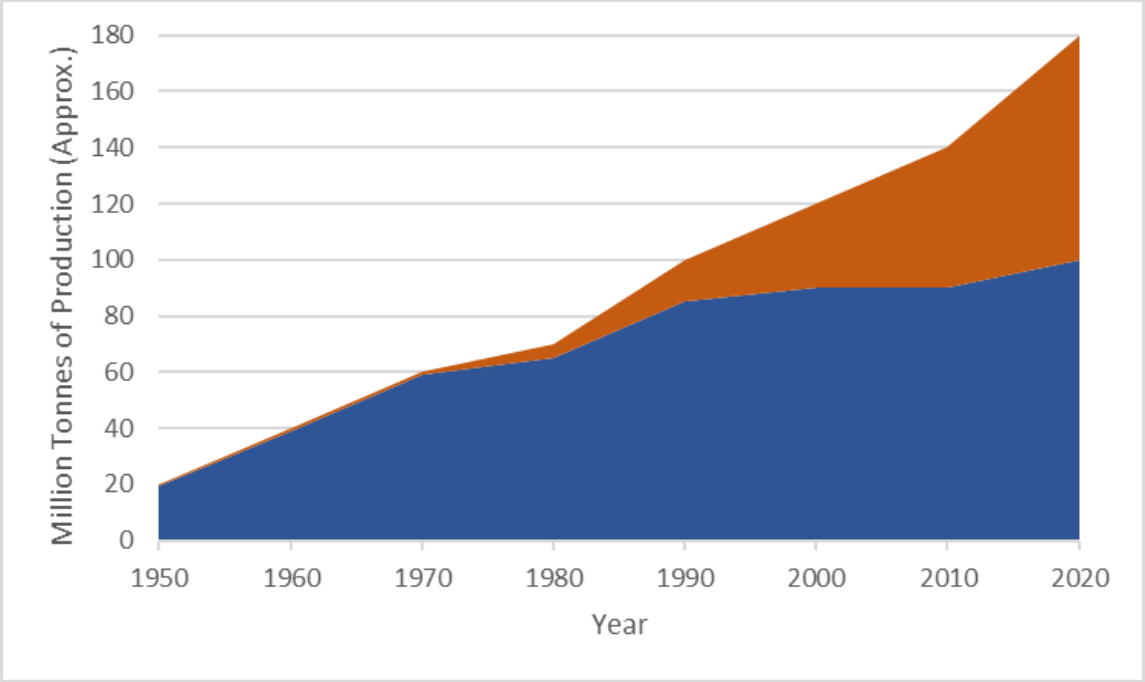

Figure 1.2. Long-term production differences between aquaculture and capture fisheries.

Adapted from FAO (2020). Trends in global capture fisheries (blue) and aquaculture (orange) production as measured by the approximate million tonnes of output since 1950.

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Chapter Two

Impact of a yeast-based dietary supplement on the intestinal microbiome of rainbow trout, *Oncorhynchus mykiss*

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Attributions

Ian S. Hines, Ann M. Stevens, and David D. Kuhn wrote the manuscript and contributed to the data analysis, study design, performed the experiments, and reviewed the manuscript. Clay S. Ferguson and Timothy J. Bushman contributed to the data analysis, performed the experiments, and reviewed the manuscript. Stephen A. Smith contributed to the study design and reviewed the manuscript. Delbert M. Gatlin III contributed to the data analysis, study design and reviewed the manuscript, performed the experiments, and reviewed the manuscript. Roderick V. Jensen contributed to the data analysis and reviewed the manuscript.

Abstract

The microbiome, an important aspect of fish aquaculture, is influenced by exogenous factors in the rearing environment including the composition and nutrient quality of the diet. To reduce reliance on fishmeal, alternative protein sources including yeast (*Saccharomyces cerevisiae*), have been successfully used in many aquafeeds. To investigate the effect of supplemented lysed and dried yeast on the fish physiology, including the intestinal epithelial-associated microbiome composition, rainbow trout (*Oncorhynchus mykiss*) were fed a standard commercial diet or one of four additional in-house extruded experimental diets containing 0%, 20%, 40%, or 60% yeast nutrient supplement as a menhaden fishmeal substitute for 16 weeks. The commercial diet, 0%, and 20% supplement-fed fish had similar average weight gains that were significantly ($P < 0.05$) higher in comparison to groups with a higher percentage of yeast. To examine if the yeast-supplemented diet had any impact on the intestinal epithelial-associated microbiome, both phylum- and family-level comparisons of the microbial communities across treatments were made. The dominant families were *Mycoplasmataceae* and *Fusobacteriaceae* with *Mycoplasma* spp. and *Cetobacterium somerae* being the dominant organisms, respectively. Results from bioinformatics analysis showed little community variation between experimental diets, suggesting that lysed and dried yeast will serve as a dietary supplement without causing large shifts in the intestinal microbiome community.

Introduction

Conventional harvesting methods used for wild-caught fish have led to an overexploitation of segments of the seafood industry, as the supply has not kept pace with the increasing demand for fish products brought about by a growing human population (Bongaarts, 1994; FAO, 2016). The aquaculture industry was a minor contributor to fish production until the late twentieth century, but has emerged as a viable and lucrative alternative to increase the level of fish products available for consumers. In the past decade, aquaculture growth has outpaced other food production sectors such as beef, grain, and poultry (Crépin et al., 2014). Due to its rapid expansion, aquaculture accounts for about half of global fish production and is over a $100 billion (USA) industry (FAO, 2016). Considering its importance both economically and nutritionally in human diets worldwide, maintaining and improving the sustainability of aquaculture is vital.

Commercial diets for aquaculture of carnivorous fish species historically have had a large fishmeal component, which provides highly palatable and easily digestible amino acids and fatty acids for the animals (Olsen and Hasan, 2012). Unfortunately, the primary source of fishmeal comes from wild-caught fish. With a rising demand for the limited supply of fish used to produce fishmeal, the associated costs have economically burdened the aquaculture industry. To mitigate this, many sustainable alternative proteins to fishmeal have been successfully used in appreciable amounts in aquaculture feeds for finfish and shrimp without compromising animal health and production characteristics (Gasco et al., 2018; Hasan, 2001). For example, these alternative proteins sources have included land crops (Davidson et al., 2016), algae (Camacho-Rodríguez et al., 2014), and bacterial proteins (Kuhn et al., 2016; Tlusty

et al., 2017). Another alternative protein source, the yeast *Saccharomyces cerevisiae,* has also shown potential as a partial fishmeal substitute (Huyben et al., 2018, 2017; Li and Gatlin, 2003; Sealey et al., 2007; Waché et al., 2006). Because yeast can be cultured on industrial by-products such as glycerol and molasses, there is an added benefit in using them as a replacement for fishmeal (Bekatorou et al., 2006; Freitas et al., 2014; Hahn-Hägerdal et al., 2005; Packard et al., 2019).

To evaluate alternative proteins in aquaculture diets, the inclusion of these ingredients should not negatively impact animal production characteristics (e.g., survival, growth, and feed efficiency), biometrics (e.g., condition factor, muscle ratio, hepatosomatic, and viscerosomatic indices), or overall health when compared to a control/standard diet. In addition to these factors, it is important to also understand how changing the composition of a diet can impact the intestinal microbiome of its host (Giatsis et al., 2016). In turn, the microbiome that inhabits this niche plays a crucial role in the overall health, development, and physiology of the fish. Critically, these microorganisms are responsible for the acquisition and/or production of many nutrients vital to the host (Tarnecki et al., 2017) as well as enhancing the immune system and providing protection from potentially harmful bacteria (Gatlin and Peredo, 2012). In order to fully understand the impacts of altering diet composition on the health and physiology of aquaculture-raised fish, correlations with changes to the intestinal epithelial-associated microbiome need to be evaluated.

As is found in other vertebrates (i.e., mammals), the intestinal microbiome of fish enables nutrient acquisition and provides metabolite production for the host (Ghanbari et al., 2015; Llewellyn et al., 2014; Nicholson et al., 2012). For example, in fish intestines, short chain

fatty acids and lactic acid-producing bacteria are present at levels that may benefit the host (Clements et al., 2014; Ingerslev et al., 2014; Lyons et al., 2017a; Michl et al., 2017; Wong et al., 2013). Specific to trout, other organisms such as *Carnobacterium maltaromaticum* found within intestines have been shown to possess beneficial antimicrobial properties (Kim and Austin, 2008; Mansfield et al., 2010). Next-generation sequencing techniques have identified *Cetobacterium somerae* and *Mycoplasma* spp. in the intestinal tract of trout where they may comprise part of the core microbiome (P.P. Lyons et al., 2017c; Philip P Lyons et al., 2017a). Moreover, several studies have indicated the core microbiome is resilient to changes in exogenous factors such as rearing intensity, culturing sites, and diet (Lyons et al., 2017c; Wong et al., 2013).

This study sought to evaluate the impacts of using lysed and dried yeast (*Saccharomyces cerevisiae*) as a dietary supplement on the host physiology and intestinal microbiome of rainbow trout cultured in a freshwater recirculating aquaculture system (RAS). It was originally hypothesized that while lysed yeast cells would be an effective alternative to fishmeal, enabling growth rates similar to standard commercial diets, their addition as a macronutrient supplement at high levels would alter the composition of the intestinal microbiome as seen in studies using inactivated yeast, or other alternative proteins (Green et al., 2013; Huyben et al., 2017; Moutinho et al., 2017; Tapia-Paniagua et al., 2011). Our results instead indicate the opposite, that lysed and dried yeast supplementation did not cause any significant changes to the intestinal microbiome, despite differences in growth that were observed amongst the experimental diet-fed fish.

Materials and methods

Fish and animal husbandry

Juvenile rainbow trout (*Oncorhynchus mykiss*) were supplied by the Virginia Trout Company (Monterey, VA). The average weight of the fish at the beginning of the study was 70.03 ± 1.02 grams (mean ± standard deviation). After two weeks of acclimation, the mean weight of the fish used at experiment initiation was 76.0 ± 4.18 grams. For the nutrition trial, fish were fed one of the following diets: commercial feed (Zeigler Finfish, Gardners, PA) or an experimental diet with 0%, 20%, 40%, or 60% of the menhaden fishmeal replaced with lysed and dried cells of *Saccharomyces cerevisiae* on a dry-weight basis, respectively. Novozymes Biologicals Inc. and Microbiogen Pty Ltd. manufactured and processed the yeast, which is a proprietary strain that was cultured on glycerol recovered as a byproduct of ethanol production. Analysis indicated that the nutrient composition on a dry-matter basis was 41.3% crude protein and 6.0% total fat. The ingredients in the experimental diets were mixed and extruded by Texas A&M Process Engineering Research & Development Center (College Station, TX) using the feed components listed in Table 2.1 and the nutrient profiles were subsequently analyzed (Table 2.2). Diets were formulated to be isonitrogenous and isocaloric; the yeast was balanced accordingly with the ingredient that it replaced. An additional diet, a commercial feed, served as an internal control for comparison to the 0% supplement diet to test the manufacturing efficacy of the experimental replacement diets. Fecal material was examined in the animals fed the commercial diet so that information about the transient versus adherent microbes in the intestinal tract could be ascertained.

The fish were housed in a single indoor freshwater (dechlorinated municipal water) RAS outfitted with 25 220-liter tanks, two bubble bead filters and a sand filter for solids removal, a one-cubic meter fluidized-bed bioreactor for nitrification, a 130W UV unit for disinfection, a chiller to maintain temperatures at approximately 14-15º C, and diffused aeration. Throughout the duration of the trial, the system was exposed to fluorescent overhead lighting (5,000K) on a 14:10 (light:dark) h cycle with natural light (August-November) also entering the space through windows. Water quality parameters monitored in the RAS included alkalinity as calcium carbonate, dissolved oxygen, nitrate-N, nitrite-N, pH, temperature, and total ammonia-N (APHA, 2012). All parameters were maintained in accordance with the optimal conditions for rainbow trout growth (Hinshaw et al., 2004).

Ethics statement

The authors confirm that the ethical policies of the journal as noted on the journal's authors guidelines page have been adhered to and the appropriate ethical review committee approval has been received. Experimental methods were approved by Virginia Tech's Institute for Animal Care and Use Committee and performed in accordance with all relevant protocol guidelines and regulations under IACUC # 16-047.

Experimental design and sampling

For this study, all 25 tanks in the RAS were used with each of the five diets distributed across five different tanks. Each tank was stocked with ten fish creating five tank replicates for a total of 50 fish per diet. To track production data and to adjust feed amounts, all fish from each tank were weighed weekly for the duration of the trial which lasted 16 weeks. At the end of the trial, all fish were humanely euthanized using a buffered 250 mg/L buffered MS-222

tricaine methanesulfonate water bath (Western Chemical, Inc., Ferndale, WA). Next, five of the ten fish per tank (25 fish per diet) were measured and dissected to determine individual overall weight and length, and weights of the fillet, viscera, and liver. From this data, biometrics (i.e., condition factor, muscle ratio, hepatosomatic and viscerosomatic indices) were determined using standard equations (Stoneham et al., 2018).

Using ethanol flame-sterilized surgical tools, approximately 10 cm of intestine, comprised primarily of midgut tissue, was removed from six fish per diet (drawn randomly from the different tanks) for the microbiome analysis to enable identification of the core microbiome across the entire animal population. The midgut was selected due to its ease of identification and accessibility, and the fact that the microbial communities along trout intestinal tracts are relatively homogenous (Lowrey et al., 2015). Within the weigh boat, adipose tissue was removed from the exterior of the intestinal segment and the sample was weighed. Fecal material was discarded from the animals in the yeast supplement experimental groups by gently squeezing the intestinal segment to observe only the adherent microbiome. Fecal material was not removed from the intestines of the commercial diet fish to enable an analysis of both the transient and adherent microbiomes in these animals. The commercial diet- fed fish also served as an internal control for comparison only to the 0% supplement animals in the study. Each intestinal tissue was transferred into a 15-mL polypropylene conical tube (Corning, Corning, NY) and immediately frozen in a 70% ethanol-dry ice bath prior to storage at -20ºC. Between each sampling of fish, cutting boards, knives, gloves, and weigh boats were sanitized with 70% ethanol. Within six weeks, tissue samples were thawed and transferred into separate 50-mL polypropylene conical tubes (Corning) without buffer. Individual samples were then

homogenized until the homogenate contained no visibly intact tissue using an OmniTip homogenizer (Omni International, Kennesaw, GA) with a soft tissue tip that was washed in distilled water then surface-sanitized with ethanol between samples.

DNA extraction

DNA was extracted from individual tissue samples using the Qiagen PowerSoil kit (Germantown, MD) following the manufacturer's protocol with the following alterations: a 10-min incubation at 72°C after addition of C1 buffer and a 5-min incubation at 72°C prior to elution in 50 µL dH_2O. Intestinal homogenate was added to the lysis buffer within the PowerBead tubes (Qiagen) using a range of masses between 400 and 750 mg with a mean weight of 450 mg. After isolation of total genomic DNA (gDNA), the gDNA quality was checked using a nanospectrophotometer (Implen, Westlake Village, CA) at which point the samples were frozen at -20°C.

PCR amplification

DNA purified from experimental intestinal samples was used as the template to amplify the 16S rRNA gene V4 region in triplicate with a single negative water control for each primer set. PCR reaction conditions were: 10 µL of 2.5X 5Prime Hotstart Master Mix (Quantbio, Beverly, MA), 0.5 µL of 10 µM (200 nM final concentration) each of forward barcoded primer (515f; AATGATACGGCGACCACCGAGATCTACACGCTxxxxxxxxxxxxTATGGTAATTGTGTGYCAGCMGCCGCGGTAA, with the x region representing the bar code) and reverse primer (806r; CAAGCAGAAGACGGCATACGAGATAGTCAGCCAGCCGGACTACNVGGGTWTCTAAT), 2 µL DNA template of the 50 µL elution from the Qiagen column, and 12 µL PCR-grade dH_2O for a total

volume of 25 µL (Caporaso et al., 2011). Universal barcoded forward primers were created according to Caporaso et al. (2011). Thermocycler (Thermo Scientific, Waltham, MA) settings were programmed as follows: initial denaturation at 94°C for 2 min; 35 cycles of denaturation at 94°C for 45 sec, annealing at 50°C for 1 min, and elongation at 68°C for 30 sec; final elongation at 68°C for 10 min.

Gel extraction

The triplicate experimental PCR products were pooled together and subsequently visualized on a 1% agarose gel. Due to the presence of host 18S rRNA DNA, the desired 16S rRNA DNA V4 region products were excised from the gel after visualization on a UV-transilluminator and purified using the Qiagen Gel Extraction kit per the manufacturer's instructions with the following alterations: elution with 50 µL dH_2O and incubation at 72°C for 5 min prior to elution. DNA yield, as measured by the A_{260} in ng/µL, and purity, as measured by the A_{260}/A_{280} and A_{260}/A_{230} ratios, were measured on a nanospectrophotometer (Implen). An ethanol precipitation protocol was used to concentrate some gel-extracted samples with low DNA yields.

Sequencing and bioinformatics data analysis

Immediately prior to sequencing, the yield and purity of sample DNA, containing 16S rRNA gene V4 regions, were quantitated using a Qubit fluorometer at the Biocomplexity Institute at Virginia Tech. The Illumina MiSeq sequencing platform was used to generate 250 bp paired-end sequencing data from the pool of V4 region DNA samples loaded at a final concentration of 9.5 pM based on qPCR with 20% phiX spiking. The total number of sequences generated for this study was 5,437,414 reads. Individual samples with less than a total of

10,000 associated reads each were subsequently removed from the bioinformatics analyses. Groups with greater than four samples were pared down to four samples each by removing the samples with the lowest number of attributed reads to maintain an equal sample size (n = 4) for downstream analysis. These data were used to construct the phylum-level and family-level microbiome communities and diversity analyses using the Quantitative Insights Into Microbial Ecology 2 (QIIME2) version 2019.1.0 (Bolyen et al., 2018). Briefly, samples were demultiplexed prior to denoising using the DADA2 program with default parameters (i.e., q = 2 and no read trimming) (Callahan et al., 2016). Amplicon Sequence Variants (ASVs), generated from the DADA2 pipeline, associated with less than 0.001% of the total number of reads were removed due to low confidence. Taxonomy was assigned to ASVs using a pre-trained, 16S rRNA gene V4-region classifier from the Silva database version 132 (Glöckner et al., 2017; Quast et al., 2013; Yilmaz et al., 2014). The resulting ASV table containing taxonomic information and abundance was filtered to remove those ASVs associated with contaminating host tissues (i.e., mitochondria). Finally, to evenly compare the relative ASV abundances between the 20 individual samples used for bioinformatics analysis, the sample with the lowest number of sequences was used to define the rarefaction threshold. The total number of sequences in the remaining samples was therefore rarefied to the rarefaction threshold (53,642 reads) resulting in a total of 152 unique ASVs. Following rarefaction, a taxonomic bar plot representing the relative abundance of the top 10 most abundant ASVs (comprising five assigned phyla and eight assigned families) identified within each diet (average of four fish) was created using the QIIME2R, phyloseq, tidyverse, dplyr, and ggplot2 R packages (Bisanz, 2018; McMurdie and Holmes, 2013; R Core Team, 2019; Wickham, 2017, 2016; Wickham et al., 2019). The

sequences associated with ASVs of interest were further analyzed using the Basic Local Alignment Search Tool (BLAST; NCBI, Bethesda, MD). The BLAST search provided genus- and species-level information to confirm taxonomic information assigned using the Silva database.

Alpha diversity analyses were calculated using the Shannon, Simpson, and Chao1 diversity indices for the averaged set of four fish from each diet. The number of observed ASVs per treatment group was also calculated. Beta diversity analyses were calculated using the unweighted UniFrac and weighted UniFrac metrics for the averaged set of four fish from each diet and visualized in a Principal Coordinates of Analysis (PCoA) plot. To better visualize the estimated separation between the microbiomes of different treatment groups, ellipses were plotted using a T-distribution with a 99% confidence interval. Both beta diversity analyses were plotted in R using the QIIME2R, tidyverse, ggplot2, and vegan packages (Oksanen et al., 2019).

Statistical analysis

Fish response data from the feeding trial in which incremental levels of yeast were substituted for fishmeal were subjected to one-way analysis of variance (ANOVA) to determine if significant ($P<0.05$) differences were detected. The distribution was analyzed using the Levene's test for homogeneity prior to the ANOVA tests. If significant differences were detected, then the Tukey's post-hoc test was applied to determine where those differences occurred. Statistical analyses performed on fish response data was carried out using JMP (JMP® Pro, v.14.0.0, Cary, NC).

For each of the alpha diversity indices (i.e., Shannon, Simpson, Chao1, observed ASVs), an ANOVA was performed. The ANOVA was followed by the Tukey's post-hoc test to determine if significant differences were observed between treatment groups. Similarly, a permutational

ANOVA (PERMANOVA) was used to test for any significant differences in beta diversities between the different diets. Statistical analyses used for alpha and beta diversities were done using R and internally through QIIME2, respectively. An ANCOM differential abundance analysis was done to confirm there were no significant changes in the abundance of ASVs between treatment groups (Mandal et al., 2015).

Results

Fish responses

To investigate the effect(s) of replacing portions of fishmeal with lysed and dried yeast (*Saccharomyces cerevisiae*), physiological parameters were measured and compared between groups of fish fed different yeast-supplemented diets (Table 2.3). The feed efficiency as measured by the food conversion ratio (FCR) was not significantly different among fish fed the various diets. Factors HSI and VSI, proxy measurements of energy retention and fat accumulation, respectively, were also not found to be significantly different among fish fed the various diets. As a relative measure of the weight and length of fish within the study, the calculated K condition factors for each diet indicated neither significant decreases nor increases in overall condition among fish fed the diets. The groups fed the diet without yeast supplementation (0% supplement diet) and the 20% supplement diet each had significantly ($P<0.05$) higher weight gains compared to those fed the 40% and 60% supplement diet-fed groups. On the other hand, the 0% supplement diet surprisingly led to significantly lower fish survival levels than fish fed the other diets for unknown reasons. There were no gross clinical signs of infection in the population of fish, so a dietary issue was considered to be the likely cause of the higher levels of mortality. Therefore, this 0% supplement diet group was also

compared to an internal control group fed a commercial diet; there was no significant change in weight gain nor FCR between them (Table 2.4). However, the survival in the 0% supplement group was also significantly decreased compared to the commercial diet group.

Intestinal epithelial-associated microbiome

The fish shared a similar intestinal epithelial-associated microbiome regardless of the dietary treatment (Figure 2.1, Figure 2.2, and Figure A.1). Overall, the beta diversity calculations indicated little change in the microbial community present within the fish intestinal microbiome (Figure 2.1). Further, the intestinal microbiomes all clustered together regardless of diet when phylogenetic distances without or with relative abundance were considered via unweighted UniFrac or weighted UniFrac, respectively. A PERMANOVA confirmed this similarity ($P>0.05$).

No significant differences in microbial composition were observed between treatment groups for the various alpha diversity indices (Table 2.5). Additionally, the alpha diversities reported here correlated to the observed dominance by just a few taxa (Figure 2.2). The microbiomes were analyzed at both the family (Figure 2.2) and phylum levels (Figure A.1) with the top 10 most abundant ASVs comprising organisms in eight assigned families and five assigned phyla.

Within these fish, the bacterial families *Mycoplasmataceae* and *Fusobacteriaceae*, associated with the phyla Tenericutes and Fusobacteria, respectively, dominated the intestinal microbiome. Further, a BLAST search using the ASV sequences associated with the most abundant organisms in these tissues indicated these sequences belonged to *Mycoplasma* spp. (~60%) and *Cetobacterium somerae* (~30%). In contrast to these dominant organisms, the

remainder of the top ten ASVs accounted for less than 10% of the total microbiome. These ASVs were associated with the families *Bacillaceae*, *Enterobacteriaceae*, *Vibrionaceae*, *Brevinemataceae*, *Ruminococcaceae*, and *Desulfovibrionaceae* (or the phyla Firmicutes, Proteobacteria and Sprirochaetes). The family *Bacillaceae* accounted for a larger portion (~20%) of the intestinal microbiome only in fish fed the commercial diet, where fecal matter remained, suggesting that these bacteria were transient in nature in the system. These commercial diet-fed fish still harbored a high abundance of both *Mycoplasmataceae* (~30%) and *Fusobacteriaceae* (~50%) in their intestinal tracts.

Discussion

One way to increase the sustainability of raising fish in aquaculture is by using alternative protein sources from a variety of feedstuffs including those of plant, animal and microbial origin. Some examples of these include cottonseed meal, soybean products, and other plant-based ingredients such as corn gluten meal, and algal meals (Camacho-Rodríguez et al., 2014; El-Sayed, 1999). High-level replacement of fishmeal with yeast has been correlated with decreased fish growth (Huyben et al., 2018; Vidakovic et al., 2020). In the present study, fish fed diets with 40% or 60% of fishmeal replaced with a lysed and dried yeast product displayed significantly ($P<0.05$) decreased weight gains compared to those fed the 20% supplement diet and 0% supplement diet (Table 2.3). The observed difference in weight gains might be attributed to an increased presence of antinutritional factors present in the higher supplemented dietary groups. For example, nucleic acids can lead to decreased feed efficiency and growth in trout (Huyben et al., 2017; Rumsey et al., 1992, 1991; Sánchez-Muniz et al., 1982). The low survival rate of the animals fed the 0% supplement diet may have been due to

an error in the diet manufacturing. However, there were no significant differences observed from the production or microbiome analyses of the surviving animals in comparison to the fish fed the commercial diet control.

As a vital component to fish health, it is important to understand how the intestinal microbiome is shaped by exogenous factors such as the diet. Within this study, the intestinal microbiome was not dramatically altered by the replacement of fishmeal with lysed and dried yeast (*Saccharomyces cerevisiae*) (Figure 2.2), even at high levels. This is similar to a study with live yeast (Huyben et al., 2018) where up to a 40% replacement of fishmeal had little impact on the bacterial microbiome. However, this is opposite to the findings of another study using inactivated, but partially culturable yeast cells as a dietary supplement where 40-60% replacement of fishmeal caused significant changes in the microbiome (Huyben et al., 2017). In our study, the phyla Tenericutes and Fusobacteria and the families *Mycoplasmataceae* and *Fusobacteriaceae*, respectively, remained dominant in the fish across all diets as reflected by low alpha diversities representing the lack of intra-sample diversity with consideration of relative abundance (Table 2.5). The beta diversity findings also demonstrated a high degree of similarity among the microbiomes across all diets. The presence of *Bacilliaceae*, within the phylum Firmicutes, at higher levels in the commercial diet may have been due to transient fecal material, but this did not significantly impact either the alpha or beta diversity findings. In addition, the physiological parameters of the fish fed the commercial diet were similar to those fed the 0% supplement diet.

One of the dominant families found in the microbiome across all diets, *Mycoplasmataceae*, represented here by the genus *Mycoplasma*, is a group of bacteria

harboring a unique morphology in that they do not contain a cell wall. In addition to this, they are difficult to culture without cholesterol supplied by the host (Razin, 1967). This illustrates the importance of amplifying 16S rRNA genes from host-derived DNA samples versus culture-grown isolates to provide a more accurate representation of the microbiome composition (Amann et al., 1995; Staley and Konopka, 1985). Different species of *Mycoplasma* favor colonization of distinct tissues including gills (Kirchhoff and Rosengarten, 1984) and the intestinal tract (Bano et al., 2007; Ransom, 2008). Though this organism is considered to be the causative agent of some respiratory diseases in humans (Razin, 1967), it appears to act as a commensal in the intestinal tracts of various fish (Holben et al., 2002; Huyben et al., 2018; Llewellyn et al., 2016). For example, *Mycoplasma mobile* can colonize the fish intestinal tract without causing disease, though it also has no known metabolic genes beneficial to the host (Bano et al., 2007). The functional role of *Mycoplasma* within the intestine of rainbow trout remains to be determined.

Taxa within the second most abundant family in the microbiome, *Fusobacteriaceae*, have been extensively shown in human studies as pathogenic organisms associated with inflammation and disease states of the host under anaerobic to microaerophilic conditions such as periodontal disease, colorectal cancer, and Crohn's disease (Bajaj et al., 2012; Castellarin et al., 2012; Peterson et al., 2013). These anaerobic bacteria are known to undergo fermentation to produce short chain fatty acids such as acetic acid and butyrate involved in some host signaling pathways (Olsen, 2014; Yarza et al., 2008). The dominant *Fusobacteriaceae* species in this study was identified as *Cetobacterium somerae*. Found in other freshwater fishes such as grass carp and tilapia, these vancomycin-resistant bacteria were previously classified as

Bacteroides A and are known to produce vitamin B12 (Tsuchiya et al., 2008). Though this organism is associated with disease in humans, it appears to serve as a commensal, or possibly even a beneficial organism, within fish intestinal epithelial-associated microbiomes including those in our system (Larsen et al., 2014).

In conclusion, supplementation of diets with nutrients provided by addition of lysed and dried yeast, as a replacement for fishmeal, did not dramatically affect the intestinal epithelial-associated microbiome of rainbow trout. Despite the presence of a consistent microbiome, levels of yeast supplementation above 20% appeared to negatively impact some physiological aspects of the fish, including a significant decrease in growth rates. Curiously, other physiological parameters such as HSI, VSI, muscle ratio, FCR, and K condition factor remained largely unaltered. Nevertheless, a diet with 20% or lower yeast supplementation would effectively reduce feed costs while maintaining production outputs similar to traditional diets.

Data availability

Paired-end sequencing reads from the 20 intestinal samples used for bioinformatics analysis were deposited into the NCBI Sequence Read Archive (SRA) with accession number pending (bioproject ID PRJNA574839).

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Conflict of interest

The authors declare no conflict of interest.

Table 2.1. Ingredient levels used for each experimental diet.

	Treatment diets [g/100 g dry matter]			
Ingredients	**0% Supplement**	**20% Supplement**	**40% Supplement**	**60% Supplement**
Menhaden Meal	32.0	25.6	19.2	12.8
Yeast	0.000	7.59	15.2	22.8
Menhaden Oil	16.1	16.9	17.6	18.4
0360 Vitamin Premix	0.500	0.500	0.500	0.500
5TSZ Mineral Premix	0.100	0.100	0.100	0.100
Alphacel (Polysaccharide)[a]	5.88	3.92	1.96	0.000
Stay-C (Vitamin C Source)	0.0200	0.0200	0.0200	0.0200
Soy Protein Concentrate	11.0	11.0	11.0	11.0
Dicalcium Phosphate	1.00	1.00	1.00	1.00
Chicken By-product meal	11.0	11.0	11.0	11.0
Taurine	0.500	0.500	0.500	0.500
Soy Lecithin	0.500	0.500	0.500	0.500
Lysine HCl	0.350	0.350	0.350	0.350
DL-Methionine	0.400	0.400	0.400	0.400
Wheat flour	20.6	20.6	20.6	20.6

[a] served as an inert filler ingredient

Table 2.2. Analyzed nutrient profile of each experimental diet (dry-matter basis).

	Treatment diets [g/100g dry-matter basis]			
Ingredients	**0% Supplement**	**20% Supplement**	**40% Supplement**	**60% Supplement**
Proximates and Minerals				
Protein (crude)	41.4	42.0	40.9	40.4
Fat (crude)	19.2	19.7	20.2	19.8
Acid detergent fiber	5.20	4.10	2.50	1.90
Ash	10.5	10.6	10.3	10.5
Calcium (Ca)	2.65	2.47	1.93	1.63
Phosphorus (P)	1.67	1.94	1.95	2.17
Ca:P	1.59	1.27	0.990	0.750
Potassium	0.760	1.01	1.08	1.25
Magnesium	0.160	0.220	0.240	0.300
Fatty Acids				
Saturated (SFA)	7.05	†	†	7.09
Polyunsaturated (PUFA)	7.23	†	†	7.42
Omega-3	5.94	†	†	6.13
Omega-6	1.21	†	†	1.22

Monounsaturated (MUFA)	4.89	†	†	2.12
Omega-9	2.28	†	†	2.33
Essential Amino Acids				
Arginine	2.79	†	†	2.50
Histidine	1.15	†	†	1.04
Isoleucine	1.41	†	†	1.61
Leucine	2.71	†	†	2.60
Lysine	3.51	†	†	2.86
Methionine	1.44	†	†	1.20
Phenylalanine	1.81	†	†	1.63
Threonine	1.80	†	†	1.73
Tryptophan	0.300	†	†	0.360
Valine	2.03	†	†	1.87

† Values not determined.

Table 2.3. Physiological parameter measurements in rainbow trout (Oncorhynchus mykiss) fed yeast-supplemented diets (error (+/-) represents the standard deviation).

Treatment	0% Supplement	20% Supplement	40% Supplement	60% Supplement
Average Weight Gain (g)	252 ± 24.9[a]	227 ± 14.0[ab]	185 ± 12.4[b]	180 ± 4.93[b]
FCR[†]	1.05 ± 0.0724	1.08 ± 0.0450	1.19 ± 0.0850	1.21 ± 0.0205
K condition factor	2.30 ± 0.0479	2.26 ± 0.0961	2.43 ± 0.0298	2.31 ± 0.0343
VSI[‡]	11.2 ± 0.278	11.0 ± 0.424	11.3 ± 0.364	11.5 ± 0.188
HSI[§]	1.65 ± 0.158	1.41 ± 0.131	1.38 ± 0.105	1.23 ± 0.0447
Muscle ratio (%)	45.4 ± 0.978	48.4 ± 0.534	47.3 ± 1.24	47.7 ± 0.620
Survival (%)	66.0 ± 4.00[a]	94.0 ± 4.00[b]	88.0 ± 2.40[b]	96.0 ± 2.50[b]

[†] Feed conversion ratio

[‡] Viscerosomatic index

[§] Hepatosomatic index

[ab] Different superscript letters denote $P<0.05$ within a horizontal row after one-way analysis of variance followed by Tukey's Honest Significance Difference test

Table 2.4. Comparison of growth parameters of rainbow trout (Oncorhynchus mykiss) following supplementation of a basal formulation and a commercial diet (error (+/-) represents the standard deviation).

Treatment	0% Supplement	Commercial
Average Weight Gain (g)	252 ± 24.9	228 ± 9.36
FCR[†]	1.05 ± 0.0724	1.08 ± 0.0583
K condition factor	2.30 ± 0.0479	2.20 ± 0.180
VSI[‡]	11.2 ± 0.278	11.32 ± 0.200
HSI[§]	1.65 ± 0.158[a]	0.88 ± 0.0545[b]
Muscle ratio (%)	45.4 ± 0.978	40.18 ± 3.78
Survival (%)	66.0 ± 4.00[a]	94.0 ± 2.40[b]

[†] Feed conversion ratio

[‡] Viscerosomatic index

[§] Hepatosomatic index

[ab] Different superscript letters denote $P<0.05$ within a horizontal row after two-tailed t-test was calculated.

Table 2.5. Treatment group alpha diversities (error (+/-) represents the standard deviation†).

Diet[2]	Shannon	Simpson	Chao1	Observed ASVs
0% Supplement	1.26 ± 1.32	0.319 ± 0.315	30.3 ± 31.3	30.3 ± 31.3
20% Supplement	0.948 ± 0.601	0.347 ± 0.225	18.0 ± 7.93	17.8 ± 7.85
40% Supplement	0.629 ± 0.444	0.244 ± 0.186	9.50 ± 2.38	9.50 ± 2.38
60% Supplement	1.14 ± 0.620	0.385 ± 0.194	27.9 ± 22.1	27.8 ± 22.0
Commercial	0.630 ± 0.589	0.202 ± 0.204	8.75 ± 3.40	8.75 ± 3.40

† Four fish were analyzed for each diet.

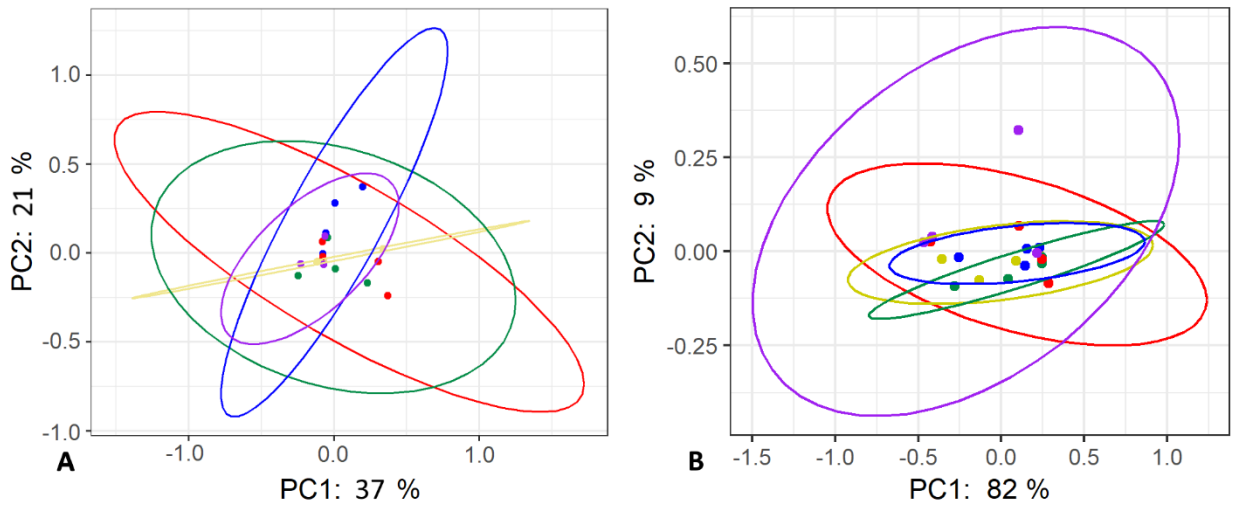

Figure 2.1. Principal coordinates of analysis (PCoA) plots representing the beta diversity distances between the intestinal microbiomes of trout fed different diets. Distances were calculated by (A) Unweighted UniFrac and (B) Weighted UniFrac. Diet treatment groups of four fish each are represented by red (0% supplement), yellow (20% supplement), green (40% supplement), blue (60% supplement), purple (commercial diet). Ellipses are defined by the Student's T test distribution of the four samples in each diet.

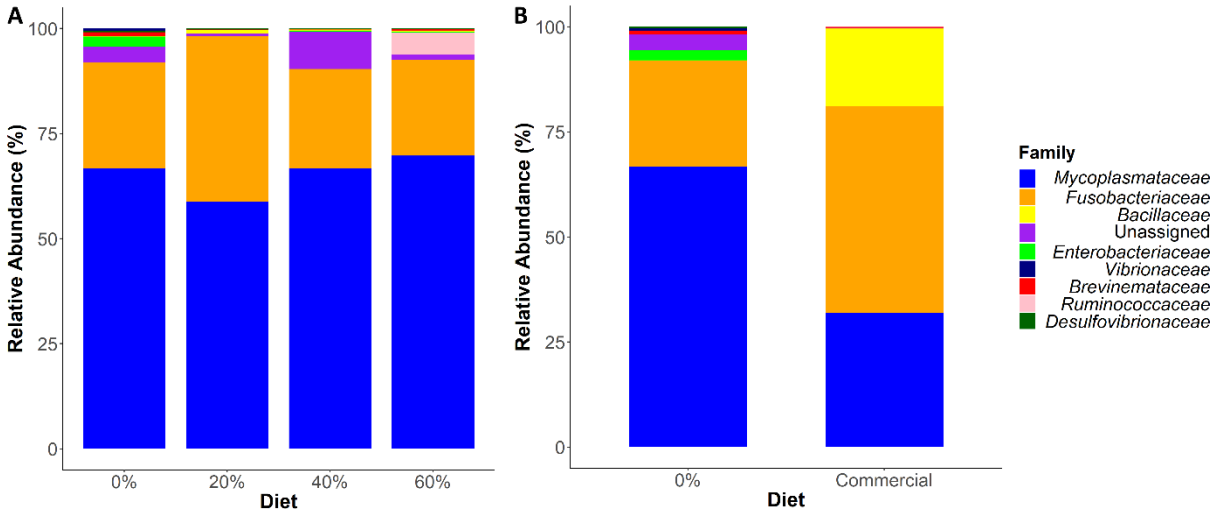

Figure 2.2. Trout intestinal epithelial-associated family-level microbiome. The average reads generated from sequencing the 16S rRNA genes for each treatment group of four fish are plotted to illustrate the relative diversity of the most abundant bacterial families for the (A) 0% to 60% yeast supplement diets and (B) the 0% supplement and commercial diet groups. Diet treatment groups include: 0% supplement (0%), 20% supplement (20%), 40% supplement (40%), 60% supplement (60%), commercial diet (Commercial).

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Chapter Three

Probiotic exposure during the early developmental stages of steelhead trout (*Oncorhynchus mykiss*) enhances fish production

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Attributions

Ian S. Hines and David D. Kuhn wrote the manuscript and contributed to the data analysis, study design, performed the experiments, and reviewed the manuscript. Ann M. Stevens wrote the manuscript and contributed to the data analysis, study design, and reviewed the manuscript. Stephen A. Smith contributed to the study design and reviewed the manuscript. Clay S. Ferguson contributed to the data analysis, performed the experiments, and reviewed the manuscript. Kevin D. Santiago-Morales contributed to the data analysis, study design, performed the experiments, and reviewed the manuscript. Jireh Clarington contributed to the data analysis, study design, performed the experiments, and reviewed the manuscript. Meaghan Thompson contributed to the data analysis, performed the experiments, and reviewed the manuscript. Meghann Rauschenbach contributed to the data analysis, performed the experiments, and reviewed the manuscript. Uri Levine contributed to the data analysis, study design, performed the experiments, and reviewed the manuscript. David Drahos contributed to the data analysis, study design, and reviewed the manuscript. Frank O. Aylward contributed to the data analysis and reviewed the manuscript.

Abstract

Sustainable aquaculture practices can help meet the increasing human demand for seafood, while easing pressures on natural fish populations. Studies aimed at increasing fish production in aquaculture have included supplementary dietary probiotics that often promote general health and enhanced growth rates by altering the microbiome of the host. Steelhead trout (*Oncorhynchus mykiss*) is an anadromous form and is a fast-growing subspecies of rainbow trout making it an attractive fish to the aquaculture industry. In this study, the impact of feeding a *Bacillus subtilis* probiotic on the microbiome of steelhead trout was examined temporally across several stages of animal development in relation to physiological measures. Diets included: commercial feed without probiotics as a control (A), continually-fed probiotic top-coated on commercial feed (B), commercial then switch to probiotic feed (C), or probiotic then switch to commercial feed (D). Validation of probiotic concentrations on feed and in fish tissues was performed using CFU/g and qPCR, respectively. Fish growth was measured and samples for microbiome analyses were collected at multiple timepoints during fish development. Fish fed diet D yielded higher weights than the other three diets, with little impact on other biometric parameters. Microbiome analysis indicated an increasing trend of overall alpha diversity from the egg stage (day -19) to day 29 for fish fed the various diets. Early developmental fish intestinal microbiomes are distinct from later timepoints with a clear influence of diet by the final harvest as evidenced by beta diversity metrics. Following day 29, fish fed diets A and D maintained a high alpha diversity in contrast to a decreased trend for fish fed diets B and C. As expected, fish fed diets B and C harbored a significantly higher relative abundance of *Bacillus* sp. in their total microbiomes (feces + mucosa). However, the mucosal-

only microbiome indicated little variation between the four groups of fish. Therefore, probiotic supplementation significantly affects the transient communities, but not the adherent mucosal microbiome. Feeding the probiotic earlier in development, during the hatchery phase, to influence microbiome development rather than later after the microbiome has been established, appears to be more effective aquaculture practice to produce higher fish yields.

Introduction

Fish are an important global agricultural resource, especially by providing a valuable source of animal protein in the human diet. However, wild-caught fishing practices are increasingly unable to meet the demands of a growing human population and this has led to over-exploitation of some fishing sources (FAO, 2020). The aquaculture industry has proved to be an effective alternative means of supplying fish products and it is now the fastest growing sector of food production, estimated to be over a $250 billion industry (FAO, 2020). With this increased reliance on aquaculture, sustainability is an important priority. Sustainability is dependent on animal welfare and health, which in turn are dependent on the associated microbiome.

Bacteria that inhabit the various external and internal environments of fish hosts constitute the fish bacterial microbiome. These microbial communities play vital roles in maintaining gastrointestinal homeostasis (Butt and Volkoff, 2019; Merrifield and Rodiles, 2015; Xu et al., 2020; Yu et al., 2021), protection against pathogens (de Bruijn et al., 2018; Gomez et al., 2013), and nutrient acquisition (Brugman et al., 2018; Hanning and Diaz-Sanchez, 2015). Exogenous agents, such pre- and probiotics, can also increase the efficacy of these benefits (Kumar et al., 2008; Pirarat et al., 2011; Sookchaiyaporn et al., 2020; Tuan et al., 2013). Prebiotics are beneficial molecules generated through microbial metabolism, while probiotics are beneficial live microbial organisms. One such probiotic, *Bacillus subtilis*, the focus of this study, is capable of helping to increase fish growth rates (Park et al., 2017) while supporting the host immune system (Galagarza et al., 2018; Kamgar et al., 2013; Newaj-Fyzul et al., 2007; Standen et al., 2015; Tang et al., 2019) when is it added as a dietary supplement.

During fish intestinal development there exists a flux in the types and abundance of bacteria inhabiting the internal microbiome (Hansen and Olafsen, 1999). This flux is the result of the developing intestine being colonized by environmental microorganisms competing for and metabolizing the nutrients supplied by the gastrointestinal tract of the fish. Microbial communities within the developing fish intestine are strongly affected by diet and rearing conditions (Michl et al., 2017; Wilkes Walburn et al., 2019). There is some evidence that communities can be "seeded" via early dietary measures, with microbiome changes persisting into maturity (Parata et al., 2020). However, as the fish mature, the gastrointestinal microbiome will remain in flux, to some degree, as the host system continually selects for the most appropriate populations in the community (Bakke et al., 2015; Ingerslev et al., 2014; Li et al., 2017; Stephens et al., 2016). Therefore, it is vital that probiotics are administered in such a manner to facilitate effective colonization in the host. Unfortunately, exogenously-fed probiotics such as *B. subtilis* are typically unable to compete with an established native microbiome long-term to persist in the host tissues (Casula and Cutting, 2002; Giatsis et al., 2016) and must be supplied for the duration of fish culturing. Persistence of probiotic organisms in the microbiome of animals following probiotic cessation is an important attribute to consider during probiotic selection (Pérez-Sánchez et al., 2014).

For the present study, the impact of a probiotic on the earliest stages of fish development, when the microbiome is first developing, versus later stages of growth, after an initial microbiome has been established, was examined. The salmonid, steelhead trout (*Oncorhynchus mykiss*), was chosen as the host animal for analysis, in part, due to its palatability and relatively understudied nature. Unlike rainbow trout (also *Oncorhynchus*

mykiss), steelhead trout are anadromous and spend part of their life cycles in marine environments, but spawn in freshwater like salmon. It was hypothesized that exposure to probiotics at the early stages of intestinal development (i.e., first feeding) would lead to more effective intestinal colonization, and enhanced animal production. Overall, feeding the probiotic exclusively during early intestinal development led to the highest individual fish weights and impacted the microbiome.

Materials and methods

Fish husbandry

Approximately 2000 steelhead trout eggs were supplied by Riverence Brood LLC (Olympia, WA, USA) and distributed evenly onto three vertical tray fish incubators (MariSource, Burlington, WA, USA). Fish incubators were situated on existing holding tanks as part of a single-system recirculating aquaculture system (RAS) using dechlorinated municipal water. The RAS system was set up as described in Hines et al. (Hines et al., 2021) with natural light (August – March) and fluorescent lighting on a 12h light:12h dark cycle. RAS water quality was monitored for temperature and dissolved oxygen on a daily basis, total ammonia-N and nitrite-N every other day, and nitrate-N and alkalinity on a weekly basis (AOCS, 2010; APHA, 2012). A water temperature of ~11°C was held until eggs hatched at which point the temperature was maintained at ~13°C for the duration of the study. Animals were maintained according to Virginia Tech IACUC #20-084.

Microbiome samples and fish weights were collected at multiple timepoints throughout the study (Figure 3.1). The first timepoint as defined by the receipt of the eggs was denoted as T-1 (day -19). Exogenous feeding was initialized 19 days after T-1 (T0; day 0) at which point the

fish were separated into two tanks wherein each was fed one of two diets: a commercial feed control (A) or a probiotic-coated feed (B). The probiotic used in this study was *Bacillus subtilis* 086 (NZ86, Galagarza et al., 2018) supplied by Novozymes Biologicals Inc. (Salem, VA, USA) that was top-coated on to a commercial feed (Zeigler Bros. Inc, Gardners, PA, USA) at a concentration of 10^8 CFU/g feed. Half of the fish from each tank (A and B treatment groups) were transferred into a new tank 29 days after first feeding (T1, day 29). At T1, 50% of the A treatment group continued on diet A, while the other half of the fish transferred from diet group A were switched to a probiotic diet (C). Similarly, 50% of the B treatment group continued on diet B, while the other half of the fish transferred from diet group B began a feeding regime using the commercial feed diet (D). From T1 to T2 (day 86), only one tank per diet was employed. The production period began at T2, wherein half of the fish from each diet A through D (n≈76) were transferred into new tanks. Fish were re-distributed two more times at day 115, increasing from two to three tanks per diet, and again at day 128, increasing from three to four tanks per diet. The study was completed with a final harvest at T3, day 184.

Each tank of fish was fed the same amount of feed to satiation during the early hatchery phase. The amount of feed used during the early hatchery phase was calculated by determining the mean weight of fish across all tanks. Fish were weighed bi-weekly on a per-tank basis starting at day 115. Tank densities (i.e., accounting for the total fish populations and average weights) were used as a basis to adjust the feeding regime to maintain adequate growth based on a dynamic percent body-weight feeding model additionally defined by the ambient water temperature, ~13°C (Hinshaw, 1999). Following each bi-weekly weighing, a bodyweight-based daily feeding model was adjusted to include the new data. The daily feeding

rate decreased from 4.9% to 3.9% of bodyweight over the course of the production period as the trout grew. Additionally, the feed conversion ratio (FCR) was calculated using the bi-weekly growth and feed weight data.

Sample acquisition strategy

Beginning at T-1 (receipt of eggs), 20 eggs were set aside on a sterile cheesecloth suspended over a beaker for microbiome sampling. Ten of these eggs were surface disinfected briefly with 1 mL 25 ppm iodine solution and rinsed with 2 mL of sterile phosphate buffered saline (PBS) for approximately 5 sec per egg; the remaining ten eggs were similarly rinsed only with sterile PBS. Each egg was individually homogenized for microbiome processing using pestles (Bel-Art, South Wayne, NJ, USA) surface-disinfected with 100% ethanol. Immediately prior to initial exogenous feeding at T0 (day 0), ten fish were anesthetized in 250 mg/L buffered MS-222 (Western Chemical Inc., Ferndale, WA) water bath, surface disinfected with 70% ethanol, and then rinsed for ~5 sec with 2 mL sterile PBS. The heads and gills were removed from these fish prior to homogenization of the remaining tissues, including the intestines, with a hand-held tissue homogenizer (Bel-Art) for microbiome sampling. Then at T1 (day 29) ten fish each from diets A and B were processed in the same manner as the T0-processed samples described above. After euthanizing ten fish of each diet at T2 (day 86), whole intestinal segments (with pyloric ceca and rectums removed), including feces, were dissected out of each animal and subsequently homogenized for microbiome processing. Fish intestinal segments were homogenized using a surface-disinfected OmniTip homogenizer (Omni International, Kennesaw, GA, USA). A final harvest at T3 (day 184) included microbiome samples from diets A through D harvested in the same manner as T2. In addition, another ten intestinal segments

per diet at T3 were extracted and manually cleared of fecal material by gentle squeezing then swabbed with sterile cotton-tipped swabs (Fisher brand, Pittsburgh, PA, USA) to obtain adherent microbiome samples. Water column samples were also collected at the various timepoints by applying vacuum-filtration (Corning 0.22 µm filter, Corning, NY, USA) to 2.5 L of tank water. Sterile cotton swabs were used to collect the filter retentate.

Separately, 32 fish per diet (eight fish per tank from four tanks) were harvested at T3 and measured for the following biometrics: weight and length, viscerosomatic index (VSI), hepatosomatic index (HSI), fillet and ribless fillet yields, and muscle ratio for all diets A-D.

Probiotic ingestion

Additional samples separately collected at T1 and T2 (processed in the same manner as the microbiome samples) and aliquots from the T3 microbiome homogenate samples were used for qPCR analysis of probiotic consumption. First, genomic DNA was isolated from the tissue homogenates using the Qiagen PowerLyzer PowerSoil kit and Qiagen Qiacube (Germantown, MD, USA). After slicing the homogenates into smaller pieces and bead beating using PowerBead tubes, 750 µL PowerBead solution was added. Following addition of solution C1, the PowerBead tubes were transferred onto the FastPrep system and set to shake at 1600 rpm for 1 min. Samples were then subjected to 13,000 x g centrifugation after which 450 µL of supernatant was transferred to a Qiacube cuvette. The Qiacube protocol was followed per the manufacturer's procedure. Samples were then stored at -20°C until ready for qPCR-amplification.

Reaction concentrations for qPCR-amplification were as follows: 2 µL of the final 100µL elution volume of gDNA isolated via the Qiacube protocol, 200 nM each of the forward

(CTGTTCTCATGAACTGGGGC) and reverse (GCTAACTCTGCAGGTACCCC) primers targeting the *B. subtilis* strain 086, 100 nM of the probe ([FAM]AAGGTCGAAGTTGAGGCAAA[BHQ1a~6FAM]), 10 µL of LightCycler 480 Probes Master (Roche, Rotkreuz, Switzerland) and 5 µL of dH_2O for a final volume of 20 µL. qPCR thermal cycler (Roche) settings included: initial denaturation at 95°C for 10 min, 36 cycles including denaturation at 95°C for 10 sec and annealing at 61°C for 30 sec, and a final cooling step at 37°C for 30 sec. At T1, 18 and 12 samples were taken from diet A and diet B fish, respectively. Timepoints T2 and T3 18 samples were obtained from diet A fish and 12 samples from fish fed diets B, C, and D. Six additional samples were collected from fish fed diet A (no probiotic) than the other diets at each timepoint and artificially spiked with known concentrations of the *B. subtilis* 086 probiotic to create a standard curve prior to qPCR analysis so qPCR results could be correlated with CFU probiotic/gm fish homogenates. Probiotic detection in the water column was accomplished by taking 1 mL water samples from the system for analysis.

DNA extractions for microbiome analysis

Genomic DNA (gDNA) was isolated from tissue homogenates and water column samples using the Qiagen PowerSoil kit per the manufacturer's protocol with alterations including a 10-min incubation at 72°C after addition of C1 buffer and a 5-min incubation at 72°C prior to elution in 50 µL dH_2O. Tissue homogenates were added to the PowerBead (Qiagen) tubes using a range of weights between 10 and 60 mg. Water column filter retentate swabs and T3 intestinal swabs were added directly to the PowerBead tubes. Prior to sample storage at -20°C, total gDNA quantity and purity (i.e., A_{260}/A_{280} and A_{260}/A_{280}) were analyzed via a nanospectrophotometer (Implen, Westlake Village, CA).

PCR amplification for microbiome analysis

The gDNA of tissue and water column samples was used as template to PCR-amplify the V4 region of the bacterial 16S rRNA gene (Table B.1). Amplification reactions were done in triplicate with a separate negative water control. Universal barcoded forward primers were created according to Caporaso et al., (2011).

Egg and water column samples harvested at T-1 were PCR-amplified using the following conditions: 10 µL of 2.5X Quantabio 5 Prime hot start master mix (Beverly, MA, USA), 200 nM forward barcoded primer (515f; AATGATACGGCGACCACCGAGATCTACACGCTxxxxxxxxxxxxTATGGTAATTGTGTGYCAGCMGCCGCGGTAA, with the x region representing the bar code), 200 nM reverse primer (806r; CAAGCAGAAGACGGCATACGAGATAGTCAGCCAGCCGGACTACNVGGGTWTCTAAT), 325 ng of gDNA template, and PCR-grade dH_2O to bring the total volume up to 25 µL. Settings for the thermal cycler (Thermo Scientific, Waltham, MA, USA) were programmed as follows: initial denaturation at 94°C for 2 min; 35 cycles of denaturation at 94°C for 45 sec, annealing at 50°C for 1 min, and elongation at 68°C for 30 sec; final elongation at 68°C for 10 min.

Remaining tissue and water column samples harvested from T0 to T3 were PCR-amplified with the following conditions: 12.5 µL of 2X Q5 Master Mix (New England Labs, Ipswich, MA, USA), 500 nM each of forward universal barcoded primer and reverse primer as noted for the T-1 samples, a variable amount of gDNA template dependent upon primer optimization for each tissue type (Table B.1), and PCR-grade dH_2O to bring the total volume up to 25 µL.

Gel extraction

Samples, PCR-amplified in triplicate tubes, were pooled together prior to visualization on a 1% agarose gel. Due to the presence of contaminating host 18S rRNA DNA, bands associated with V4 region amplicons were manually gel extracted and purified using the Qiagen Gel Extraction kit per the manufacturer's instructions with the following alterations: elution with 50 µL dH_2O and incubation at 72°C for 5 min prior to elution.

Bioinformatics

Gel-purified samples were quantified via Qubit fluorometry (Invitrogen, Waltham, MA, USA) prior to sequencing using the Illumina 250-bp paired-end MiSeq platform at a final concentration of 14 pM and 16 pM for runs 1 and 2, respectively. Two MiSeq runs were used and set up by combining half of the samples from each diet into separate pools to be sequenced individually. PhiX was also spiked in the MiSeq runs at a concentration of 20% and 47%, for runs 1 and 2 respectively, for quality control. Sequencing generated a total of 10,071,326 and 7,999,706 reads for runs 1 and 2, respectively. Bioinformatics-based microbiome analysis was accomplished using Quantitative Insights Into Microbial Ecology (QIIME2, v. 2020.2.0) (Bolyen et al., 2019). All reads associated with the tissue samples (i.e., both eggs and fish tissues) were denoised using DADA2 (Callahan et al., 2016) including parameters to retain the full 250 bp forward reads while trimming the reverse reads starting at 105 bp. The reverse read trimming was done after visual inspection of the reads indicated a drastic reduction in read quality before 105 bp (median quality scores ~ 2) in comparison to after 105 bp (median quality scores > 30). Amplicon sequence variants (ASVs) were produced following DADA2 denoising and further filtered to remove low frequency ASVs (less than

0.001% of the total reads, similar to previous approaches (Bokulich et al., 2013; Prodan et al., 2020; Xue et al., 2018)), and to remove host DNA (e.g., mitochondrial and chloroplast) resulting in 1,381 unique ASVs. Taxonomy was assigned using a classifier specific to the 16S rRNA gene V4 region from the frequently-updated and robust SILVA database version 138 (Glöckner et al., 2017; Quast et al., 2013; Yilmaz et al., 2014). Two samples with very low sequence counts (< 30 reads) were removed from further analysis, one from the unprocessed egg group and another sample from the diet C intestinal swab group (Table B.2), to prevent relative abundance biases. Filtered ASVs were then used to create taxonomic figures by collapsing the ASVs to shared Phylum or Family levels. Individual ASVs were used for alpha and beta diversity analyses following rarefaction to 1,297 reads per sample (one sample from the diet A intestinal swab group associated with less than 250 reads was removed during rarefaction). Differentially abundant bacterial families were identified using Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) (Lin and Peddada, 2020) within R v.4.1.0 (R Core Team, 2019).

Taxonomic figures were created in R using several packages including qiime2R v.0.99.6, phyloseq v.1.27.6, vegan v.2.5-7, ggplot2 v.3.3.5, complexheatmap v.2.9.1 (Bisanz, 2018; Gu et al., 2016; McMurdie and Holmes, 2013; Oksanen et al., 2019; Wickham, 2016). Alpha diversities were calculated using the Shannon metric (measurement of the overall diversity with relative abundance factored in), evenness metric (determination of the relative dominance by ASVs), and observed ASVs (total number of ASVs present after filtering for each group) generated via QIIME2. Beta diversity non-metric multidimensional scaling (NMDS) visualization plots were created using inter-group microbiome dissimilarities calculated by the unweighted UniFrac (phylogeny-based beta diversity metric without relative abundances) and weighted

UniFrac (phylogeny-based beta diversity metric including relative abundances) metrics, respectively within the phyloseq package in R.

Statistical analyses

Fish production data was analyzed using the vegan package in R. Analyses included Student's T-test for biometrics measured at the final timepoint, and the parametric one-way analysis of variance (ANOVA) for the production period data, following confirmation of variance homogeneity via Levene's test ($P>0.05$). The Tukey's post-hoc was implemented if the ANOVA test identified significant results ($P<0.05$).

Alpha diversities were compared using the non-parametric one-way Kruskal-Wallis test, and statistically significantly different results were further identified using Dunn's post-hoc test ($P<0.05$ considered significant). UniFrac-based beta diversities were compared using permutational multivariate analysis of variance (PERMANOVA) with pairwiseAdonis v.0.0.1 (Arbizu, 2017) R package for post-hoc tests.

Results

Fish production data

Survival for all diets was greater than 98% and the introduction of probiotics didn't significantly impact the survival rate of steelhead trout (Table 3.1). The system was able to provide a healthy water quality environment for the fish for the duration of the study.

On a per-tank basis, all four treatment groups (A to D) entered the linear phase of growth around the same period of time (day 128) and followed a similar growth curve pattern (Figure B.1). Throughout the course of the trial, fish fed diet D had a consistently higher growth

rate. This is in contrast to the lowest growth rates observed in fish fed diet C. However, following a one-way analysis of variance (ANOVA) of the overall fish weights, no group was significantly higher nor lower in average fish weight ($P>0.05$; Figure B.1), on a per-tank basis.

As a measure of overall feed efficiency, the bi-weekly feed conversion ratio (FCR) remained under 1 for the majority of the production period (Figure B.2). The only statistical difference in FCR, calculated by ANOVA on a per-tank basis, was between fish fed diets D and C observed at day 115 ($P<0.05$). In regard to the overall FCR, each group was highly similar, and all values were below one (Table 3.1). These FCR values demonstrated an appropriate rate of fish feeding specific to each tank was implemented during the production period.

Fish physiology

Individual animal biometric data (n=32 fish) comparisons were statistically assessed using multiple pairwise comparisons between fish fed diets A through D. Fish fed diet D (probiotic then commercial feed) achieved a significantly higher ($P<0.05$) weight than diet C fish (commercial feed then probiotic) (Table 3.2), on an individual basis. Generally, fish in the diet D group trended higher than those fed other diets regarding weight, length, fillet, and ribless fillet yields. Further, the ribless fillet yield of diet D fish was ~13% higher ($P<0.05$) than that of diet A fish (continually-fed commercial feed). To indicate relative weight gains and control for fillet sampling bias, the Fulton's condition factor (K) was employed ($K = (\text{weight(g)}/\text{full length (cm)}^3)*100$, (Nash et al., 2006)). These results also indicated the Fulton's condition factor of the individual fish fed diet D were trending higher than fish fed diet A, adding confidence to the difference in fillet yields.

Apart from muscle ratio, diet C fish generally exhibited poorer biometric conditions than the fish fed other diets on an individual basis (Table 3.2). The VSI, which is a broad measure of energy retention in the visceral tissues, was significantly lower ($P<0.05$) in these fish compared to diet A fish. The lower VSI compounded with the lower growth implies the inability of the diet C fish to store enough energy to supply sufficient growth. Moreover, the Fulton's condition factor for fish fed diet C was significantly lower than that of fish fed diet B.

Probiotic ingestion

Fish fed diet B (continually-fed probiotic) consistently harbored the highest concentration of probiotic as measured by qPCR analysis of tissue homogenates across all of the time points sampled (T1 to T3; day 29 to day 184; Table 3.3). The second highest ingested concentration of probiotic was measured in fish fed diet C (commercial feed then probiotic) at T2 (day 86) and T3. In comparison, the fish fed diet A (continually-fed commercial feed) harbored no probiotic organisms detectable by qPCR until T1 (day 29). At day 86, while detectable, the amount of probiotic in fish fed either diet A or D (probiotic then commercial feed) is several orders of magnitude lower than diet B and C groups actively being fed the probiotic. The fact that the probiotic was detectable in animals never fed the probiotic (diet A) may indicate possible uptake of low levels of the probiotic, below the limit of detection, from the environment (e.g., originating from the air, water, equipment, etc.). This is further supported by observations that the probiotic was below qPCR-detectable levels in the water column from day 31 to 125.

Taxonomic identification of the microbiome

The microbiome was analyzed at the phylum (Figure 3.2), family (Figure 3.3) and genus taxon levels (Table B.3). The steelhead trout intestinal microbiome is mostly comprised of Proteobacteria during early development (T-1 to T1; day -19 to day 29) (Figure 3.2A-C). Furthermore, the effect of in-lab iodine-treatment on the exterior of the eggs did not dramatically alter the phylum-level microbiome structure of the eggs indicating the majority of recovered ASVs at this timepoint originated from internal tissues. However, there is noticeable variation between the first three timepoints within the family-level phylogeny (Figure 3.3). Initially dominated by *Methylophilaceae* and *Oxalobacteraceae* at the egg stage (T-1), the fish become dominated by *Moraxellaceae*, *Comamonadaceae*, and *Pseduomonadaceae* by the first feeding at T0 (day 0). *Acinetobacter* sp. (Table B.3) represented the most dominant genus at T0 within *Moraxellaceae* and they continued to represent a major genus of the internal microbiome of the fish through T1 (Table B.3). By T1, the fish were no longer dominated by just a few families and begin to exhibit diversified microbiomes. For example, there were more families associated with the phylum Bacteroidota in higher relative abundance at T1 than at T0 (e.g., *Bacteroidaceae*, *Porphyromonadaceae*, and *Spirosomaceae*; Figure 3.3). Additionally, more families within Proteobacteria comprised a greater percentage of the microbiome from T0 to T1 including two families associated with fish pathogens (*Yersiniaceae* and *Aeromonadaceae*) (Figure 3.3). Fish fed the probiotic (diet B) from T0 to T1 still contained a relatively large proportion of *Acinetobacter* sp. (within *Moraxellaceae*; Figure 3.3) with ~5% relative abundance at T1 (Table B.3). However, they had a much higher proportion of *Yersinia* sp. (within *Yersiniaceae*; Figure 3.3) with ~31% relative abundance (Table B.3) and *Bacillus* sp.

(within *Bacillaceae*; Figure 3.3) with ~19% relative abundance (Table B.3) than diet A control-fed fish with ~2% and <1% relative abundances of *Yersinia* sp. and *Bacillus* sp., respectively (Table B.3).

Between T1 and T2 (day 86), the phylum-level taxonomic structure appeared similar to T1 with Proteobacteria, Firmicutes, and Bacteroidota representing the three main phyla (Figure 3.2C-D). The dietary effect on the Firmicutes' relative abundance was more exaggerated by T2. For instance, fish fed diets A and D harbored a lower proportion of Firmicutes than fish being fed probiotic diets B and C. The dominant Firmicutes families at T2 were *Lachnospiraceae* (with the most dominant organism, *Oribacterium* sp., at <3% of the total microbiome; Table B.3) for fish fed diets A or D. For fish fed diets B or C, the dominant Firmicutes family was *Bacillaceae* (with the most dominant organism, *Bacillus* sp. at 41-46% of the total microbiome (Table B.3; Figure 3.3). At T2, diet A- and D-fed fish harbored higher numbers of *Bacteroidaceae* (represented by *Bacteroides* sp.; Table B.3) in their internal microbiomes compared to T1 (Figure 3.3). Importantly, the relative abundance of *Bacteroidaceae* in fish fed diets A and D was not as high as the *Bacillaceae* (represented by *Bacillus* sp.; Table B.3) observed in diets B- and C-fed fish (Figure 3.3).

At the final timepoint (T3; day 184), the total internal microbiomes within all sampled fish were dominated by the phyla Firmicutes and Proteobacteria (Figure 3.2E), comprised mostly of the families *Bacillaceae* and *Lactobacillaceae* within Firmicutes and *Erwiniaceae* within Proteobacteria, respectively (Figure 3.3). By T3, many families appear to be differentially represented, dependent upon administration of the probiotic. For example, probiotic administration using *Bacillus subtilis* correlates to the increased presence of its associated

taxonomic family, *Bacillaceae*, in fish fed diets B and C compared to A and D (Figure 3.3). In contrast, the family *Lactobacillaceae* appeared at higher relative abundances within fish fed diets A and D compared to fish fed diets B and C. Similar to the relative abundance pattern observed with the Firmicutes-associated family *Lactobacillaceae*, Proteobacteria-associated families including *Erwiniaceae*, *Moraxaellaceae*, and *Comamonadaceae* appeared at higher levels within fish fed diets A and D compared to fish fed diets B and C (Figure 3.3).

Interestingly, the adherent microbiomes (i.e., swabbed intestinal samples) between fish fed all four diets appeared fairly similar (Figure 3.2F). Interestingly, the adherent microbiome of fish fed diet D harbored a higher proportion of Firmicutes, represented mostly by *Bacillus* sp. (Table B.3), than fish fed the other three diets (Figure 3.2F). The second highest phylum in the adherent microbiomes was Proteobacteria, represented by the *Enterobacteriaceae* family (Table B.3).

The ANCOM results, measuring the ASV abundance differential between diet groups, for the T3 intestinal homogenates indicated the following families were enriched ($P<0.05$) in fish fed diets A and D compared to fish fed diets B and C: *Lachnospiraceae, Clostridiaceae, Microbacteriaceae,* and *Lactobacillaceae*. Conversely, the family *Bacillaceae* was highly enriched ($P<0.001$) in fish fed diets B and C compared to diets A and D. No significant shifts in differential abundance were detected for the mucosal microbiomes collected via sterile swabs.

Microbiome community diversity analysis

Diet A-fed fish exhibited their highest overall alpha diversities, reflecting the number and relative representation of different ASVs in a sample, at T1 (day 29) versus later timepoints, as calculated by the Shannon index (H; 5.64) and 156 observed ASVs (Table 3.4). Following T1,

a decline in H (5.05 by T3) and observed ASVs (87 by T3) was observed for the animals fed diet A. A similar trend was identified for fish fed diet D from T2 (day 86) to T3 (day 184) with a decreasing trend in H (5.27 to 5.22) and observed ASVs (119 to 90.6) (Table 3.4).

Fish fed diets B or C also exhibited decreasing trends in H and observed ASVs as identified in fish fed diets A or D from T1 to T3. However, H was significantly lower in these fish than fish fed diets A or D (Table 3.4). In fact, the Shannon diversities of fish fed diets B and C at later time points were lower than that of the egg samples collected at the beginning of the study. The number of observed ASVs for diet C-fed fish were statistically similar to the other three groups at T2 and T3, albeit the values were trending lower than fish fed diets A or D.

One distinction for the alpha diversity results between the four diets was observed in the evenness metric, a relative measure for the representation of individual taxa (i.e., microbiomes dominated by a few taxa will have lower evenness values than microbiomes harboring taxa with similar relative abundances). Between T2 and T3, the evenness results increased 0.752 to 0.780 for fish fed diet A and 0.766 to 0.808 for fish fed diet D. On the other hand, a decrease in evenness from T2 to T3 was observed in fish fed diets B (0.517 to 0.334) or C (0.463 to 0.374).

All calculated alpha diversities of the intestinal mucosa at T3, sampled using sterile swabs, were similar between the four diets (Table 3.4). Notably, the overall alpha diversities (H, observed ASVs, and evenness) were lower in the mucosal microbiome compared to the total intestinal homogenate microbiome for fish fed diets A and D. This observed decrease in alpha diversities did not appear to be mirrored in fish fed diets B and C.

Beta diversities were calculated for each diet across the five timepoints, excluding the intestinal swabs, using the unweighted UniFrac method which compares dissimilarities between microbiomes without factoring in relative abundance (Figure 3.4). Unweighted beta diversities can help to identify the degree to which microbiomes share microorganisms. Prior to initial feeding, the unweighted UniFrac beta diversities of unfed fish (the original animals shared by all treatments and collected at T-1 and T0) indicated highly similar microbiomes following a PERMANOVA pairwise test (P ~1, Figure 3.4 A-C). However, the unweighted UniFrac results between the later timepoints (i.e., T1, T2, and T3) showed a high degree of dissimilarity ($P<0.05$) for all diets (Figure 3.4). Further, the microbiomes between unfed timepoints (i.e., T-1 and T0) and fed timepoints (i.e., T1, T2, and T3) were highly dissimilar ($P<0.05$) for all diets. Regardless of diet, the unweighted microbiomes appear to transition from T1 to T2 to T3 (i.e., T1 clusters closer to T2 than T3 and T2 clusters closely to both T1 and T3).

Weighted UniFrac-calculated beta diversities, which factor in relative abundance to compare the overall trends between microbiomes, indicated the microbiomes within fish fed diets A or C were distinct between each timpoint ($P<0.05$; Figure 3.5A and 3.5C). Fish fed diets B or D also had distinct microbiomes over the course of the study with the exception between T1 to T2 ($P>0.05$; Figure 3.5B and 3.5D), wherein the microbiomes at T1 and T2 were significantly similar for fish fed diets B or D. Fish fed diet D also appeared to have more similarities between their microbiomes present at T1, the timepoint exhibiting the highest overall diversities (Table 3.4), and T3 (Figure 3.5D).

Intra-timepoint dietary effects were also analyzed for beta diversity differences. All diets/treatments exhibited a high degree of similarity ($P>0.05$) for the unweighted UniFrac

metrics (Figure B3 and Figure 3.6A) at each timepoint. The weighted microbiomes, however, indicated distinct microbiomes ($P<0.05$) present at T1 (between groups A and B; Figure B.3) and T2 (between groups A and C; Figure B.3). However, the intestinal homogenates' microbiomes clustered into two groups ($P<0.05$) with regard to the probiotic feeding regime in place by the end of the study at T3. In other words, the microbiomes of fish fed diets A or D were highly similar, and the microbiomes of fish fed diets B or C were highly similar (Figure 3.6B). Comparison between fish fed diets A or D and fish fed diets B or C indicated a clear distinction between the two clustered groups.

Discussion

Probiotic use in aquaculture has been implemented to provide several key benefits to cultured fish. One probiotic organism in particular, *Bacillus subtilis,* has been extensively studied for its impact on aquatic host growth, immune functioning, microbiome modulation, and pathogen protection among other benefits (Cheng et al., 2019; Kumar et al., 2008; Merrifield et al., 2010; Standen et al., 2015). The findings of this study demonstrated that exposure to a *B. subtilis* probiotic exclusively during the initial stages of intestinal development (diet D) benefitted the fish significantly better than feeding probiotic at a later stage of growth (diet C). In fact, fish fed probiotic only during the later developmental stages tended to perform worse than all the other treatment groups. The poorer performance of diet C fish may be linked to their lower VSI (Table 3.3) in comparison to fish not fed any probiotic (diet A) (Adhami et al., 2021; Liu et al., 2021). There is evidence that probiotic administration may actually diminish the health of fish under some growth conditions (Ramos et al., 2017). However, continued delivery of the probiotic without cessation (diet B) in this study did not

appear to negatively impact fish growth compared to fish fed no probiotic (diet A), nor did it benefit the fish any more than limited early exposure to the probiotic (diet D) in regard to the fish biometrics.

Bacillus subtilis probiotic ingestion by fish that were fed it was confirmed via qPCR analysis (Table 3.3). The amount of *B. subtilis* present in fish groups B and C was similar, suggesting near equal feeding and consumption. Taxonomic analyses also agree with the qPCR findings by indicating a large proportion of the diet B- and C-fed fish microbiomes were dominated by *Bacillaceae*, the family with which *B. subtilis* is associated (Figure 3.3). Curiously, the *Bacillus subtilis* probiotic was detected via qPCR in the control fish (diet A) by day 29 (T1) and in both diet A- and D-fed fish at days 86 (T2) and 184 (T3) (Table 3.3). Further, *Bacillaceae* was present in the microbiome analysis of diet A- and D-fed fish at all timepoints, albeit at a much lower relative abundance than diets B- and C-fed fish (Figure 3.3). The source of *Bacillus* sp. in the cases where the fish weren't actively being fed probiotic could have originated from the commercial feed (Wilkes Walburn et al., 2019) or via cross contamination from the water column at a level below our limit of detection for qPCR analysis (10^2 CFU/g).

Though diet D-fed fish had significantly increased fish growth over the other three groups on an individual basis, the total microbiome (i.e., feces + mucosa) of these animals was similar ($P>0.05$; Figures 3.6, Figure B.3) to fish never exposed to probiotic (diet A). In contrast, continued administration of the probiotic after T1 (diets B and C) led to a reduction in the number of observed ASVs compared to fish fed diets A and D (Table 3.5), and it also led to dominance by much fewer taxa (including a majority by *Bacillus* sp.; Table B.3) thus reducing Shannon diversities. In addition to the higher alpha diversities, fish fed diets A and D exhibited

an increased relative abundance of other bacterial families, notably *Lactobacillaceae* (Figure 3.3), compared to fish fed diets B and C. Several bacteria in the *Lactobacillaceae* family have been isolated from trout and identified as potential probiotics (Chapagain et al., 2019; Mohammadian et al., 2019; Soltani et al., 2019), including *Lactobacillus plantarum* that has been used as effective immune-supporting probiotics in trout cultures (Balcázar et al., 2008, 2007; Vendrell et al., 2008). Thus, the increased microbiome diversity in the diet A and D fish may have afforded benefits to the animals.

In comparison to the microbiome in the total intestinal homogenates, the mucosal-only based microbiome (collected via swabs) showed a high degree of similarity between all four diets indicating no significant impact of the probiotic treatments on the adherent microbiomes. Though statistically similar, diet D-fed fish had the highest levels of Firmicutes (Figure 3.2F), which was dominated by *Bacillus* sp. (Table B.3), compared to the other three diets, suggesting some level of colonization by the probiotic. Thus, the mucosal colonization by the probiotic appears to be more effective when exposed to the fish earlier in their development (diet D), compared to fish consistently exposed to the probiotic or fed later in development (diets B and C, respectively). Additionally, the majority of microbial families shed in the feces (total microbiome minus mucosal microbiome) of fish fed diets A or D were not classified as *Bacillaceae*; transient bacteria shed in the feces of diet A- and D-fed fish were much more diverse compared to the fish fed diets B and C.

Because the diet-based separation of microbiomes, as examined through weighted beta diversity analysis, was not as apparent until day 184 (Figures 3.6 and B.3), host age may also be an important factor in microbiome development in addition to diet. Temporal shifts in the

microbiome communities occurred as the fish matured, illustrated by a trend from low diversity microbiomes (Table 3.4) dominated primarily by Proteobacteria (Figure 3.2), specifically *Methylophilaceae* (Figure 3.3), at the egg stage towards a high diversity microbiome dominated by Proteobacteria, Firmicutes, and Bacteroidota at T1 (day 29). These three phyla likely make up a portion of the core microbiome of several different fish species including trout (Hines et al., submitted; Tarnecki et al., 2017). The initial increasing shifts in microbiome constituency from unfed (T-1) to fed (T1) fish ended at T1 as illustrated by the alpha diversities (Table 3.4). Microbiome constituencies then appear to transition from T1 to T2 to T3 in a more ordered and overlapping fashion for all four diets (Figure 3.4). This slower change in the microbial community suggests some establishment by persistent ASVs following the peak overall diversity at day 29. Interestingly, fish fed diet D appear to harbor microbiomes at T3 more similar to T1 than the other dietary groups. This is reflected in the overall highest Shannon diversity present at T3 compared to the other groups of fish. Thus, the early exposure of the probiotic may have lasting benefits that aid in maintaining high diversity microbiomes that aren't dominated by just a few taxa.

Conclusions

In conclusion, while the amount of research surrounding probiotic efficacy in fish feed is extensive, the efficacy of timed probiotic exposure is less well understood. In the present study, different treatment groups were defined by probiotic administration that was varied with respect to fish development, starting with eggs. Temporal shifts in the microbiome were observed, with the highest level of microbiome community diversity present after 29 days across all diets. The study results suggest implementation of probiotic only during initial

intestinal development will increase the overall growth of the fish, increase overall microbial diversities within the intestinal tract, and possibly allow for better establishment of the probiotic within the adherent microbiome. There was a significant decrease in the overall diversities of fish total internal microbiomes (i.e., feces plus mucosa) when the probiotic was being administered. The adherent mucosal microbiomes differed little between the four diets, but the faster growing diet D-fed fish had the highest adherent probiotic levels. The lack of major variability in the mucosal microbiome indicates a possible set of resilient bacteria that constitute the core microbiome for steelhead trout (Lyons et al., 2017; Roeselers et al., 2011; Tarnecki et al., 2017; Wong et al., 2013). Overall, these results indicate feeding trout a *Bacillus subtilis* probiotic is most beneficial when supplied as a short exposure early in intestinal development during the early hatchery phase, rather than beginning probiotic regimens later in fish development or continuous use. This finding has important economic implications for producers and the interpretation of results from studies administering the probiotic during later development.

Data availability statement

Raw paired-end sequencing files used for the bioinformatics analyses have been uploaded to the NCBI Sequence Read Archive (SRA) under BioProject ID PRJNA750741.

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Table 3.1. Production period measurements of steelhead trout following probiotic treatment.

Diet	Initial sample size (n)	Survival (%)[1]	FCR[2]
A (continually-fed commercial)	206	98.7	0.937 ± 0.046
B (continually-fed probiotic)	168	99.4	0.934 ± 0.012
C (commercial to probiotic)	169	99.4	0.971 ± 0.028
D (probiotic to commercial)	191	100	0.899 ± 0.015

[1] No error is presented here due to the step-wise expansion from two to four tanks during the production period; instead this represents the mean survival for each diet across the entire production period

[2] Feed conversion ratio (FCR) calculated as mean ± standard error mean on a per tank basis

Table 3.2. Physiological biometric measurements of individual steelhead trout at day 184[1].

Diet	A	B	C	D
Sample size (n)	32	32	32	32
Weight (g)	125 ± 4.97	129 ± 6.31	121 ± 6.45[a]	138 ± 5.29[b]
Length (cm)	21.3 ± 0.26	21.2 ± 0.31	21.1 ± 0.31	21.9 ± 0.30
Fillet (g)	34.4 ± 1.53	35.8 ± 1.94	34.1 ± 1.97	38.1 ± 1.59
Ribless fillet (g)	30.4 ± 1.35[a]	32.2 ± 1.82	30.7 ± 1.84	34.9 ± 1.52[b]
HSI	1.58 ± 0.06	1.71 ± 0.05	1.70 ± 0.03	1.62 ± 0.07
VSI	16.2 ± 0.39[a]	15.3 ± 0.42	15.0 ± 0.39[b]	16.1 ± 0.42
Muscle ratio (%)[2]	54.8 ± 0.76	55.3 ± 0.76	56.1 ± 0.41	55.4 ± 1.04
Fulton's condition factor	1.27 ± 0.02	1.31 ± 0.02[a]	1.24 ± 0.02[b]	1.30 ± 0.02

[1] Error represented by the mean ± standard error mean

[2] Ratio of total fillet yield (both sides of the fish) to total fish weight

[ab] Different superscript letters represent $P<0.05$ within a horizontal row after multiple pairwise comparisons using the t-test

Table 3.3. Steelhead trout probiotic ingestion as detected by qPCR analysis[1].

	Sampling day					
Diet	**3[3]**	**7[3]**	**11[3]**	**29 (T1)[4]**	**86 (T2)[5]**	**184 (T3)[6]**
A	ND[2]	ND	ND	8.09E+01 ± 3.46E+01	2.22E+02 ± 6.05E+01	1.45E+02 ± 1.53E+01
B	1.51E+02 ± 8.78E+01	3.08E+03 ± 2.13E+03	8.99E+03 ± 8.64E+03	6.43E+04 ± 1.60E+04	3.88E+05 ± 9.72E+04	2.74E+05 ± 5.49E+04
C	N/A	N/A	N/A	N/A	3.33E+05 ± 6.08E+04	1.65E+05 ± 2.24E+04
D	N/A	N/A	N/A	N/A	8.32E+02 ± 7.46E+02	5.80E+02 ± 4.22E+02

[1] Error represented by the mean probiotic concentration (CFU/g) ± standard error mean

[2] Not detected (ND), below the limit of detection for the qPCR analysis

[3] n = 4 fish (diets A and B)

[4] n = 18 fish (diet A), 11 fish (diet B)

[5] n = 18 fish (diet A) or 12 fish (diets B, C, and D)

[6] n = 12 fish

Table 3.4. Summary of alpha diversity analyses (mean ± SEM).

Timepoint	Diet	Shannon	Evenness	Observed ASVs
T-1	Non-disinfected	2.70 ± 0.59	0.614 ± 0.12	48.2 ± 33.2
	Disinfected	3.26 ± 0.90	0.499 ± 0.11	43.8 ± 8.70
T1	A	5.64 ± 0.45[a]	0.775 ± 0.038[a]	156 ± 25.0[a]
	B	3.95 ± 1.24[b]	0.573 ± 0.16[b]	115 ± 31.5[b]
T2	A	5.05 ± 0.41[a]	0.752 ± 0.06[a]	115 ± 33.6[ac]
	B	3.22 ± 0.82[b]	0.517 ± 0.10[b]	75.5 ± 20.0[bc]
	C	2.980 ± 1.41[b]	0.463 ± 0.19[b]	83.0 ± 23.9[bc]
	D	5.27 ± 0.52[a]	0.766 ± 0.05[a]	119 ± 26.4[ac]
T3-Homogenate	A	5.05 ± 1.47[a]	0.780 ± 0.20[a]	87.0 ± 30.8[ac]
	B	1.96 ± 0.63[b]	0.334 ± 0.10[b]	60.8 ± 17.9[bc]
	C	2.29 ± 0.60[b]	0.374 ± 0.08[b]	69.3 ± 15.1[bc]
	D	5.22 ± 0.62[a]	0.808 ± 0.11[a]	90.6 ± 16.6[ac]
T3-Swab	A	2.74 ± 1.91	0.505 ± 0.29	45.0 ± 44.4
	B	2.36 ± 1.26	0.505 ± 0.22	25.4 ± 12.2
	C	2.04 ± 0.93	0.432 ± 0.16	31.1 ± 21.2
	D	1.43 ± 1.42	0.302 ± 0.22	24.2 ± 23.9

[abc] Different superscript letters represent P<0.05 between diets at a specific timepoint within a given diversity metric following one-way Kruskal Wallis with Dunn post-hoc test

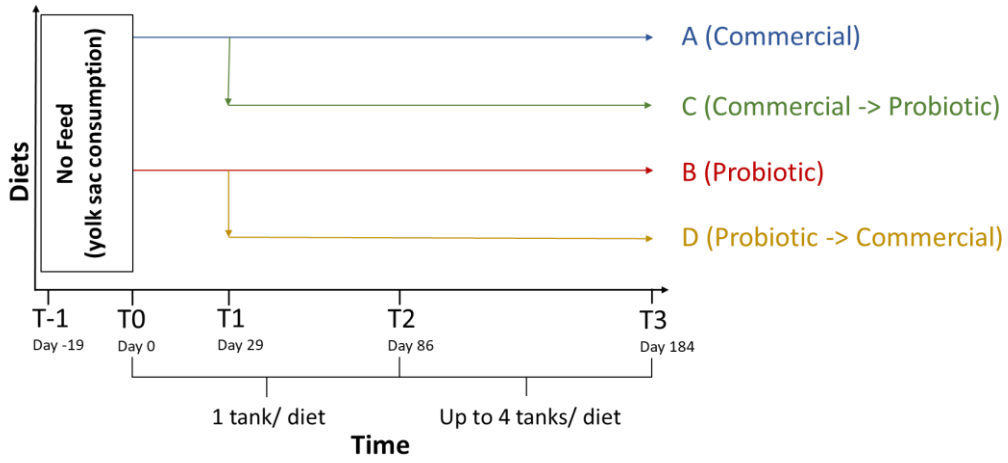

Figure 3.1. Timeline for steelhead trout harvests and feeding schedule. The water column and internal tissues of trout were collected at the indicated timepoints relative to the date of first exogenous feeding (T-1, Day -19; T0, Day 0; T1, Day 29; T2, Day 86; T3, Day 184). Fish were fed one of four diet regimens starting at T0 with the C and D diet groups differentiated at T1. The probiotic concentration for diets B, C, and D was ~10^8 CFU/g.

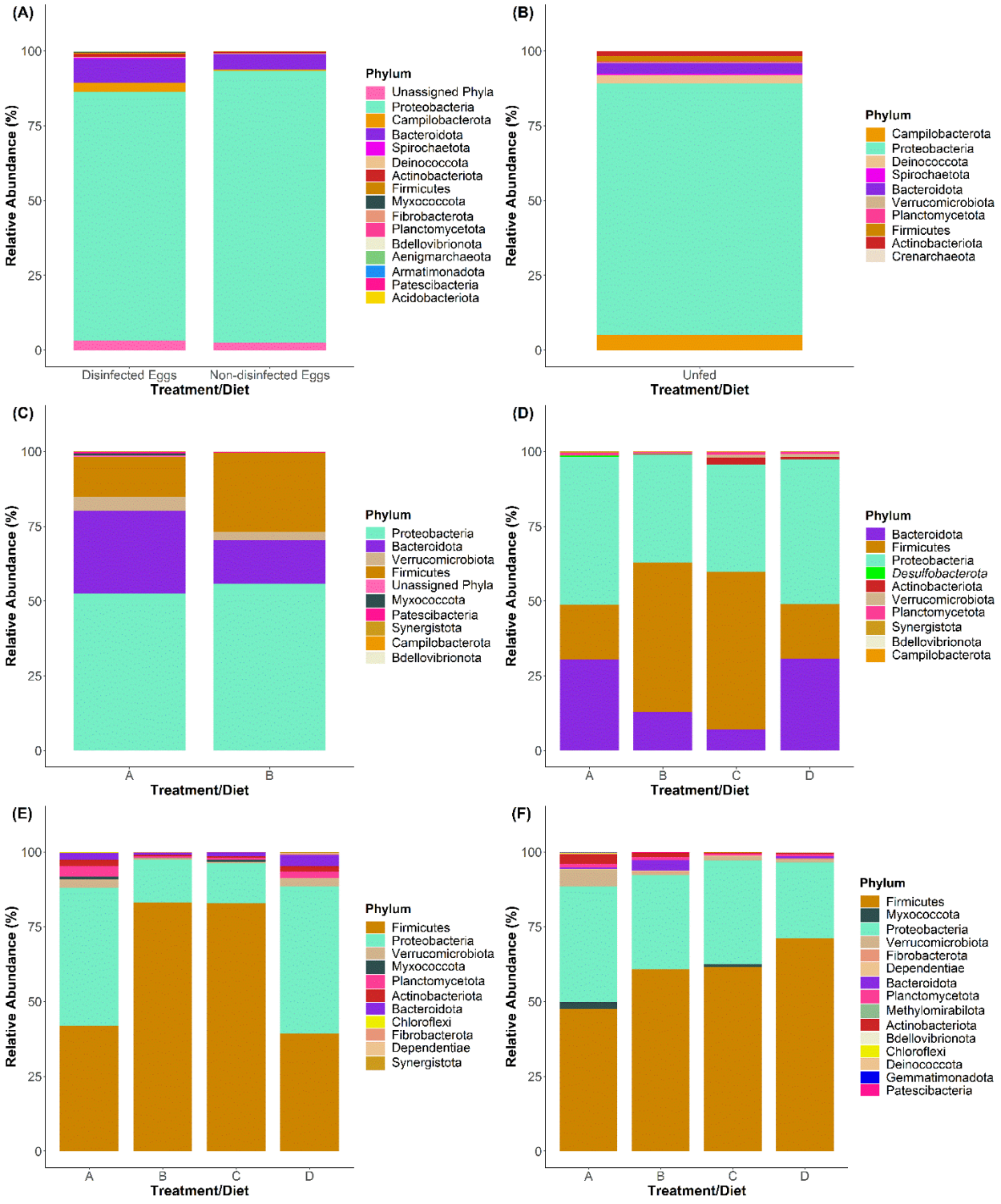

Figure 3.2. Phylum-level taxonomy of the internal microbiome of steelhead trout throughout a probiotic feeding regime. The 16S rRNA gene V4 region was sequenced at various points

throughout the early lifecycle of steelhead trout at five timepoints where the total intestinal microbiome was examined: (A) T-1, (B) T0, (C) T1, (D) T2, (E) T3 intestinal homogenate samples. The adherent intestinal microbiome was analyzed at one time point: (F) T3 intestinal swab samples. Plots represent the top phyla comprising at least 90% of the total microbiome for all treatment groups. Bars were constructed using the average reads from samples specified as: five non-disinfected eggs, six disinfected eggs, 10 T0 internal homogenates, 10 T1 internal homogenates, eight T2 intestinal homogenates, 12 T3 intestinal homogenates, 10 T3 intestinal swabs for diets A, B, and D, and nine T3 intestinal swabs for diet C.

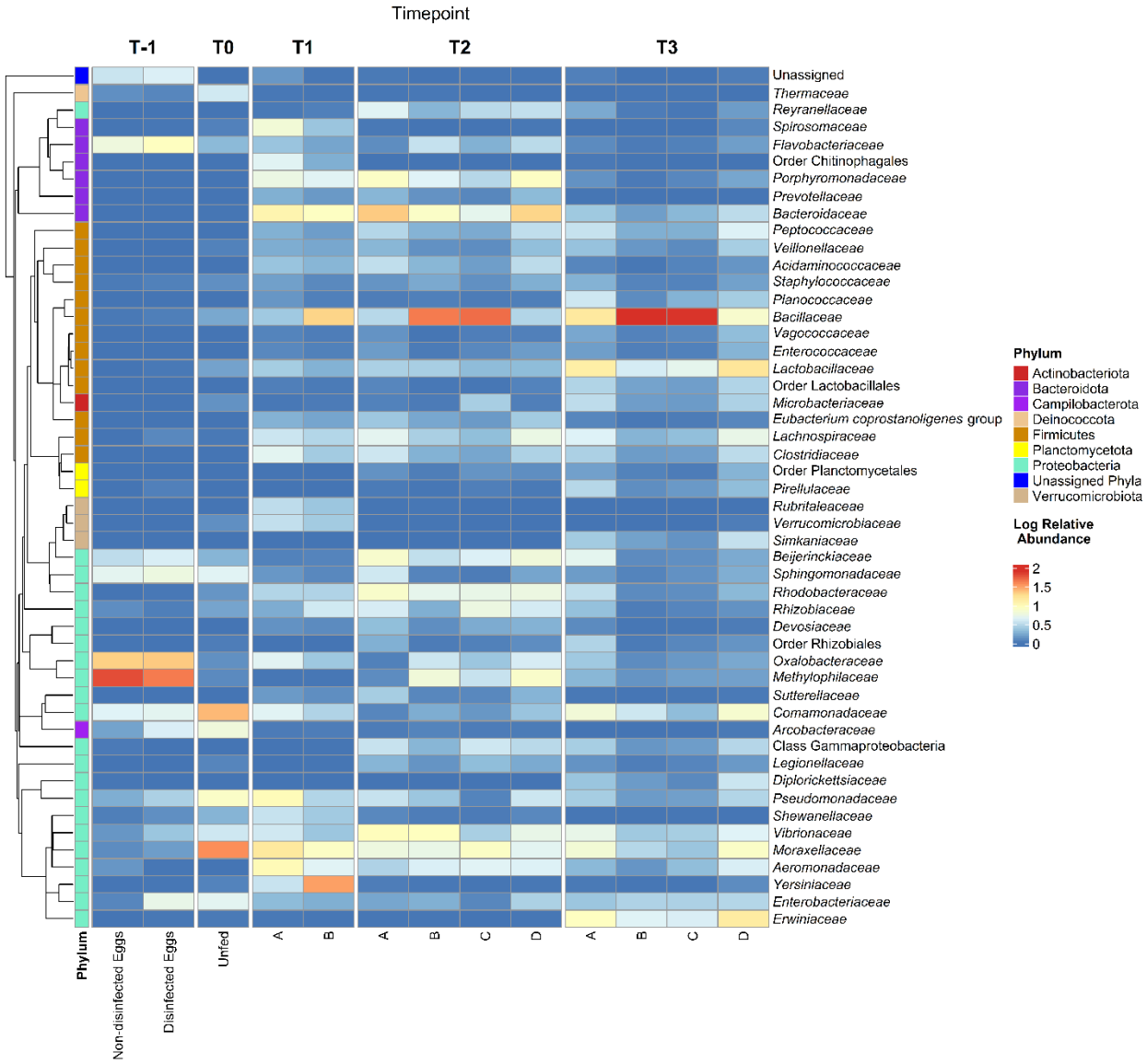

Figure 3.3. Family-level taxonomy of the internal microbiome of steelhead trout throughout a probiotic feeding regime. Following amplification of the 16S rRNA gene V4 region from fish internal GI samples collected at various timepoints during the early lifecycle of steelhead trout and fed one of four different diets (A, B, C, or D) or unfed, the amplicons were sequenced via Illumina MiSeq. The log relative abundance of family-level taxa is plotted here. ASVs without family-level assignment are designated the higher classification as necessary. A QIIME2-

generated phylogenetic tree was used to denote the phylogeny and phylum to which each family-level taxon belongs.

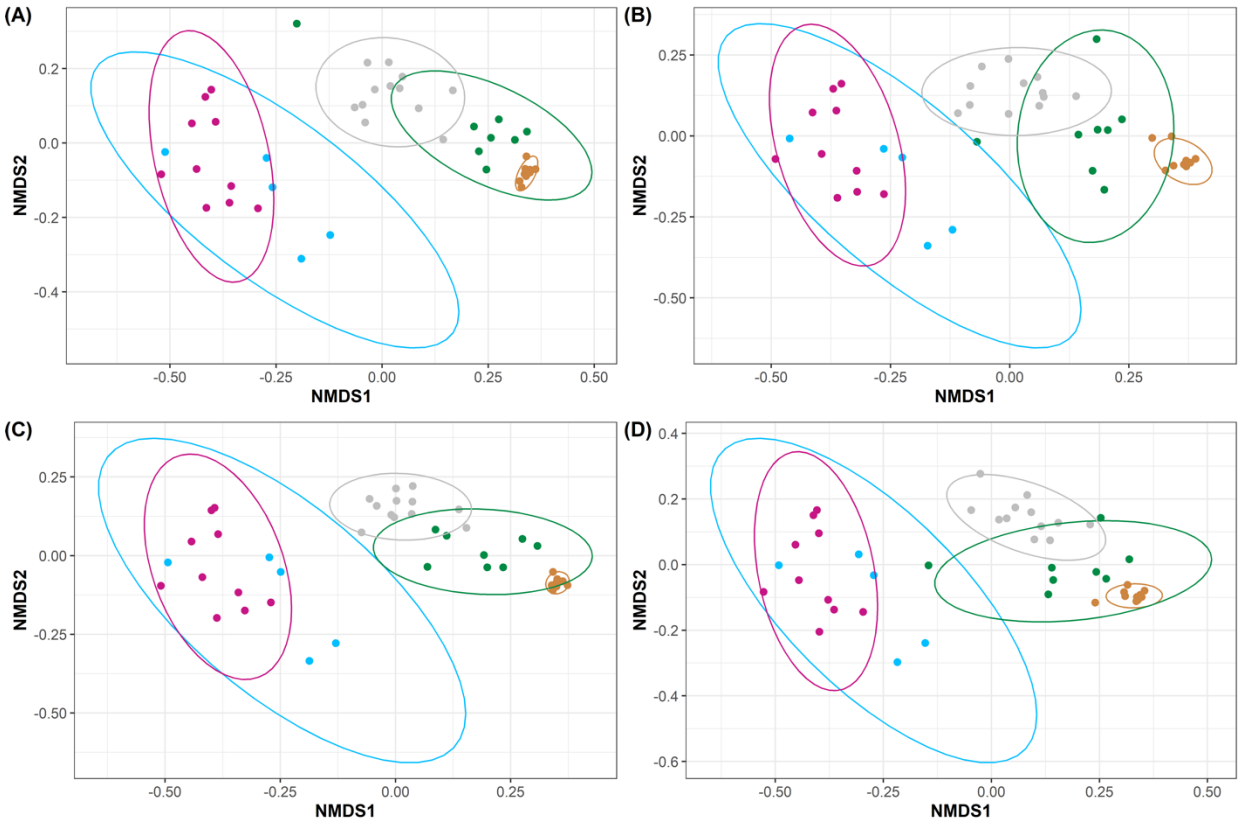

Figure 3.4. Non-metric Multidimensional Scaling (NMDS) plots representing the unweighted beta diversities of steelhead trout microbiomes for the duration of a probiotic feeding regime. Distances were calculated by unweighted UniFrac between the five timepoints for fish fed diets (A) A, (B) B, (C) C, and (D) D. Timepoints are represented by cyan (T-1), maroon (T0), orange (T1), green (T2), and gray (T3). Ellipses represent the 95% confidence intervals calculated via Student's T test for five non-disinfected eggs, 10 T0 internal homogenates, 10 T1 internal homogenates, eight T2 intestinal homogenates, and 12 T3 intestinal homogenates.

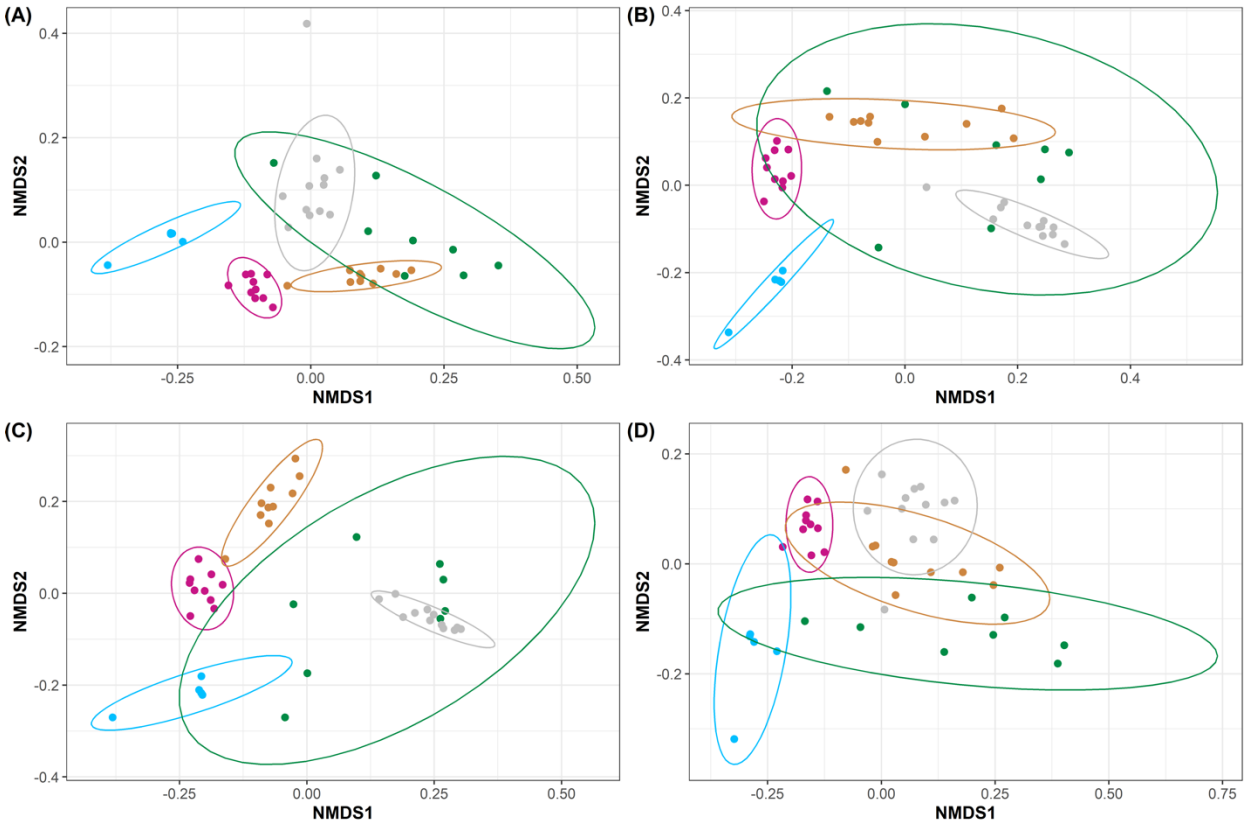

Figure 3.5. Non-metric Multidimensional Scaling (NMDS) plots representing the weighted beta diversities of steelhead trout microbiomes for the duration of a probiotic feeding regime. Distances were calculated by weighted UniFrac between the five timepoints for fish fed diets (A) A, (B) B, (C) C, and (D) D. Timepoints are represented by cyan (T-1), maroon (T0), orange (T1), green (T2), and gray (T3). Ellipses represent the 95% confidence intervals calculated via Student's T test for five non-disinfected eggs, 10 T0 internal homogenates, 10 T1 internal homogenates, eight T2 intestinal homogenates, and 12 T3 intestinal homogenates.

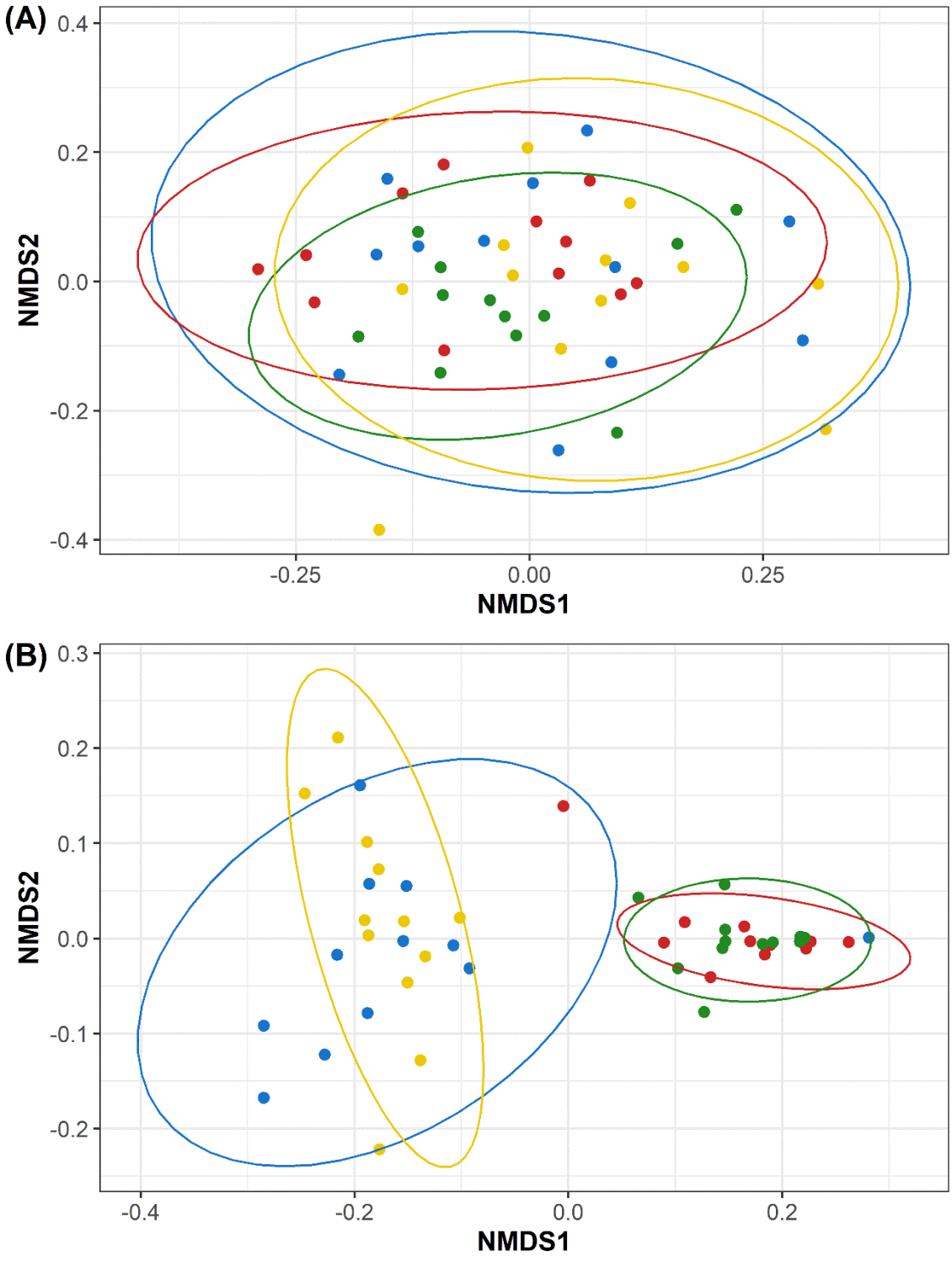

Figure 3.6. Non-metric Multidimensional Scaling (NMDS) plots representing the T3 beta diversities between steelhead trout fed different concentrations of a probiotic. Distances

were calculated by (A) unweighted UniFrac and (B) weighted UniFrac between four diets: A (blue), B (red), C (green), and (yellow). Ellipses represent the 95% confidence intervals calculated via Student's T test for 12 T3 intestinal homogenates.

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Chapter Four

Key bacterial constituents in the core intestinal microbiome of rainbow trout (*Oncorhynchus mykiss*)

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Attributions

Ian S. Hines wrote the manuscript and contributed to the data analysis, study design, performed the experiments, and reviewed the manuscript. Ann M. Stevens wrote the manuscript and contributed to the data analysis, study design, and reviewed the manuscript. David D. Kuhn wrote the manuscript and contributed to the study design and reviewed the manuscript. David D. Kuhn wrote the manuscript and contributed to the data analysis, study design, performed the experiments, and reviewed the manuscript. Maggie A. Marshall wrote the manuscript and contributed to the data analysis, study design, performed the experiments, and reviewed the manuscript.

Abstract

Fish aquaculture has become the fastest growing sector in global food production. Thus, ensuring the sustainability of aquaculture practices is of the utmost importance. Studies in higher vertebrates (i.e. mammals) have demonstrated the importance of the host microbiome on several processes from nutrient acquisition to protection from pathogens. Therefore, analysis of fish microbiomes is an important factor to consider with regard to overall animal health and welfare. Rainbow trout (*Oncorhynchus mykiss*) are an economically valued fish cultured worldwide. Several studies have identified microbial constituents inhabiting the intestinal tract of rainbow trout. To better elucidate some of the core constituents of the rainbow trout intestinal microbiome, this review analyzed the relative abundance results from 25 articles published on the rainbow trout intestinal microbiome from 2017 to 2021. Bacteria classified within the phyla Firmicutes and Proteobacteria were observed in every study. At the family level, *Lactobacillaceae* was consistently observed. Additionally, bacteria in the Actinobacteria, Bacteroides, and Tenericutes phyla were identified in at least 50% of the studies. Interestingly, *Mycoplasma* spp. were occasionally the most dominant organisms present in the microbiome. Overall, the results here identify bacteria that are important members of the rainbow trout core intestinal microbiome.

Introduction

Expansion of the human population has led to an increased food demand, including seafood products. Because fish provide a substantial source of animal protein in the human diet (FAO, 2020; Godfray et al., 2010), the need for this specific food product has risen sharply. Traditional sources of fish (i.e. wild-caught fisheries) are overexploited and unable to keep up with the current rate of human seafood consumption (Godfray et al., 2010; Ye and Gutierrez, 2017). As a farmed source of fish, the aquaculture industry can alleviate some of the strain on depleted natural fisheries. In fact, aquaculture has become the fastest growing industry in the food production sector (Crépin et al., 2014). Moving forward, one major avenue of research in the aquaculture industry is related to maintaining its sustainability by ensuring the health and welfare of the farmed fish. The microbiomes inhabiting fish aid the animal in multiple ways and can be a good indicator for overall health of the animals (Banerjee and Ray, 2017; de Bruijn et al., 2018; Legrand et al., 2020; Tarnecki et al., 2017). With the introduction of next generation sequencing and improved bioinformatic methods, a growing number of studies have been performed to analyze the fish microbiome.

Rainbow trout (*Oncorhynchus mykiss*) are typically carnivorous fish (with some omnivorous characteristics). They dwell in freshwater ecosystems, though anadromous stock (living in marine environments, but spawning in freshwater) exist as steelhead trout, and they are endemic to the Pacific northwest of North America (Hinshaw et al., 2004). Farming of rainbow trout began relatively recently (compared to other species like Nile tilapia), around the late 19th century (Hardy, 2002). However, their incorporation in the aquaculture industry worldwide has led to rainbow trout currently being one of the top 15 most farmed fish (FAO,

2020). This increased farming potential for rainbow trout has led to increased human consumption and a subsequent rise in demand (Hinshaw et al., 2004). The expansion of rainbow trout farming has helped to elucidate optimal farming parameters. For example, these fish grow best at colder water temperatures (~15°C) and require exposure to ample aeration (Hardy, 2002). Additional work to analyze the role of the microbiome of rainbow trout on the health and welfare of the animal will help to ensure efficient farming in the future.

Communities of microorganisms that inhabit a particular environmental niche comprise the microbiome. Like higher vertebrates (i.e. mammals), fish contain various tissue-specific microbiomes, such as the microbiomes associated with the skin and gills (Larsen et al., 2013; Legrand et al., 2018; Rosado et al., 2019; Xu et al., 2013) or the gastrointestinal tract (Clements et al., 2014; Tarnecki et al., 2017; Wang et al., 2018). These microorganisms provide several key benefits for its fish hosts. For instance, the bacteria present in the intestinal tract microbiome play an important role in maintaining energy homeostasis (Butt and Volkoff, 2019; Nicholson et al., 2012). Host digestive processes are also aided by the ability of the microbiome to facilitate nutrient acquisition from the diet and provide essential nutrients (e.g. vitamins) for the host (Chatterjee et al., 2017; Ghanbari et al., 2015; Llewellyn et al., 2014; Nicholson et al., 2012; Tarnecki et al., 2017). During host immune system development, the microbiome helps guide the ability to recognize self from non-self and modulate immune system regulation (Brugman et al., 2018; Kelly and Salinas, 2017). Lastly, the microbiome of fish confers some protection against fish pathogens, such as *Aeromonas* spp. (Nya and Austin, 2009; Park et al., 2017), *Streptococcus agalactiae* (Silva et al., 2020), *Flavobacterium* spp. (Mohammed and Arias, 2015; Nematollahi et al., 2003), and *Yersinia ruckeri* (Ingerslev et al., 2014), by occupying space,

competing for nutrients, and in some cases directly inhibiting their growth (Chatterjee et al., 2017; Hai, 2015).

Exogenous factors influence the dynamics of fish colonization by microbes. In contrast to most mammals, fish are constantly and directly exposed to environmental microorganisms in the aquatic environment. Because of their immersion in the water column, the microbiomes of fish are strongly affected by changes in temperature (Element et al., 2020; Huyben et al., 2018), salinity (Element et al., 2020; Zhang et al., 2016), and, specifically important for aquaculture-raised animals, stocking density (Du et al., 2019; Parma et al., 2020). This aquatic, high-density lifestyle creates more opportunities for exposure to pathogens which can alter the fish microbiomes. The aquaculture industry, like other agricultural and livestock sectors in food production, has historically employed the use of antibiotics in order to not only combat pathogens, but promote growth. The use of prophylactic antibiotics is partly to blame for the increased prevalence of antibiotic-resistant pathogens (Cabello, 2006). Antibiotics can also have a major impact on the microbiomes of fish and ultimately risk the health of the fish stocks (Cabello, 2006; Carlson et al., 2015; He et al., 2017; Schmidt et al., 2017). To provide a viable alternative to prophylactic antibiotic administration, which is still done in some regions outside of the United States, an increasing amount of research has been conducted on the efficacy of probiotics (live microbes) and prebiotics (compounds metabolized by microbes) in promoting a healthy microbiome. These dietary supplements have the capability to support healthy fish physiology and microbiomes while also inhibiting pathogen proliferation (Al-Hisnawi et al., 2019; Dawood et al., 2020; Hooshyar et al., 2020; Mora-Sánchez et al., 2020; Park et al., 2017; Zabidi et al., 2021). Dietary changes especially affect the inhabitants of the gastrointestinal

tract. One such change has been aimed at reducing reliance on the unsustainable use of fishmeal by supplementing or outright replacing it with alternative protein sources (Olsen and Hasan, 2012; Turchini et al., 2019). Through these efforts, many studies have also investigated the impact of alternative feeds on the fish microbiome such as plant-based alternatives (Blaufuss et al., 2020; Estruch et al., 2015; Lim and Lee, 2009) and insect meals (Huyben et al., 2019; Rimoldi et al., 2021; Terova et al., 2021) among other sources (Gasco et al., 2018; Rimoldi et al., 2018; Tlusty et al., 2017).

Fish microbiomes are also impacted by intrinsic physiological factors. Unlike herbivorous and omnivorous fish species, the intestinal tracts of carnivorous fish, like trout, are less complex morphologically, being shorter in length and straighter in comparison to other fish (Al-Hisnawi, 1947; Elliott and Bellwood, 2003). In fact, it has been observed that the intestinal tract of rainbow trout is more microbially homogenous than other mucosal sites (Lowrey et al., 2015). Development of the intestinal tract as the fish matures also has a significant impact on the structure of the microbial communities. The colonizing members of the microbiome can shift depending on the stage of animal development (Li et al., 2017). Though less studied than host age, host genetics and phylogeny have also been implicated as possible drivers in the selection of the microbiome inhabitants (Liu et al., 2016; Tzeng et al., 2015).

Regardless of the factors influencing the rainbow trout microbiome, some studies have identified microorganisms that are consistently persistent (Dehler et al., 2017; Gajardo et al., 2016; Ricaud et al., 2018; Roeselers et al., 2011; Tarnecki et al., 2017; Wong et al., 2013). This core set of microbes could illustrate the “healthy” microbiome and therefore be used to establish targets for beneficial manipulation. Therefore, identifying the core set of microbes

that inhabit the intestinal tract of rainbow trout will be helpful in advancing the aquaculture-raised rainbow trout industry. This review compiles an overview of the recent pertinent literature in the field to identify potential key members of the rainbow trout core intestinal microbiome.

Methods

Article inclusion criteria

This review was accomplished by analyzing microbiome data from recently published articles (i.e. 2017- July 2021) that utilized 16S rRNA gene-based approaches. Moreover, reviewed papers were selected such that a variety of testing variables (e.g. diet, location, etc.) were included as part of the analysis (Table 4.1). Papers that did not include a clear description of the relative abundances of individual phyla were filtered out for the quantitative analysis reported in Table 4.2.

Core constituent analyses

The taxonomic data were analyzed using Microsoft Excel. The frequency of each phylum observed, out of the total 25 papers analyzed, was reported as a percentage. No minimum relative abundance value was used as a criterion to omit phyla. Instead, a "phylum-positive" identification was defined by a simple observation of an individual phylum within the referenced study; bacterial phyla associated with rainbow trout intestines were reported here when observed in the reviewed paper. Key parameters impacting the microbiome including different treatment groups (e.g. diet) and the effect of tissue type (see below) were also analyzed. Briefly, the "total" category is defined by the percentage of papers reporting phyla

that were associated with either digesta (i.e. feces) or mucosa out of the 25 total papers. In other words, a positive result reported for the "total" is not constrained by the presence of a particular phylum in both tissue types, a phyla only needed to be present in at least one of the tissues. Conversely, the tissue-specific percentages represent the frequency a particular phylum was identified in a tissue type (i.e. digesta, mucosa, or the combination of digesta plus mucosa at the point of genomic DNA extraction) out of the total number of papers that analyzed the particular tissue of interest.

Of the 25 total papers included for this review, ten reported the relative abundances of intestinal phyla in clearly formatted numerical formats. The relative abundance values are defined as the number of sequences associated with a specific taxon normalized to the total number sequences within a sample (represented as a percentage of the total sequences). These reported relative abundances corresponding to each phylum were averaged together between different studies regardless of tissue type. For studies that investigated more than one treatment, the relative abundances associated with each treatment were averaged together prior to inclusion within the full dataset reported here. The standard error mean (SEM) represents the error associated with the averaged relative abundances corresponding to the number of papers that observed the phylum of interest.

Not all articles reviewed here reported the family-level (or lower classification) taxonomy. Family-level analysis was accomplished by calculating the frequency with which a particular family was observed out of the total number of papers that identified its associated parent phylum classification (i.e. phylum-positive). Tissue-specific analysis is also reported as either the total (representing an identification in either tissue, digesta or mucosa), digesta,

mucosa, or the combination of digesta and mucosa (tissues combined prior to genomic DNA extractions).

Results and discussion

Identifying bacterial members of the core intestinal microbiome

Studies of fish intestinal tracts analyzed in this review tested the impact of various parameters including diet, pathogens, host genetics, environment, and host age on the gastrointestinal microbiome of rainbow trout (Table 4.1). These studies encompassed locations around the globe with fish originating from several different farmed sources. Regardless of the variables, a core set of bacteria appeared to be consistently present within the trout gastrointestinal tract in every study. Bacteria belonging to the phyla Firmicutes and Proteobacteria were always present in the trout intestinal microbiome (Figure 4.1). Also identified in a majority of the studies (> 50% of the 25 studies investigated here) were the phyla Actinobacteria, Bacteroidetes, and Tenericutes.

Although bacteria in the phyla Firmicutes and Proteobacteria were present in every study, the relative abundances of each phylum can be vastly different depending on the study. Most studies indicate the microbiome is dominated by these two phyla, while other studies find these two phyla to exist in much lower abundances relative to other more dominant organisms such as Fusobacteria and Tenericutes (Brown et al., 2019; Farzad et al., 2021; Hines et al., 2021; Huyben et al., 2018; Lyons et al., 2017a, 2017b; Mora-Sánchez et al., 2020; Terova et al., 2021). On average, bacteria classified as Firmicutes account for ~19% of the relative abundances and bacteria classified as Proteobacteria account for ~ 29% of the relative abundances (Table 4.2).

Firmicutes

Firmicutes are a phylum of bacteria that typically have a low G+C content in their genomes and most, but not all of them, have a Gram-positive cell wall structure. The family *Lactobacillaceae* is one of the most common constituents of the Firmicutes phylum observed (Figure 4.2). Overall, the family *Lactobacillaceae* is more commonly identified within the trout digesta, largely defining the allochthonous microbiome (transient bacteria), as opposed to the mucosal layer (Figure 4.2). This heterogenic group of bacteria includes several species that can provide probiotic effects for their fish hosts (Claesson et al., 2007). Specifically, some *Lactobacillus* species confer immune-stimulatory and antioxidant properties for fish (Adeshina et al., 2020; Dawood et al., 2020; Hooshyar et al., 2020; Pérez-Sánchez et al., 2011). Two other common Firmicutes families, *Streptococcaceae* and *Clostridiaceae*, were also more prevalent in the digesta than mucosa of the rainbow trout (Figure 4.2). The *Streptococcaceae* family contains several fish-specific pathogens including the *Lactococcus garvieae* analyzed by Pérez-Sánchez et al. (2020). This group may also encompass opportunistic pathogens that are otherwise common constituents of the rainbow trout microbiome. It is possible the constituents belonging to the *Lactobacilliacae* family may be acting as probiotics to inhibit further proliferation by opportunistic pathogens such as those in the *Streptococcaceae* family (Harikrishnan et al., 2011; Heo et al., 2013; Kim et al., 2019; Son et al., 2009). Similar to members of the *Streptococcaceae* family, constituents belonging to the *Clostridiaceae* family represent several higher vertebrate pathogens occasionally associated with an increased level of microbiome dysbiosis and disease (Muñiz Pedrogo et al., 2019; Picchianti-Diamanti et al., 2018; Scarpa et al., 2011). The role of *Clostridiaceae* in the internal microbiome of trout is not

well known, however, some strains have actually been shown to act as probiotics for the fish host (de Bruijn et al., 2018; Sakai et al., 1995).

Proteobacteria

Proteobacteria are a major phylum of Gram-negative bacteria. Within Proteobacteria, the most common families identified in the rainbow trout intestine were *Enterobacteriaceae*, *Pseudomonadaceae*, and *Moraxellaceae*. The family *Enterobacteriaceae* is a broadly encompassing group of bacteria that includes pathogens like *Yersinia ruckeri* and *Aeromonas* sp. and commensal organisms like *Escherichia coli* (Conway and Cohen, 2015). One major benefit posed by *Enterobacteriaceae* members is the production of short chain fatty acids via sugar fermentation similar to *Clostridiaceae* members (Gottschalk, 1986; Wüst et al., 2009). Certain species in the *Enterobacteriaceae* family can act as protective organisms against pathogenic infections in trout (Schubiger et al., 2015). The families *Pseudomonadaceae* and *Moraxellaceae* include several fish pathogens (Austin and Austin, 2012), notably those of the *Acinetobacter* genus within *Moraxellaceae* (Rossau et al., 1991). Though *Acinetobacter* was identified as being present in the study, the cultured fish did not exhibit disease caused by these organisms. A protective system generated by lactic acid bacteria, similar to their antagonistic nature against *Streptococcaceae*, may be inhibiting excessive proliferation by these potentially pathogenic bacterial families.

Actinobacteria

The phylum Actinobacteria is comprised of mostly Gram-positive bacteria that primarily occupy soil and water environments. Though not found in every study, bacteria belonging to the phylum Actinobacteria are identified in at least 75% of the studies reviewed (Figure 4.1).

Actinobacteria are also typically present at lower relative abundances compared to the more common Firmicutes and Proteobacteria (Table 4.2). However, its levels vary at different stages in the life cycle of rainbow trout. Specifically, Actinobacteria appear to dominate the microbiome of trout during the early life stages compared to fish during adulthood wherein a much smaller relative abundance is observed (Ceppa et al., 2018; Piazzon et al., 2019). Therefore, age appears to be a driving factor that influences the selection of microbial constituents throughout the life of the fish.

The most commonly identified Actintobacteria family, *Corynebacteriaceae* (Figure 4.2), encompasses bacteria prevalent in the microflora of fish species including salmonids (Hartviksen et al., 2014; Izvekova et al., 2007; Ruohonen et al., 2014). Though it consists of human and fish pathogens such as *Corynebacterium diphtheriae*, the studies used for the taxonomic analysis in this review did not indicate any disease associated with infection by *Corynebacterium* spp.

Bacteroidetes

Bacteroidetes are non-spore forming, anaerobic or aerobic, rod-shaped bacteria found in a variety of environments such as soil and marine water environments, as well as the microbiome of animals and humans. Bacteria associated with the Bacteroidetes phylum are identified in at least 50% of the studies analyzed in this review and are typically in lower relative abundances compared to Firmicutes, Proteobacteria, and Actinobacteria (Figure 4.1, Table 4.2). The dominant Bacteroidetes family, *Flavobacteriaceae* (Figure 4.2), includes important fish pathogens such as *Flavobacterium columnare* and *Flavobacterium psychrophilum* (Mohammed and Arias, 2015; Nematollahi et al., 2003). *F. psychrophilum* may pose a serious issue due to

the prevalence of antibiotic resistance (Dalsgaard and Madsen, 2000), which when compounded with the high frequency of identification in trout microbiomes, indicates the importance of controlling this opportunistic pathogen.

Tenericutes

The Tenericutes phylum is dominated by bacteria associated with the *Mycoplasmataceae* family, a group of bacteria lacking cell walls (Figure 4.2), per the reviewed studies. However, it should be noted that *Mycoplamataceae* has recently been reclassified within the Firmicutes phylum (Parks et al., 2018). Tenericutes was present in at least 60% of the papers analyzed here (Figure 4.1). When present, this group of bacteria typically dominate the microbiome compared to the other organisms (Table 4.2). *Mycoplasma*, a common genera within the *Mycoplasmataceae* family, can sometimes dominate the trout intestinal microbiome (Al-Hisnawi et al., 2019; Brown et al., 2019; Farzad et al., 2021; Lyons et al., 2017a, 2017b). Uniquely, this group of bacteria are usually associated with a host due to their increased vulnerability resulting from the lack of a cell wall (Razin, 1967). This phenomenon is further validated by the results presented here indicating a higher abundance of *Mycoplasmataceae* found in the autochthonous microbiome (mucosal adherent bacteria) versus transient digesta microbiome present in trout feces. Within fish, *Mycoplasma* spp. are increasingly being identified as commensal organisms instead of pathogens, though some species such as *Mycoplasma mobile* are known to cause disease (Stadtländer and Kirchhoff, 1990; Stadtländer et al., 1995). Further studies are necessary to further characterize the role of this bacterium within rainbow trout.

Niches within the gastrointestinal tract

Specific to trout, bacterial communities are distinctive to their respective internal or external tissues (Lowrey et al., 2015). However, the microbiota dwelling within intestinal tracts of trout may be relatively homogenous. This relative microbial homogeneity is due, in part, to a relatively shorter and linear intestinal tract with consistent resting levels of effectors (i.e. intestinal ions and pH), regardless of intestinal topography (Bucking and Wood, 2009; Fard et al., 2007; Lowrey et al., 2015). However, within the gastrointestinal tract, some members of the microbiome colonize the mucosal epithelial layer and are considered adherent, whereas others are transient as they are sloughed off into the lumen and/or are contained within the fecal material that comprises the digesta. The sampling method within the gastrointestinal tract (i.e. digesta only, mucosa only, or intestinal mucosa combined with digesta) (Table 4.1) had a direct impact on the organisms that were observed in the microbiome. Notably, most of the taxa were identified across all sample types, though some patterns were apparent. For instance, Firmicutes tended to be identified more often in the digesta than mucosa (Figure 4.1). On the other hand, Proteobacteria were identified more in the mucosa than the digesta. *Mycoplasmataceae* were reported in both digesta and mucosa types, but this family of bacteria were present in the mucosa five times more often than in the digesta (Figure 4.2). Taxa that are more common in the digesta but not in the mucosa, such as *Lactobacillaceae*, *Leuconostocaceae*, and *Corynebacteriaceae,* indicates transient organisms, including potential *Lactobacillus* probiotics, that may be unable to colonize the host's mucosa (O'Toole and Cooney, 2008; Walter, 2008). Conversely, the taxa present to a high degree in the mucosa but not in the digesta (e.g. *Flavobacteriaceae*, *Brevinemataceae*, *Mycoplasmataceae*, and

Enterobacteriaceae) indicates adherent organisms that likely colonized the host's intestinal mucosa and thus are able to persist, despite otherwise detrimental conditions such as pH fluctuations (Banerjee and Ray, 2017). However, it's important to note that the increased abundance of a particular organism within the mucosa does not necessarily guarantee long term mucosal colonization, likely due to competitive exclusion (Frese et al., 2012). The sloughing of dead bacterial cells may contribute to these taxa reported from both sample types (Ventura et al., 2009).

Limitations of methods used to assess microbiome constituents and future approaches

Microbiome analysis based on sequencing of the 16S rRNA gene has continued to provide a metric to analyze microbial populations independent of culture-based techniques. With the advent of next generation sequencing (NGS), high-throughput 16S rRNA gene-based studies have generated a plethora of microbiome data. The majority of studies reviewed here utilized Illumina MiSeq NGS which generates short-read sequences. These short-read sequences are useful for comparing reads aligned to the shorter sequences available within the hypervariable regions of the 16S rRNA gene. Each hypervariable region presents differing resolutions between bacterial taxa. Because of these differential resolutions, it's important to choose the most appropriate target region for microbial community analysis. A standard method proposed by the Earth Microbiome Project (Thompson et al., 2017) involves targeting the fourth hypervariable region (V4). Though most of the studies here also targeted the V4 region, about half also included the V3 region during amplification. Inclusion of the V3-V4 data may represent a higher resolution bias resulting from the more precise primer sets used

(Almeida et al., 2018; Fuks et al., 2018). However, any introduced biases would be more apparent at lower taxonomic classes (i.e. genus and species levels). A potential solution to overcome biases introduced using individual hypervariable regions and even utilization of the full 16S rRNA gene (e.g. bias introduced by copy number) for microbial community analyses could be the implementation of metagenomic sequencing. While the costs and specific experience required for running metagenomic analyses are greater than that of 16S-based community analyses, the additional insights gained from metagenomics can greatly increase taxonomic classification confidence. Potentially, a more precise bacterial function within the microbiome can be inferred by successfully identifying bacteria at the species and even strain level. As the expense of these more robust analyses decreases and application increases, microbial community studies should begin to incorporate metagenomics.

Assigning taxonomic information to sequences also inherently introduces bias (Mysara et al., 2017; Schmidt et al., 2015), though this bias reduces with the inclusion and verification of more reference genomes. The majority of studies used for this review analyzed microbiome data by assigning taxonomy to artificially created operational taxonomic units (OTUs). Most of the OTUs here were defined by 97% similarities. As sequencing technologies continue to advance with reduction of inherent errors and bioinformatic tools develop around increasing the confidence of filtered sequences (e.g. DADA2 (Callahan et al., 2016)), the frequency of implementing OTU clustering will continue to decrease. Additional work in this field would also be benefitted by the consistent publication of taxonomic relative abundance tables for ease of comparison.

Concluding thoughts

All studies used for this review analyzed the microbiomes of fish raised in aquaculture. As shown here, a core set of intestinal microbial constituents exists independent of study variables including age, specific intestinal sampling site and geographical location. However, it would be informative to investigate how culturing trout affects the microbiome compared to wild-caught fish and if this impacts the members of the core microbiome. Additional analyses to define the core microbiome can involve identifying taxa shared between testing groups based upon more advanced criteria than the simple presence or absence of taxa (e.g. abundance-based, persistence, connectivity; (Shade and Handelsman, 2012)). However, the methods used in this review have enabled the elucidation of some core bacterial constituents of the aquaculture-raised rainbow trout intestinal microbiome.

Table 4.1. Parameters specified in referenced studies.

Reference	Study Variables	GI Site[1]	Trout Culturing System	Final Fish Weight	16S rRNA Gene Hypervariable Region	Geographic Location
Michl et al., 2017	Diet/Age	Combo	RAS[2]	1-4g	V6-V8	Germany
Lyons et al., 2017b	Diet	Combo	Flow-through	117-137g	V4	United Kingdom
Gonçalves and Gallardo-Escárate, 2017	Diet	Mucosa	RAS	Not Specified	V4	Chile
Pérez-Sánchez et al., 2020	Diet/Pathogen	Mucosa	Not Specified	44-48g	V1-V3	Spain
Brown et al., 2019	Genetics/Pathogen	Mucosa	Tanks	194g	V1-V3	West Virginia, USA
Ricaud et al., 2018	Genetics	Digesta	Tanks	87-90g	V3-V4	France
Lyons et al., 2017a	Exploratory	Combo	Farm/Flow-through	99g[3]/191g[4] (aquarium)	V4	Scotland

Huyben et al., 2018	Diet	Both	Flow-through	Not Specified	V4	Sweden
Betiku et al., 2018	Environment/Diet	Both	RAS	103-123g	V3-V4	Montana, USA
Terova et al., 2019	Diet	Digesta	Flow-through	216-223g	V3-V4	Italy
Al-Hisnawi et al., 2019	Diet	Both	RAS	Not Specified	V1-V2	United Kingdom
Parshukov et al., 2019	Pathogen	Combo	Farm	240-850g	V3-V4	Russia
Blaufuss et al., 2020	Diet	Digesta	RAS	406-488g	V3-V4	Idaho, USA
Rimoldi et al., 2018	Diet	Combo	Flow-through	252-298g	V3-V4	Italy
Yildirimer and Brown, 2018	Environment	Digesta	Raceway	Not Specified	V4	Northwest, USA
Huyben et al., 2019	Diet	Combo	Flow-through	74-82g	V4	Sweden
Gatesoupe et al., 2018	Diet	Mucosa	Tanks/Farm	230-345g	V3-V4[5]	France
Etyemez Büyükdeveci et al., 2018	Diet	Mucosa	Pond	171-186g	V4	Turkey
Mora-Sánchez et al., 2020	Diet/Pathogen	Mucosa	Tanks	36-39g	Not specified	Spain

Ceppa et al., 2018	Diet/Age	Digesta	Tanks	1050g	V1-V3	Italy
Pelusio et al., 2020	Diet/Environment	Digesta	RAS	300-320g	V3-V4	Italy
Rimoldi et al., 2021	Diet	Combo	Flow-through	Not Specified	V4	Italy
Terova et al., 2021	Diet	Mucosa	Flow through	312-353g	V3-V4	Italy
Farzad et al., 2021	Diet	Mucosa	RAS	115-122g	V4	Virginia, USA
Hines et al. 2021	Diet	Mucosa	RAS	180-250g	V4	Virginia, USA

[1] Gastrointestinal (GI) site utilized for microbiome analysis. Combination of mucosa and digesta (combo); Mucosa and digesta separately analyzed (both).

[2] Recirculating Aquaculture System (RAS)

[3] Farmed animals

[4] Flow-through tank-raised animals

[5] DNA product was reverse-transcribed from 16 rRNA

Table 4.2. Average phyla-level relative abundances.

Phylum	Mean relative abundance (%)[1]	Reported papers[2]
Acidobacteria	2.00 ± 0.00	2
Actinobacteria	6.28 ± 3.66	8
Bacteroidetes	5.62 ± 3.76	7
Firmicutes	18.7 ± 4.10	10
Fusobacteria	14.3 ± 12.7	6
Proteobacteria	29.0 ± 7.49	10
Spirochaetes	5.94 ± 4.01	3
Tenericutes	46.5 ± 15.7	6

[1] Error is represented by the mean relative abundances of that particular phylum across the specified number of "reported papers" ± SEM

[2] Number of studies wherein the phylum-level relative abundances were clearly reported (out of ten total papers)

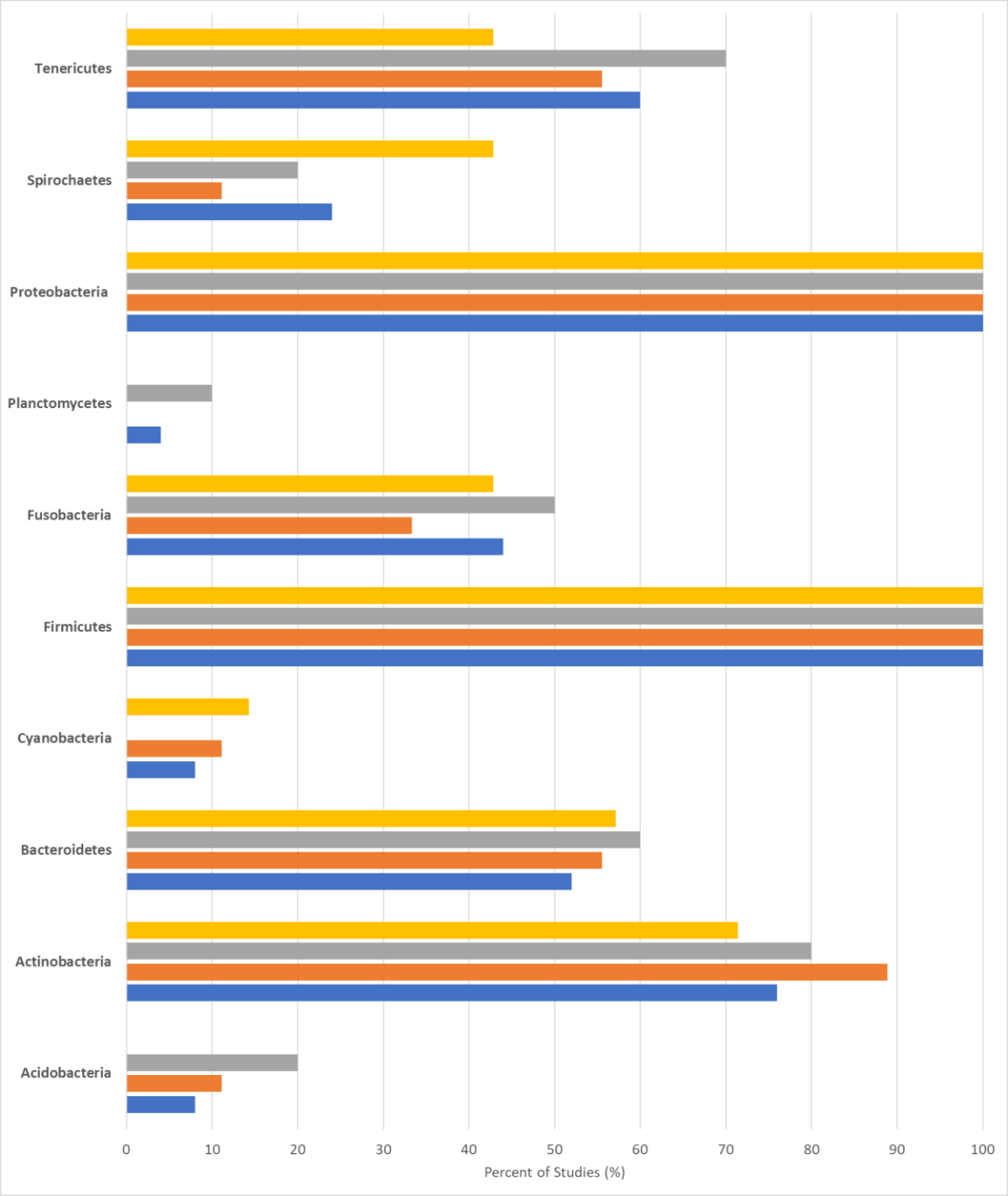

Figure 4.1. Common phyla present in rainbow trout gut microbiomes. Bars represent the percent frequency of positive phylum identification in fish tissue samples on a per study basis. Positive phylum identifications (phylum-positive) denote the phylum was observed in each

study, regardless of relative abundance, in the particular tissue. Frequencies are measured as the percent of phyla present within (blue; N=25) all papers used for this review, (orange; N=9) papers that analyzed the digesta, (gray; N=10) papers that analyzed the mucosa, or (yellow; N=7) papers that analyzed the combination of both mucosa and digesta.

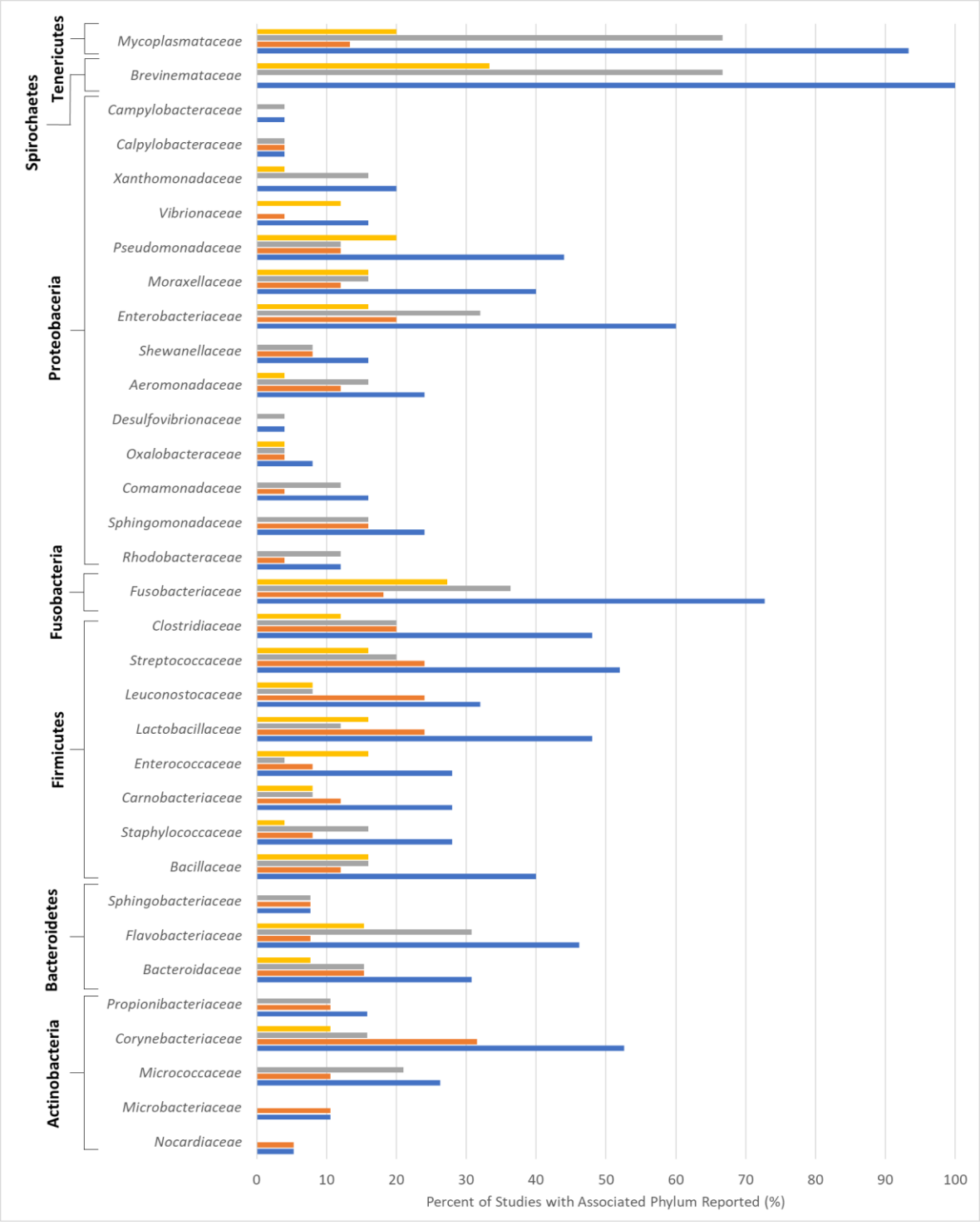

Figure 4.2. Common bacterial families identified in the core phyla of rainbow trout gut microbiomes. Bars represent the percent frequency of family presence in fish tissue samples. Positive phylum identifications (phylum-positive) denote the phylum was observed in each

study, regardless of relative abundance, in the particular tissue. Frequencies are defined as the percentage of phylum-positive papers wherein a family is identified. Colors represent the percentage of (blue; N=25) all phylum-positive papers used for this review, (orange; N=9) phylum-positive papers that analyzed the digesta, (gray; N=10) phylum-positive papers that analyzed the mucosa, or (yellow; N=7) phylum-positive papers that analyzed the combination of both mucosa and digesta.

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Chapter Five

Development of a controlled laboratory-scale inoculation system to study *Vibrio parahaemolyticus*-oyster interactions

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Attributions

Ian S. Hines and Ann M. Stevens wrote the manuscript and contributed to the data analysis, study design, performed the experiments, and reviewed the manuscript. David D. Kuhn wrote the manuscript and contributed to the data analysis, study design, and reviewed the manuscript. Stephen A. Smith contributed to the study design and reviewed the manuscript.

Abstract

Prevalence of seafood-borne gastroenteritis caused by the human pathogen *Vibrio parahaemolyticus* (VP) is increasing globally despite current preventative measures. The United States Centers for Disease Control (CDC) has designated VP as a reportable emerging human pathogen. The Eastern oyster (*Crassostrea virginica*) is a natural reservoir of VP in marine environments, but little is actually known regarding interactions between oysters and VP. Therefore, a laboratory-scale Biosafety Level-2 (BSL2) inoculation system was developed wherein Chesapeake Bay region oysters harvested during summer and winter months, were exposed to the clinical VP RIMD2210633 strain carrying a chloramphenicol-selective marker (VP RIMDmC). Homogenized whole oyster tissues were spread on selective and differential agar medium to measure viable VP RIMDmC levels. Endogenous *Vibrio* spp. cell numbers were significantly reduced followed chloramphenicol treatment and this likely contributed to higher VP RIMDmC inoculation levels, especially using colder weather animals. Warmer weather oysters had significantly higher existing *Vibrio* levels and a lower level of artificial VP RIMDmC inoculation. Thus, the pre-existing microbiome appears to afford some protection from an external VP challenge. Overall, this system successfully enabled controlled manipulation of parameters influencing VP-oyster interactions and will be useful in the testing of additional pertinent environmental variables and potential mitigation strategies.

Introduction

Vibrio parahaemolyticus (VP), a Gram-negative proteobacterium indigenous to marine coastal and estuarine environments, is the most common causative agent of seafood-borne acute gastroenteritis in humans worldwide (Joseph et al., 1982; Kaneko and Colwell, 1977; Sarkar et al., 1985; Thompson and Vanderzant, 1976). VP infections typically lead to a debilitating gastroenteritis, albeit acute and self-limiting, but certain strains are lethal in susceptible patients (Blake et al., 1980; Food and Drug Administration, 2005; Gomez-Jimenez et al., 2014; Iwamoto et al., 2010; Kim et al., 1999). Though VP is classified as an emerging pathogen by the United States Centers for Disease Control (CDC) and epidemiologically reportable (Cronquist et al., 2006), there remain few non-palliative treatments available for the gastroenteritis it causes. Increasing coastal water temperatures due to global warming are contributing to higher VP disease incidence (Chen et al., 2017; Daniels et al., 2000), and current efforts aimed to prevent disease (e.g., monitoring of oysters for VP and closure of oyster beds associated with outbreaks of illness) have not curtailed the rising disease incidence (Froelich and Noble, 2016).

Eastern oysters (*Crassostrea virginica*) play a critical role in maintaining water quality, and they are also an economically important food resource, especially within the Chesapeake Bay watershed area (Beckensteiner et al., 2020; Parker et al., 2020; Schulte, 2017). In fact, the farm gate value of Virginia-cultured oysters was $15.9 million (U.S. Dollars) in 2017 (Beckensteiner et al., 2020; Hudson, 2018). A major challenge for the shellfish industry is the impact of human illnesses, specifically those caused by VP. Ingestion of raw or undercooked shellfish, especially oysters, is the most common route of human VP infection (Blake et al.,

1980; Letchumanan et al., 2014; Odeyemi, 2016). Following harvest, batches of oysters are analyzed for the presence of pathogenic VP and harvest beds closed in response to VP outbreaks. In order to avoid closures, some farmers elect to subject the animals to post-harvest treatments to reduce anticipated pathogenic VP (Spaur et al., 2020). Although treatments such as temperature reduction, gamma radiation, or high-pressure pasteurization (Spaur et al., 2020) can reduce pathogenic VP-associated oysters, these processes may also impact the food production quality and thereby the profitability of the raw half-shelled oysters (Melody et al., 2008; Muth et al., 2013). Other forms of mitigation such as seasonal sales restriction can also reduce the profitability of the industry (Iwamoto et al., 2010). Therefore, research focused on an understudied aspect of the VP life cycle, the adult oyster host-microbe relationship, may lead to the development of future alternatives in VP disease mitigation strategies.

Methods of artificial oyster inoculation have included directly injecting the bacterium via holes bored in the shells (Feng, 1966; Kaysner et al., 1989), larval inoculation (Wendling et al., 2014), inoculating oysters within a container other than the rearing environment (Ye et al., 2012), and culturing oysters within large, inoculated aquaria including some with recirculating water (Andrews et al., 2000; Koo et al., 2006; Liu et al., 2009; Richards et al., 2012; Volety and Chu Fu-Lin, 1994). The ability to safely inoculate animals using Biosafety Level-2 (BSL-2) pathogens, such as clinical strains of VP, relies on a tightly managed system. Unfortunately, large inoculated, aerated tanks are difficult to maintain within a typical laboratory's biosafety cabinet. Moreover, physical manipulation of oysters may physiologically stress the animals and impact findings. To improve upon some of the previously developed experimental systems, a

simple, modifiable fermentation jar inoculation system was developed for use within a BSL-2-certified biosafety cabinet to study oyster-VP interactions. In addition, use of a genetically modified VP strain expressing chloramphenicol resistance enabled selection of this strain within the microbial community. The new inoculation system can be used to effectively and safely measure the pathogenic VP levels within oysters to help better understand the factors driving their association within an oyster host.

Materials and methods

Bacterial strains and plasmids

A plasmid that encodes for chloramphenicol (Cm) resistance and mCherry fluorescence, pRMJ3 (E. Stabb, University of Illinois-Chicago; (Stabb and Ruby, 1994)), was conjugated into *Vibrio parahaemolyticus* RIMD 2210633 (VP RIMDmC) (Makino et al., 2003). The transconjugation was achieved using a diparental mating with *Escherichia coli* S17 harboring the pRMJ3 plasmid and *Vibrio parahaemolyticus* RIMD 2210633. For jar inoculations, the VP culture was grown to mid-exponential phase (OD_{600} ~0.5; ~10^7 CFU/mL) in trypticase soy broth (TSB; Fisher) + 2% NaCl + 10 µg/mL Cm (Cm10) and transferred into glycerol aliquots (~20% (*v*/*v*) final glycerol concentration) for long term storage at -70°C as starter freezer stocks.

VP *in vitro* growth curve

A growth curve for the VP RIMDmC strain was also established (Figure C1). Briefly, an overnight culture was used to inoculate culture flasks containing TSB + 2% NaCl + 10 µg/mL Cm (Cm10) by diluting the overnight culture to a starting OD_{600} of 0.02. The OD_{600} was subsequently measured every 30 min in duplicate experiments.

Experimental animals

Consumer-ready oysters that were harvested in the coastal regions of the Chesapeake Bay area (Table C.1) were purchased from a local grocery store in a refrigerated condition. Summer 2020 oysters were acquired during late July and early August, and winter 2021 oysters were acquired during January. For each season, six total oysters per treatment group were used in experimental trials performed on two or three animals per treatment at a time (i.e., duplicate or triplicate experiments were performed). The oysters were gradually acclimated to the laboratory environment (i.e., room temperature at ~23°C) within a cooler filled with pre-chilled (~4°C) 20 ppt (g/L synthetic sea salt) artificial seawater (Crystal Sea Marinemix, Marine Enterprises International, Baltimore, MD).

Jar inoculation system

Approximately 24 hr after acclimation to room temperature, oysters that had opened and were actively filtering, were transferred to modified one-gallon glass fermentation jars (www.woodyshomebrew.com) within a class II A2 Biosafety Level-2 (BSL-2) cabinet. The jars were filled with 1 L 20 ppt artificial seawater per oyster, up to 3 L (maximum of three oysters). For some treatment groups, Cm10 was added to the artificial seawater to reduce native VP levels prior to inoculation with laboratory strains. The inoculation jar lids supplied by the manufacturer with one hole for the gas trap were modified by adding two additional holes (Figure C.2). The CO_2 trap was modified by adding dH_2O and a cotton ball to reduce aerosolization of VP (Figure C.2). The second hole was connected to air stones in the jar via ~45 cm long sections of ~5 mm diameter plastic tubing that facilitated aeration for the animals within the jar system. Up to four jars were controlled by the same air pump (Second Nature

Challenger Special, Willenger Bros Inc., Oakland, NJ). Finally, secondary tubing attached to a 10 mL syringe was inserted through the third hole for the purpose of water collection or addition of solutions without ceasing air flow (Figure C.2).

Exposure of oysters to VP

Jar treatments included: (A) oysters not treated with Cm10 or VP, (B) oysters treated with Cm10 but not VP, (C) oysters treated with VP but not Cm10, and (D) oysters treated with Cm10 and VP. On Day One, ~24 hr following transfer of oysters from the cooler to jars, the jars were inoculated with an individual VP culture. Culture flasks containing TSB supplemented with 2% NaCl and Cm10 were inoculated with thawed starter stocks. Cultures were incubated at 30°C for ~8 hours with shaking. Once the cultures reached late exponential/early stationary phase growth (OD_{600} ~5.0; ~10^9 CFU/mL), the cell cultures were washed. Briefly, cultures were centrifuged at 5000 rpm (Beckman Coulter, rotor JS-5.3, Brea, CA) for 6 min at room temperature (~18°C), resuspended in an equal volume of sterile phosphate buffered saline (PBS), centrifuged again using the same conditions, and again resuspended in an equal volume of PBS. PBS-washed cultures were then added to the seawater in the jars at a 1:100 dilution (~10^6 - 10^7 CFU/mL final concentration in the water column). VP RIMDmC levels within the water column following inoculation were then enumerated by serially diluting 1 mL water samples in sterile PBS and subsequently spreading onto *Vibrio*-selective and differential thiosulfate bile salts sucrose (TCBS) medium supplemented with Cm10 to confirm the ~10^6 - 10^7 CFU/mL final concentration. After ~24 hr, during which visible clearing of the seawater occurred, the animals were moved to a jar with fresh seawater (without Cm) such that the animals resumed filtration within an aerated environment, and particulate matter was

exchanged once in the closed system. Briefly, all oysters were transferred into new jars with one liter of freshly-prepared 20 ppt artificial seawater per animal to remove transiently associated VP. All animal tissues were harvested 24 hr after initiation of the seawater exchange period within the biosafety cabinet.

Oyster tissue harvest and *Vibrio* enumeration

On the harvest day, whole oyster tissues were homogenized for VP enumeration. Briefly, oysters were individually shucked using 70% ethanol-sanitized gloves and shucking tools in the BSL-2 cabinet. Once shucked, the oysters were briefly rinsed in the half shell (~5-10 sec) using freshly prepared 20 ppt artificially seawater applied via squirt bottle to further remove any transiently associated VP. Rinsed whole animals were then transferred to 50 mL conical tubes (USA Scientific, Ocala, FL) containing 10 mL sterile PBS and assessed for tissue weights prior to homogenization with 100% ethanol-sanitized OmniTip soft tissue probes attached to an OmniTip homogenizer (Omni International, Kennesaw, GA). Oyster homogenates were then serially diluted in sterile PBS and plated on TCBS medium (Fisher) to obtain countable CFU/mL values. Total *Vibrio* spp. and VP RIMDmC levels within oysters were enumerated using the TCBS medium without and with supplemented Cm10, respectively. VP inoculation efficiency was measured by comparing the level of VP colony forming units (CFUs) on the TCBS+Cm10 plate to the total *Vibrio* CFUs observed on the TCBS plate (VP RIMDmC/ total *Vibrio* spp. population).

Statistical analyses

Bacterial CFUs were normalized to the animals' weights on a per gram basis (reported as CFU/g). Normalized bacterial counts within the oysters were compared between treatment

groups using a Wilcoxon signed-rank test within the vegan package (v.2.5-7) of R (v.4.1.0) (Oksanen et al., 2019; R Core Team, 2019).

Results

Ability of inoculation system to increase VP levels in oysters

Total *Vibrio* CFUs from Summer 2020 samples, as observed by counting colonies of all colors on the TCBS-only medium, were around 10^5 -10^6 CFU/g for treatment groups A, C, and D, with group B having lower levels ~10^4 CFU/g. Groups A and B demonstrate the native *Vibrio* levels in the animal without and with exposure to Cm, respectively, and groups C and D demonstrate the levels of *Vibrio* after exposure to VP without and with exposure to Cm, respectively. Group B animals that were treated with Cm10 but not inoculated with VP had significantly lower ($p < 0.05$) *Vibrio* levels (~10^4 CFU/g) than untreated group A oysters (~10^6 CFU/g) (Figure 5.1). No VP RIMDmC was detected in the non-VP inoculated control groups A or B as observed by the lack of any colonies on the TCBS+Cm10 medium, thus indicating no cross contamination with the VP-inoculated jars and no background Cm-resistance in the native *Vibrio* population. Animals exposed to VP RIMDmC but not treated with Cm10 (group C) harbored the highest total *Vibrio* CFUs based on growth using TCBS without Cm, but the level of VP RIMDmC only accounted for ~10% of the *Vibrio* microbiome as measured by growth of green colonies on TCBS with Cm. The levels of VP RIMDmC were significantly lower ($p < 0.05$) than the total *Vibrio*-specific microbiome in Group C oysters. Group D oysters exposed to both Cm10 and VP RIMDmC, on the other hand, harbored VP RIMDmC CFUs closer to the total *Vibrio* CFUs (~25%), but this was not significantly different than the total *Vibrio* specific microbiome in Group D oyster or the VP RIMDmC levels in group C oysters.

Effects of season on VP RIMDmC levels in oysters

A comparison of the results of experiments performed with oysters harvested in the summer (Figure 5.1) with animals harvested in the winter (Figure 5.2) demonstrated some interesting differences. In the winter animals, the overall *Vibrio* CFUs for treatment groups A-C were ~1-2 orders of magnitude lower than the summer animals. Specifically, *Vibrio* levels in group A animals were significantly lower ($p < 0.05$) when collected in the winter compared to the summer (group A Figure 5.1 versus Figure 5.2). Similar to the animals harvested in the summer, addition of Cm to the winter animals led to a significant ($p < 0.01$) decrease in the level of total *Vibrio* sp. (groups A versus B, Figure 5.2). The inoculation efficacy, as measured by the number of VP RIMDmC that constitute the total *Vibrio* microbiome (~10%), was similar between the summer and winter data sets for treatment group C oysters taking into account the lower overall levels of vibrios in the winter animal. Interestingly, the reduced *Vibrio* CFUs measured in the cold weather oysters appears to facilitate higher inoculation efficacy rates by VP RIMDmC in the oysters when the existing *Vibrio* microbiome is further reduced following exposure to Cm (treatment group D). The rate of inoculation efficacy in treatment group D oysters was close to 100% which was significantly higher ($p < 0.01$) than the 10% inoculation efficiency observed in the group C animals.

Discussion

Human VP infections from consumption of raw or undercooked shellfish continue to rise despite current mitigation efforts (Chen et al., 2017; Daniels et al., 2000). Better understanding the interactions between oysters and VP may provide key insights into developing more effective human disease prevention strategies. In order to consistently test different

parameters impacting the microbe-host interactions, a laboratory-scale BSL-2 jar inoculation system was developed, permitting for an examination of laboratory-controlled VP inoculations of Eastern oysters. Using relatively simple components, in addition to built-in biosafety cabinet safety measures, the system can be applied to many avenues of pathogenic VP-oyster research. In the present study, the impact of seasonal harvest conditions (i.e., summer versus winter) on the inoculation efficiency of VP within consumer-ready oysters was investigated. In line with previous observations, untreated oysters harvested from the Chesapeake Bay area during the warmest months (i.e., July-August) harbored the highest levels of *Vibrio* spp. compared to untreated oysters harvested during the coldest months (i.e., December-January) (Figures 5.1 & 5.2; (Genthner et al., 1999; Parveen et al., 2008)). As has been established by CDC surveillance data, consumption of oysters harvested during the warmer months can lead to higher rates of VP infection likely due to increased *Vibrio* numbers and growth rates during the warmer temperatures (Parveen et al., 2013). Importantly, *Vibrio* levels in oysters persisted after an acclimation period of a single water exchange in the present lab-scale study at a level that can be considered an infectious dose (Martinez-Urtaza et al., 2010). This persistence may indicate colonization of the oyster by the VP. Additional experiments using the inoculation system may help determine the molecular mechanism by which the bacterium associates with the oysters and lead to the identification of potential mitigation targets.

Introduction of the antibiotic chloramphenicol to oysters consistently resulted in a significant ~1-2 log reduction in the total number of *Vibrio* within the oysters compared to untreated oysters, regardless of season. Importantly, this reduction in total *Vibrio* numbers appears to concurrently result in increased levels of VP RIMDmC after inoculation. For

instance, the summer-harvested oysters that were not treated with Cm10 but were inoculated with VP RIMDmC maintained a *Vibrio* population that consisted of only 10% artificially inoculated VP RIMDmC. However, summer oysters treated with Cm10 prior to VP RIMDmC inoculation harbored a *Vibrio* population consisting of 25% VP RIMDmC. The data presented here agrees with previously established notions that the pre-existing microbiome affords its oyster host some resilience to artificial inoculation (Brugman et al., 2018; Clavijo and Flórez, 2018; King et al., 2019; Perry et al., 2020). The microbiomes of the animals used in our study were likely altered through the post-harvest processing methods to make them consumer-ready. Determination of the composition of this microbiome is of interest as it might suggest appropriate probiotic microbes that could be used to prevent VP colonization.

Similar to the summer-harvested animals, untreated oysters harvested in the winter and exposed to VP RIMDmC maintained a *Vibrio* population with only 10% represented by VP RIMDmC. However, when Cm10 pretreatment was used, VP RIMDmC appears to become the only culturable representative of the *Vibrio* population. VP are known to enter a viable but not culturable state (VBNC) under unfavorable conditions (i.e., cold temperatures; (Wong and Wang, 2004)). Since the average water temperature of the Chesapeake Bay during the harvests for the winter 2021 animals was ~4-7°C (Table C.1) (compared to ~26-28°C during summer 2020; (NOAA)), it is reasonable to consider the possibility that some of the VP within the winter oysters entered the VBNC state and were held in this state by refrigeration following harvest and processing. Resuscitation from the VBNC state into a fully functional vegetative state may take up to ~24 hours (Falcioni et al., 2008) at which point the oysters were exposed to the

bacteriostatic Cm in the jar inoculation system. This may in part explain why the winter animals had lower levels of total VP and were more efficiently inoculated.

Conclusion

A viable laboratory-scale system to safely test different pathogenic VP inoculation strategies on Eastern oysters has been established. Seasonal changes, including the existing microbiome, impacted the effectiveness of external inoculation. The oyster microbiome present in the summer oysters afforded them protection against artificial inoculation; additional studies are needed to further investigate the relationship between the oyster host and its microbial constituents. This novel VP-oyster inoculation system will permit for an examination of the impact of environmental parameters on the pathogenicity of microbes under controlled and safe (BSL-2) laboratory-scale conditions in shellfish, including oysters, and other aquatic organisms.

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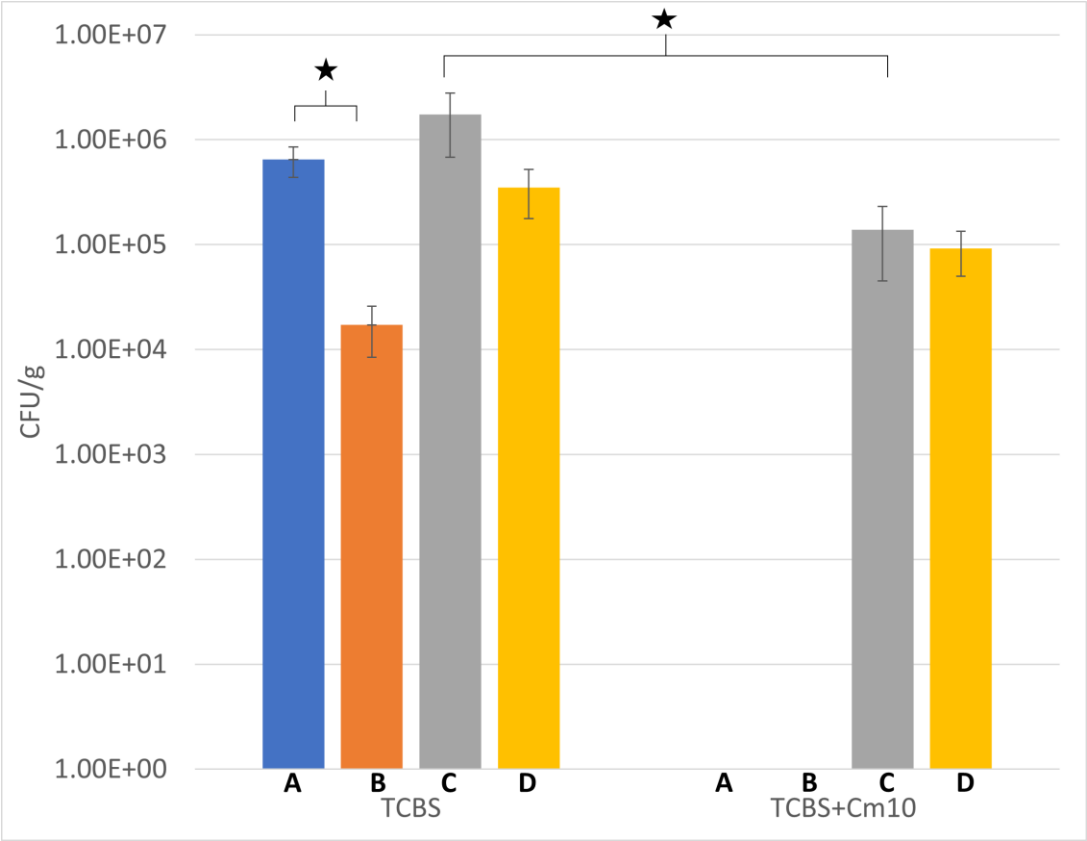

Figure 5.1. *Vibrio* levels in whole oyster homogenates (Summer 2020). Jar treatments included untreated oysters (blue, group A), oysters exposed to Cm10 (orange, group B), oysters exposed to VP RIMDmC (gray, group C), and oysters exposed to both VP RIMDmC and Cm10 (yellow, group D). Whole oyster homogenates were enumerated on TCBS without Cm10 and TCBS with Cm10 as indicated. Two stars represent $p < 0.01$ and one star represents $p < 0.05$ calculated via Wilcoxon signed-rank test. Error is represented by the mean weight-normalized CFU/g values for six total animals per treatment group ± SEM.

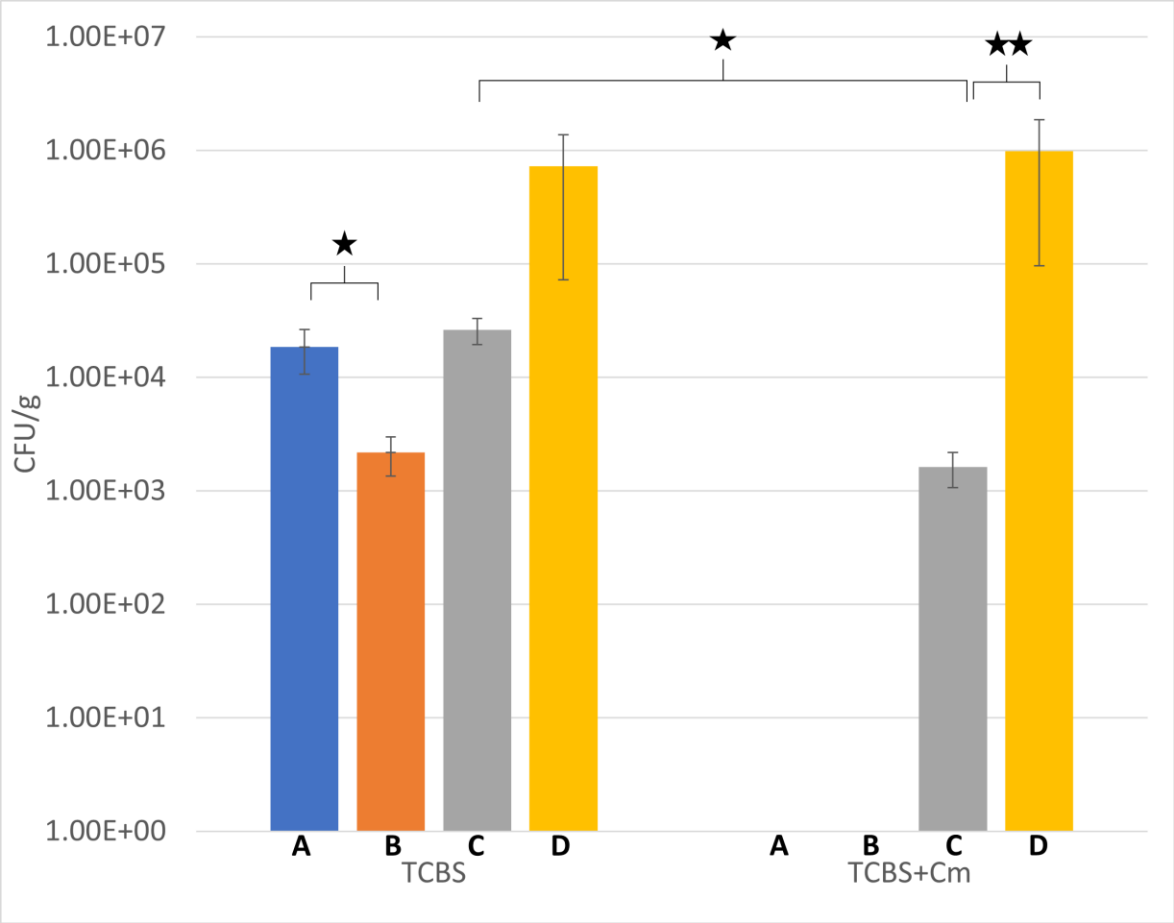

Figure 5.2. *Vibrio* levels in whole oyster homogenates (Winter 2021). Jar treatments included untreated oysters (blue, group A), oysters exposed to Cm10 (orange, group B), oysters exposed to VP RIMDmC (gray, group C), and oysters exposed to both VP RIMDmC and Cm10 (yellow, group D). Whole oyster homogenates were enumerated on TCBS without Cm10 and TCBS with Cm10 as indicated. Two stars represent $p < 0.01$ and one star represents $p < 0.05$ calculated via Wilcoxon signed-rank test. Error is represented by the mean weight-normalized CFU/g values for six total animals per treatment group ± SEM.

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Chapter Six

Seasonal impacts on the Eastern oyster microbiome

Hines, I.S., Markov Madanick, J., Smith, S.A., Kuhn, D.D., Stevens*, A.M. Seasonal impacts on the Eastern oyster microbiome**.** IN PREP.

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Attributions

Ian S. Hines and Justin Markov Madanick wrote the manuscript and contributed to the data analysis, study design, performed the experiments, and reviewed the manuscript. Ann M. Stevens wrote the manuscript and contributed to the data analysis, study design, and reviewed the manuscript. David D. Kuhn wrote the manuscript and contributed to the study design and reviewed the manuscript. Stephen A. Smith contributed to the study design and reviewed the manuscript.

Abstract

Shellfish such as the Eastern oyster (*Crassostrea virginica*) are an important agricultural commodity. Previous research has demonstrated the importance of the native microbiome of oysters against exogenous challenge by a non-native pathogen. However, the taxonomic makeup of the native oyster microbiome and factors affecting it, including seasonal time of harvest, are understudied. Research was conducted quarterly over a calendar year (winter 2020 through winter 2021) to analyze seasonal effects on the taxonomic diversity of the Eastern oyster microbiome. It was hypothesized that a core group of bacterial species would be present in the microbiome regardless of external factors including season, water temperature, and salinity. At each time point, 18 Chesapeake Bay watershed consumer-ready oysters were acquired from a local grocery store, genomic DNA was extracted from the homogenized whole oyster tissues, and the bacterial 16S rRNA gene hypervariable V4 region was PCR-amplified using barcoded primers prior to sequencing via Illumina MiSeq. Resulting sequences were analyzed for taxonomic diversity using Quantitative Insights Into Microbial Ecology (QIIME2) and R. It was found that the microbiome of the Eastern oyster had a consistent core group of permanent bacterial residents, with fluctuating phylogenetic relative abundances influenced heavily by seasonal temperature.

Introduction

The Eastern oyster, *Crassostrea virginica*, has historically been an important agricultural commodity in the Chesapeake Bay area. Increasingly, aquaculture farming of oysters has become a viable and more sustainable alternative than traditional wild-caught harvesting. This process may include genetic breeding, maintenance and/or collection of animals in a controlled fashion. Overall, the aquaculture industry for a variety of aquatic organisms has grown faster than any other sector in food production globally since the 1980s (Garlock et al., 2020). Heightened demand, decreased wild-caught supply and increased profit margins during an ongoing 16-billion-dollar seafood deficit in the United States have contributed to the increased focus on aquaculture-based methodologies (Parker and Bricker, 2020). In 2011, the National Shellfish initiative was created by the NOAA to emphasize the need for updated and streamlined aquaculture policies (Fujitani et al., 2015; Lester et al., 2018). Consequently, several states launched shellfish initiatives which aim to allocate resources and funding towards special planning, permitting and research ((NOAA), n.d.; Knapp and Rubino, 2016). In the Chesapeake Bay area over 6000 acres of brackish water was expediently permitted in Virginia and Maryland to cultivate oysters (Fujitani et al., 2015). Considering the aquaculture industry as a whole is projected to grow from $1 billion U.S. dollars (USD) to $3 billion USD by 2025, the current value of the United States shellfish industry, $323 million dollars, should follow a similar profitable trajectory (Olin et al., 2011).

To support expansion of the oyster aquaculture industry, farmers are approaching the matter holistically by often relying on innovative new techniques based on updated scientific data. The water column in which oysters reside is a microorganism-rich environment which

inevitably impacts both the oyster population, as well as the safety of the output product, as oysters are most consumed in their raw form. Modifying the microbial composition of the water in which oysters are farmed via either antibiotic or probiotic (i.e., live microorganisms) treatment can help farmers prevent disease outbreak in oyster populations (Dittmann et al., 2017). However, antibiotic treatments have been banned in the United States and are diminishing in use in the industry as a whole. Therefore, investigations into the efficacy of probiotic and prebiotic treatments (i.e., compounds used to support beneficial microbial metabolism and growth) have become more common. For example, the probiotic organisms *Phaeobacter* sp. S4 or *Bacillus pumilus* RI06-95 have been shown to decrease oyster death when probiotic-treated animals were challenged with the oyster pathogens *Vibrio tubiashii* or *Roseovarius crassostreae,* indicating the role of a probiotic-supplemented microbiome in host-pathogen defense (Karim et al., 2013; Modak and Gomez-Chiarri, 2020). Likewise, key microbiome constituents of the Pacific oyster (*Crassostrea gigas*), *Winogradskyella* sp. and *Bradyrhizobiaceae* sp. confer high resistance to disease caused by the OsHV-1 µvar virus, which is responsible for high mortality rates in oysters (King et al., 2019). On the other hand, *Photobacterium* sp., *Vibrio* sp., *Aliivibrio* sp., *Streptococcus* sp., and *Roseovarius* sp. were associated with greater susceptibility (King et al., 2019). Atrazine, a popular herbicide utilized around the Chesapeake Bay watershed has also been found to alter the microbiome of the Eastern oyster, leading to increased colonization by the oyster pathogen *Nocardia* sp. (Britt et al., 2020). Oysters are the main vector of *Vibrio parahaemolyticus* (Hubert and Michell, 2020), the number one source of seafood-borne gastroenteritis in the world. Due to the inherent significance of the oyster microbiome in protecting the animals from pathogen challenge, it is

important to better define the endogenous microbiome of *Crassostrea virginica* to establish a rationale for improvements to probiotic supplementation as a disease prevention strategy.

An increasing amount of research is being conducted on the microbiomes of aquaculture-raised animals; better understanding the effectors of the existing microbial constituents associated with animal hosts is of paramount importance to the fisheries industry. Characterization of aquaculture-raised animal microbiomes over longitudinal studies can also provide critical information describing the conditions most suitable for human pathogens (Bentzon-Tilia et al., 2016). Some literature has indicated the ambient water column temperature is a key factor driving the fluctuations of the core microbiome of Eastern oysters (Pierce et al., 2016). For example, temperature-dependent bacterial carbon source-substrate utilization may lead to fluctuations in the microbial populations (Schultz and Ducklow, 2000). Elevated temperatures, in addition to elevated carbon dioxide concentrations, have also been shown to increase microbial diversity and richness in oysters (Scanes et al., 2021). Temperature driven stress has even been shown to alter surviving oyster host microbiome to a greater extent than infection by pathogens (Lokmer and Mathias Wegner, 2015).

Therefore, this study sought to identify the microbial constituents of the Eastern oyster that persist in the animals independent of seasonal temperature. Firmicutes and Spirochaetota were highlighted as core phyla present for the duration of the year. Alternatively, the presence of other members of the microbiome was dependent on seasonal temperature; Cyanobacterota and Campliobacterota were more dominant during the warmer and colder months, respectively. Diversity analyses indicated greater bacterial community diversity during the summer, and more similar composition during the colder months. The results presented

here will help define key members of the core microbiome of Eastern oysters and guide strategies for improved oyster host-microbe interactions.

Methods

Oyster samples

Eighteen consumer-ready oysters, originally harvested from the Chesapeake Bay, were purchased from a local grocery store on five different dates spread out quarterly over a calendar year (winter 2020 to winter 2021; 90 animals total). Specific oyster source locations were obtained from the company shipment labels. Using these locations, environmental conditions, including temperature and salinity, during the time of oyster collection were recorded (Table D.1) using the NOAA buoy database (NOAA).

Genomic DNA extraction

For each timepoint, 18 oysters were shucked and an OMNI homogenizer (Omni International, Kennesaw, GA) was used to homogenize each whole oyster using ethanol sanitized equipment. Genomic DNA was isolated from tissue homogenates using the Qiagen PowerSoil kit (Qiagen, Germantown, MD) per the manufacturer's protocol with alterations including a 10-min incubation at 72°C after addition of C1 buffer and a 5-min incubation at 72°C prior to elution in 50 µL dH_2O. Tissue homogenates were added to the PowerBead (Qiagen) tubes using a range of weights between 10 and 60 mg. Prior to storage at -20°C, the quantity and purity (i.e., A_{260} /A_{280} and A_{260} /A_{280}) of the gDNA samples were analyzed via a nanospectrophotometer (Implen, Westlake Village, CA).

16S rRNA gene PCR amplification and purification

The purified gDNA obtained from oyster homogenized tissues was used as the template for PCR-amplification of the V4 region of the bacterial 16S rRNA gene. PCR conditions for each reaction were as follows: 24.5 µL of 1X Q5 Master Mix (New England Labs, Ipswich, MA) containing 500 nM each of forward universal barcoded primer (515f; AATGATACGGCGACCACCGAGATCTACACGCTxxxxxxxxxxxxTATGGTAATTGTGTGYCAGCMGCCGCG GTAA, with the x region representing the golay barcode) and reverse primer (806r; CAAGCAGAAGACGGCATACGAGATAGTCAGCCAGCCGGACTACNVGGGTWTCTAAT), 30 ng of gDNA template and a variable amount of dH_2O to bring the total volume up to 25 µL. PCR amplification of each sample was performed in triplicate along one negative water control per reaction. The universal barcoded forward primers were created according to Caporaso et al. (Caporaso et al., 2011).

Purification of PCR products

The triplicate PCR-amplified samples were pooled together prior to visualization on a 1% agarose gel to confirm product size. The PCR product was subsequently purified using the Qiagen PCR purification kit with the following alterations: 50 µl of dH_2O was used for the final elution and a 5-minute incubation at 72°C prior to final elution. Following PCR purification, amplicons were analyzed for yield and purity using Qubit fluorometry.

Bioinformatics

Purified V4 amplicons were then processed via Solid Phase Reversible Immobilization (SPRI) beads to remove unwanted lower molecular weight bands. SPRI bead-purified amplicons

were further processed using the Pippin Prep (Sage Science, Beverly, MA) to filter out contaminating higher molecular weight bands associated with host 18S rDNA while purifying the V4 bands of interest. Amplicons were then sequenced on the Illumina MiSeq platform using 500 cycles of 250 bp paired-end sequencing at a concentration of 8.5 pM. Phix was spiked in at 25% to analyze run efficiency. A total of 9,883,610 sequences were generated from MiSeq and analyzed for microbial diversity and identification using Quantitative Insights Into Microbial Ecology (QIIME2 v. 2020.2; (Bolyen et al., 2019)). Sequences were denoised using the Dada2 program (Callahan et al., 2016) to generate amplicon sequence variants (ASVs). ASVs were further filtered to remove eukaryotic-associated sequences. This filtering step resulted in a total of 7,050,516 sequences and 1,659 unique ASVs. Filtered ASVs were used to create taxonomic barplots by collapsing them to their shared Phylum and Family levels. Individual ASVs were also used for diversity analyses within the R statistics program (R Core Team, 2019) using the following packages: qiime2R v.0.99.6 (Bisanz, 2018), phyloseq v.1.27.6 (McMurdie and Holmes, 2013), vegan v.2.5-7 (Oksanen et al., 2019), ggplot2 v.3.3.5 (Wickham, 2016), complexheatmap v.2.9.1 (Gu et al., 2016). Phylum-level plots used only the 100 most abundant ASVs for visualization which represented at least 95% of the total microbiome for each season.

Statistics

The overall microbial diversities within each season (i.e. alpha diversities as analyzed by Shannon, evenness, and observed ASVs) were calculated via QIIME2. Differences between alpha diversities were analyzed for statistical significance using the non-parametric Kruskal-Wallis test with Dunn post-hoc test to identify pairwise significance. The similarities between seasonal microbiomes (i.e. beta diversity) were analyzed using weighted and unweighted

UniFrac metrics. Beta diversities were further analyzed for statistical significance using the permutational multivariate analysis of variance (PERMANOVA) followed by pairwise comparisons for the five seasons within the vegan package of R.

Results

Taxonomic identification

Several shifts in the microbiome occurred with respect to season. For example, bacteria associated with the phyla Campilobacterota and Proteobacteria have higher relative abundances during the colder winter months (Figure 6.1) with greatly reduced levels during the warmer spring and summer months. The most dominant family of Campilobacterota, *Arcobacteraceae* (Figure 6.2), is represented by *Pseudarcobacter* sp. This organism is absent from the top 50 taxa of the spring and summer animals, but it is present at ~6% and 15% relative abundance within the winter 2020 and 2021 animals, respectively (Table D.2). Conversely, Cyanobacteria, represented by *Cyanobium* sp. within the *Cyanobiaceae* family (Table D.2, Figure 6.2), are at the highest relative abundance during the warmer months and virtually absent during the colder months.

Apart from the shifts in microbiome constituents correlated with the collection season, several taxa remain present throughout the year. For example, the phyla Firmicutes and Spirochaetota (Figure 6.1), specifically the families *Mycoplasmataceae* and *Spirochaetaceae* (Figure 6.2), appear to consistently be components of the oyster microbiome throughout the year. *Mycoplasma* sp. (within the *Mycoplasmataceae* family) specifically constitute no less than 10% of the total oyster microbiome throughout the year (Table D.2). Similar to *Mycoplasma* sp., an unidentified organism within the *Spirochaetaceae* family, on average constitutes ~18% of

the total microbiome throughout the year (Table D.2). However, this organism constitutes just under 10% of the total microbiome during the spring and summer seasons. Other organisms present throughout the year with an average relative abundance of less than 3% (Table D.2) included an unidentified organism associated with the Order Chlamydiales (~2%), an unidentified organism associated with the Class Alphaproteobacteria (~1%), *Halioglobus* sp. (~0.3%), and an unidentified organism associated with the Class Gammaproteobacteria (~0.3%).

Diversity analyses

Alpha diversities were calculated via Shannon metric (H), observed ASVs, and the evenness metric (Table 6.1). Regardless of the metric used, the overall diversities for the oysters were highest in the summer. The lowest H diversities for the oysters were identified in the colder months (i.e. fall and both winters), which are all highly similar. Though statistically similar to winter 2020 oysters, the H diversities of spring oysters were significantly higher than the winter 2021 oysters ($P < 0.05$). The highest number of observed ASVs was in the summer oysters (~219 ASVs) which was similar to spring oysters (~202 ASVs). The number of observed ASVs measured during each of the warmer seasons was significantly higher than in oysters collected during the fall and winters ($P < 0.05$). Higher values resulting from the evenness metric calculations showed that summer and winter 2020 oysters were well-represented microbiomes (i.e. not dominated by just a few taxa). Winter 2021 oysters exhibited the lowest evenness over the course of a year, moreover, the evenness was significantly lower than oysters collected during the summer ($P < 0.001$). Between the two extremes of microbial representation (evenness) identified during the summer and winter 2021, respectively, oysters collected during the fall and spring exhibited highly similar evenness measurements. While not

statistically different, the year-over-year alpha diversities calculated from the winter 2020 and winter 2021 oysters, respectively, indicated a slight decrease in each diversity metric.

Microbiomes between oysters collected at each season were compared for similarities both without and with relative abundance taken into account using the unweighted UniFrac and weighted UniFrac metrics, respectively. Though oysters harvested during spring and summer appeared to share the most taxa (Figure 6.3A), a PERMANOVA indicated the two groups of microbiomes were significantly different ($p < 0.01$). In fact, the PERMANOVA indicated the oyster microbiomes were significantly different between every season (Figure 6.3). Even the year-over-year analysis indicated statistically different microbiomes from the oysters collected during the two winter timepoints regardless of UniFrac metric.

Discussion

In addition to providing several key benefits to the Chesapeake Bay ecosystem, Eastern oysters are also an important economic resource to the coastal regions of the Chesapeake Bay (Murray and Hudson, 2013; Van Senten et al., 2019). Improving sustainable aquaculture methods of culturing Eastern oysters has received government support in the USA (Parker and Bricker, 2020) and is an active area of research, including studies about the microbiome of oysters. Providing several key benefits for the host, the microbiome is considered akin to a vital organ of many animals. The structure of the microbiome has been studied in numerous invertebrate hosts such as insects (Gupta and Nair, 2020; Lanan et al., 2016), corals (Marangon et al., 2021; Rosenberg et al., 2007), crustaceans (Bouchon et al., 2016; Sison-Mangus et al., 2015), and other bivalves (Neu et al., 2021; Rubiolo et al., 2019). These studies illustrate the

functional necessity for a healthy, stable microbiome for invertebrates, including oysters as examined here.

Oysters are processed following harvest to reduce the infection potential by bacteria such as *Vibrio* spp. (Baker, 2016; Spaur et al., 2020). While the infection potential is greatly reduced from these treatments, the effect on the microbiome is not entirely understood. Importantly, the oysters used for this analysis were exposed to post-harvest methods. Therefore, the resulting microbiome data represents the bacteria most associated with consumer-ready oysters.

A group of bacteria that is present in a population of host animals regardless of external factors may constitute a core microbiome. Within oysters, a core group of bacterial resides in the microbiome at relatively consistent relative abundances (i.e.,Firmicutes and Spirochaetota; Figure 6.1). *Mycoplamataceae* and *Spirochaetaceae* are the two most consistent families present in oysters throughout the year (Figure 6.2), and the most common species within the *Mycoplasmataceae* are *Mycoplasma* spp. (Table D.2). Interestingly, *Mycoplasma* spp. are commonly associated with a host organism, including oysters, due to the lack of a cell wall (Benedetti et al., 2020; Horodesky et al., 2020). Some of them also represent a pathogenic threat to animals, including humans and fish, especially within the host mucosal layers (EI-Jakee et al., 2020; Kirchhoff et al., 1987; Razin and Jacobs, 1992). However, the oysters harvested for this study exhibited no obvious signs of clinical distress. Oyster-associated *Mycoplasma* may instead exhibit a commensal-like lifestyle. In fact, some *Mycoplasma* species may represent a potential benefit for their hosts (Bozzi et al., 2021; Cheaib et al., 2021).

Bacteria within the family *Spirochaetaceae*, have also been identified in this and other oyster microbiomes as members of a potential core microbiome family (King et al., 2020; Lasa et al., 2019; Offret et al., 2020). The Spirochaete *Cristispira* is commonly associated with mollusks, including the Eastern oyster, where it is present on the crystalline style of oysters used for feeding and may represent a core genus (Margulis et al., 1991; Tall and Nauman, 1981; Tulupova et al., 2012). If *Cristispira* is indeed the dominant unidentified *Spirochaetaceae* genus observed in this analysis, then it may represent a commensal of the Eastern oyster.

Although some core bacterial families appear being capable of maintaining their presence in the oyster microbiome independent of seasonal changes, there are also other organisms that display clear seasonal relative abundance fluctuations. For example, there is an increased relative abundance of Cyanobacteria, represented by *Cyanobiaceae* (Figure 6.2), in warm months that is virtually absent in the colder months, and, conversely, an increased relative abundance of Campilobacterota, represented by *Arcobacteraceae* (Figure 6.2) in colder months that is much lower during the warmer months (Figure 6.1). The dominant *Cyanobiaceae* organism identified here, *Cyanobium* sp., is capable of producing cyanotoxins and can be associated with cyanobacterial blooms (Das and Ranjan Dash, 2018; Li et al., 2020; Rogers et al., 2021). These blooms typically occur during warmer months and can present a hazard to both marine and human life. For example, higher levels of *Cyanobium* sp. within the water column may influence higher incidence of potentially pathogenic *Vibrio* sp. (Qiao et al., 2020).

The microbiomes of summer oysters had more unique taxa than the microbiomes form other seasons (Figure 6.3) and the overall diversity increased significantly during the summer (Table 6.1). Specifically, the number of ASVs that are observed in the summer microbiome

increased in comparison to the other seasons. This indicates new taxa, not present at the other seasons, have been incorporated into the microbiome. The presence of high levels of Cyanobacteria within the microbiome may be an important contributing factor driving the diversity increase. Importantly, the unique summer taxa are not dominating the microbiome, as the evenness does indicate an even representation by all taxa. This could indicate an increase in the total bacterial numbers during the summer, though this would need to be further quantitated.

The dominant *Arcobacteriaceae* taxon identified in this study, *Pseudarcobacter* (Table D.2), was recently reclassified from the genus *Arcobacter* (Pérez-Cataluña et al., 2018). Though little is known about the *Pseudarcobacter* genus, the *Arcobacter* genus has been identified in other shellfish including as a common commensal of the oyster microbiota (Collado et al., 2009; Lasa et al., 2019; Romero et al., 2002). *Arcobacter* also includes several species known to be pathogenic to humans (Ferreira et al., 2015; Mudadu et al., 2021; Petton et al., 2021). Curiously, the relative abundance of *Arcobacteriaceae* in the oyster microbiome was higher in the winter than the summer months (Figure 6.2), similar to other seasonal patterns of specific *Arcobacteriaceae* species (Fisher,2014). Without knowing the absolute density and species identity of *Arcobacteriaceae* within the oysters across seasons, it is difficult to determine if these taxa might represent a possible pathogenic threat to their hosts and other susceptible animals. However, the data from this study suggest that bacteria within the *Arcobacteriaceae* family are commensal members of the oyster microbiome during colder weather (i.e. winter and fall).

The year over year trend (winter 2020 compared to winter 2021) indicated a decreasing trend in the overall diversity (Table 6.1), however, the taxa present in the oysters from winter 2020 to winter 2021 appeared more similar than to other seasons (Figure 6.3). The lower overall diversity may be correlated to the lower temperature in the Chesapeake Bay during the winter 2021 harvest. This phenomenon has been observed in other bivalves showing a decrease in microbial diversity during periods of decreased temperatures (Pierce and Ward, 2019). Importantly, the similarities between the two harvests, winter 2020 and winter 2021, indicates a specialized set of bacteria that are resilient to yearly conditions.

Conclusion

Overall, the oyster microbiomes were comprised of many bacteria that exhibited fluctuating levels related to harvest season, as indicated by their variable relative abundances. However, several organisms remained at a consistently high relative abundance regardless of the harvest season and therefore may be considered members of the core microbiome. This study provides important insights into the microbiome of consumer-ready Eastern oysters in relation to the season in which the animals were harvested.

Table 6.1. Seasonal correlation to oyster microbiome alpha diversities.

Season	Shannon (H)	Evenness	Observed ASVs
Winter (2020)	4.24 ± 0.52^{ab}	0.61 ± 0.06^{ab}	137 ± 51^{a}
Spring	4.36 ± 1.01^{a}	0.57 ± 0.11^{a}	202 ± 57^{b}
Summer	5.30 ± 0.64^{c}	0.68 ± 0.06^{b}	219 ± 48^{b}
Fall	3.92 ± 1.06^{ab}	0.56 ± 0.12^{a}	136 ± 57^{a}
Winter (2021)	3.46 ± 0.56^{b}	0.52 ± 0.07^{a}	108 ± 29^{a}

^{abc} Different superscript letters represent $P < 0.05$ between seasons within a given diversity metric following one-way Kruskal Wallis with Dunn post-hoc test

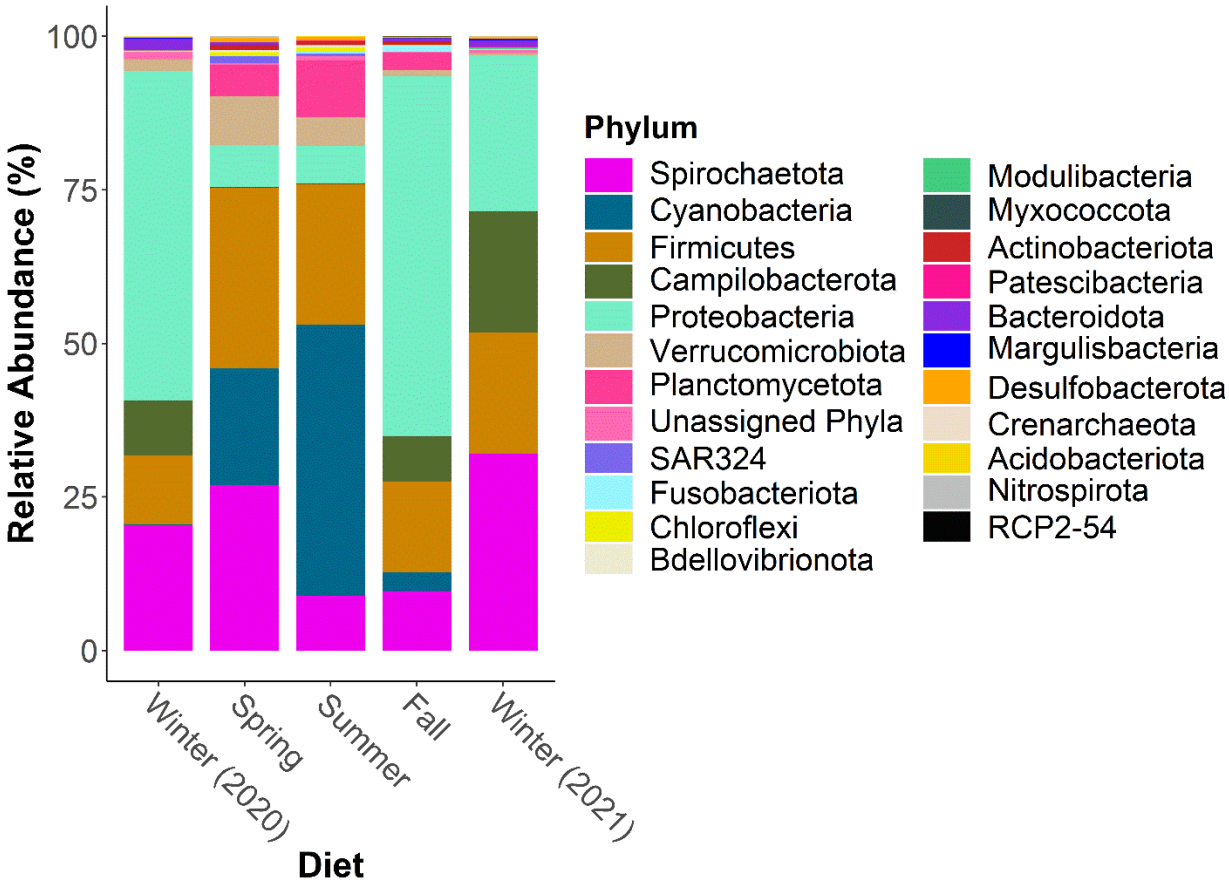

Figure 6.1. Seasonal effects on the phylum-level relative abundances within the Eastern oyster microbiome. Eastern oysters were collected during each season of the year (n=18 oysters per season) including a second harvest one year following the first harvest. The 16S rRNA gene V4 region was sequenced from the oyster homogenates wherein the microbiomes were examined. The top phyla comprising at least 90% of the total microbiome for all treatment groups are represented in the plot.

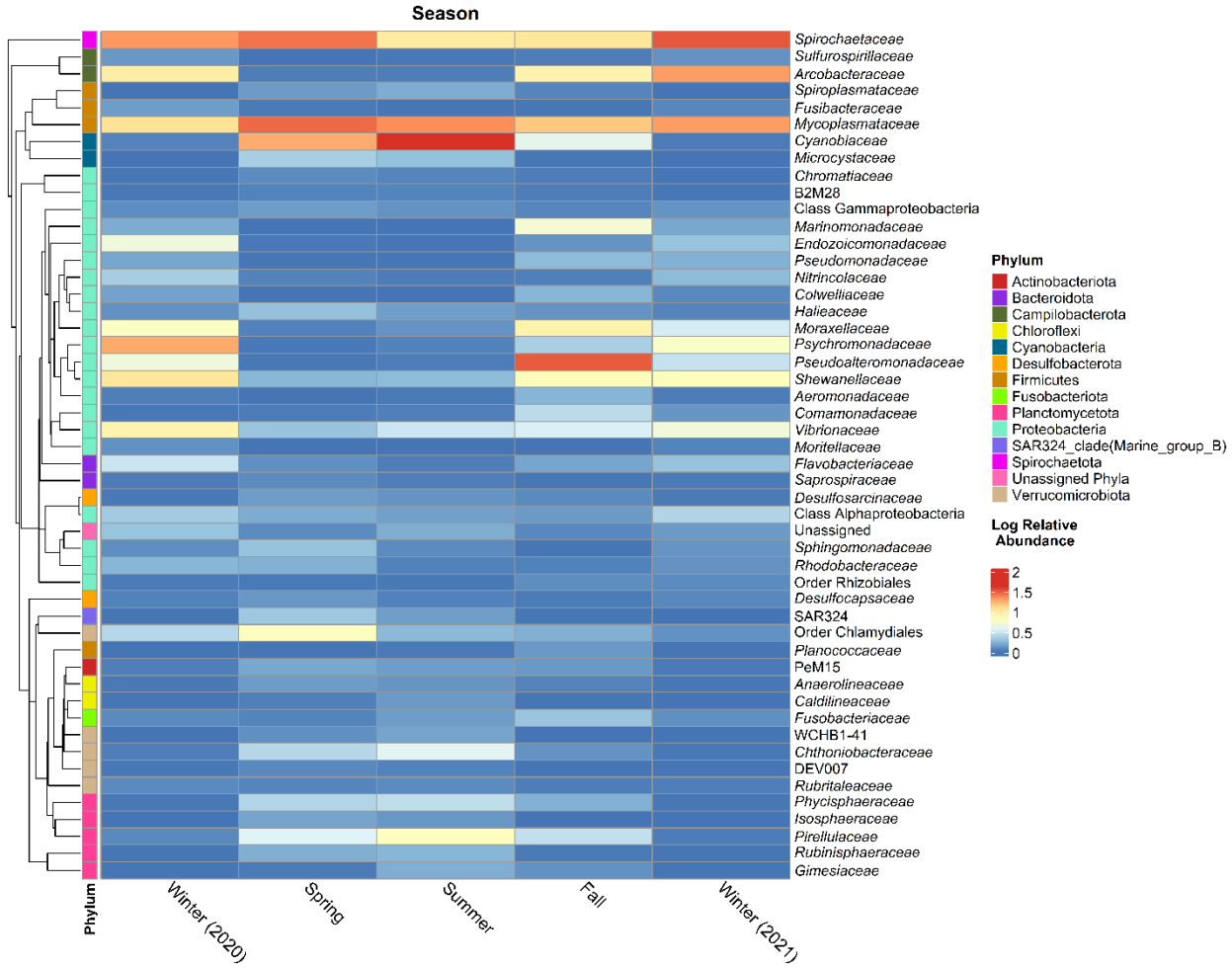

Figure 6.2. Family-level relative abundances of seasonally-correlated microbiomes within the Eastern oyster microbiome. Oyster-isolated 16S rRNA V4 region amplicons were sequenced via Illumina MiSeq. The log relative abundance of family-level taxa. The phylogenetic tree was constructed via QIIME2 to denote the phylogeny and phylum to which each family-level taxon belongs.

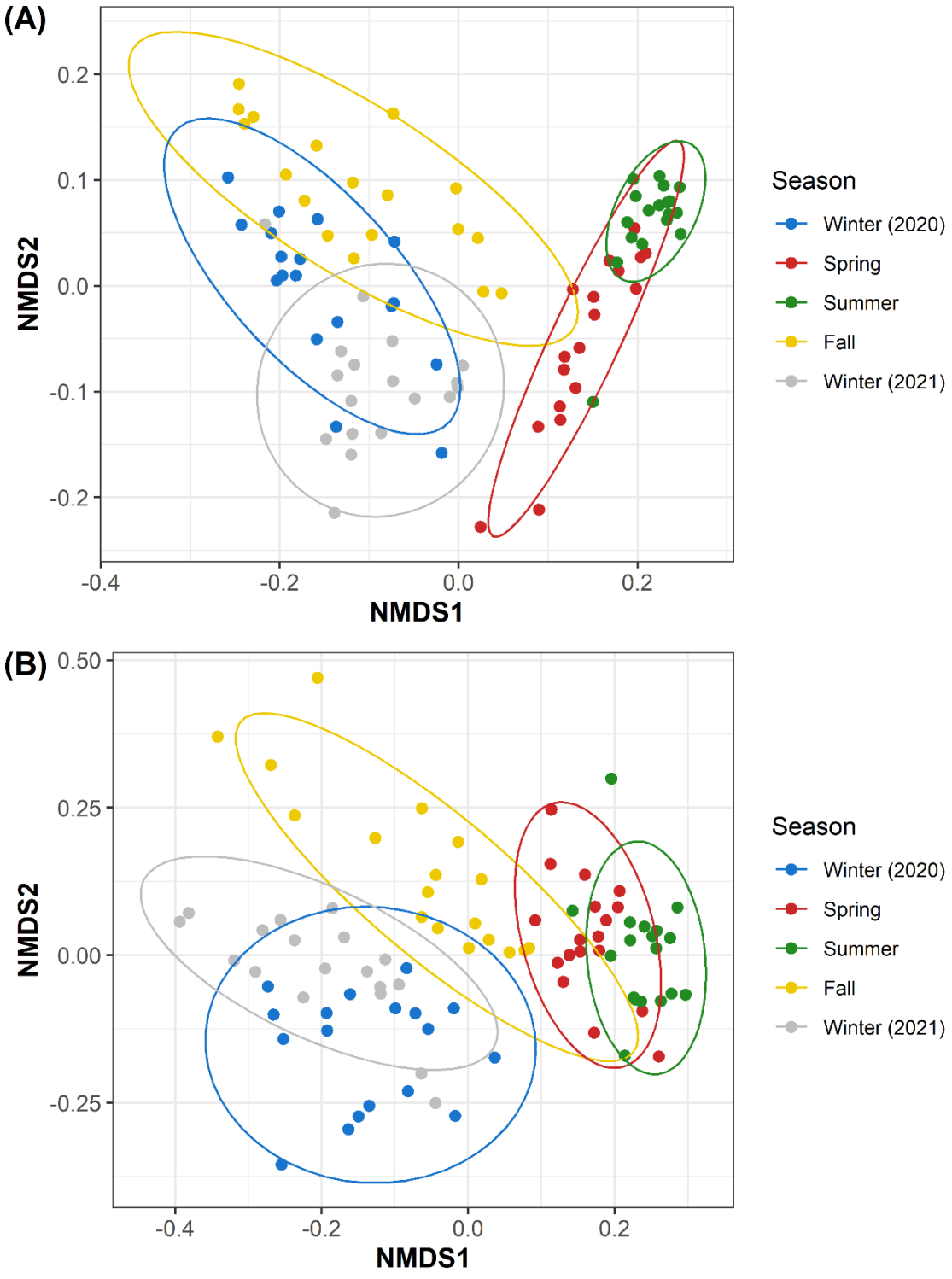

Figure 6.3. Seasonally-correlated Eastern oyster microbiome beta diversities. Non-metric multidimensional scaling was used to visualize the dissimilarities between seasonal

microbiomes of the Eastern oyster. Dissimilarities were calculated via (A) unweighted UniFrac and (B) weighted UniFrac beta diversity metrics.

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Chapter Seven

Concluding remarks

The host-microbiome relationship is vital for the well-being of the host. The microbiome of host animals is impacted by various external factors such as exposure to antibiotics, exposure to pathogens, rearing temperature, and diet. Of these factors, diet is of high importance to the aquaculture industry as it can directly impact the fish growth rates and therefore the profits of fisheries. Chapter Two examined the role of replacing a key component of fish feeds, fishmeal. Fishmeal is primarily produced from wild-caught fish, an overexploited natural resource globally. Development of alternatives to fishmeal is critical to the sustainability of this industry. The work described in Chapter Two contributed to this area of research by establishing that inclusion of higher percentages of the yeast product in the diet did have a negative impact on fish growth, however, lower percentages of yeast aided fish growth and did not dramatically alter the microbiome (i.e., result in microbiome dysbiosis). Thus, this study validated the efficacy of a lyzed yeast replacement for fishmeal in aquaculture practices by analyzing the microbiome of rainbow trout in addition to fish physiological parameters.

Microbiome dysbiosis can occur as the result of physiological stress encountered by the fish hosts. Some compounds when supplemented in fish feeds, such as selenium, are beneficial to fish health during culturing by increasing the activity of antioxidant selenium-containing enzymes. Work in this area, not included as part of this dissertation (Razieh et al., 2021), examined the impact of a selenium-available compound, selenoneine, on the intestinal microbiome of rainbow trout. This study significantly correlated the use of selenium on the increased growth rate of fish while having no impact on the microbiome. Thus, application of selenium in diets is another potential improvement fisheries could make to improve production levels.

Historically, antibiotics were used across agriculture to improve animal growth. However, antibiotics can a have a profound effect on the microbiome, leading to dysbiosis and thereby negatively impact animal physiology. Probiotics and prebiotics have emerged as potential alternatives to prophylactic antibiotic use in the field of aquaculture; some are known to inhibit proliferation of pathogenic bacteria. Inclusion of a probiotic strain of *Bacillus subtilis* in the diet of steelhead trout resulted in an increased weight gain, though only if fed during the early stages of intestinal development (Chapter Four). Interestingly, continually feeding the probiotic led to a reduction in the total intestinal (mucosa + digesta) microbiome diversity. This suggests that early application of probiotics may contribute to long term animal health and be a more economical approach in aquaculture than using probiotics at later stages of fish growth or for prolonged periods of time. Mechanistically, the *B. subtilis* probiotic could be helping to maintain a safe environment for the developing fish during a period when they are at an increased risk of infection due to their immature immune system. However, as the microbiome further develops, continued use of the probiotic may reduce the amount of nutrients available to the other microbial constituents capable of aiding in host metabolism. The microbiome may be less diverse and thereby less capable of benefitting the host if the probiotic organism consumes a large proportion of the available nutrients. It is known that *B. subtilis* probiotics can have a high germination rate (~70%; Drahos et al., 2017) within a host intestinal tract suggesting that it quickly becomes metabolically active at high levels. This may limit nutrient availability to the existing microflora, leading to reduced overall growth rates for the fish.

Another exogenous effector of the microbiome, temperature, was analyzed in Chapter Six where the impact of harvesting season on the microbiome structure of Eastern oysters was

explored. It was found that consumer-ready oysters harvested during the summer harbored the most diverse microbiomes whereas oysters harvested during the winter harbored the least diverse microbiomes; this demonstrates a strong correlation between harvest temperature and microbiome diversity. Interestingly, the beta diversities appeared to indicate a gradual shift from the coldest to the warmest timepoints and vice versa. Dominant organisms at these timepoints (i.e., *Pseudoarcobacter* sp. in the winter and *Cyanobium* sp. in the summer) may be directly affected by the environmental temperatures or other seasonal environmental variables. For instance, since *Cyanobium* sp. is associated with algal blooms, there may be a correlation between the amount of light exposure in the oysters' water column and the levels of *Cyanobium* sp. Further studies should focus on this vastly understudied relationship to determine if light exposure levels, independent of temperature, are also driving microbiome shifts within oysters and the water column as a whole.

Persistence of some microorganisms (e.g., *Mycoplasmataceae* and *Spirochaetaceae*) throughout the year as observed in the microbiomes of oysters (Chapter Six) indicates some degree of specialization with regard to their relationship with oysters. Interestingly, *Mycoplasma* spp. continue to be identified in many aquatic animal host microbiome studies including some reported in this dissertation. This group of organisms was most commonly found in the mucosa of fish intestines, therefore it is likely also closely associated with the mucosa of oysters as well. However, its role as a component of the microbiome is not yet fully understood. *Mycoplasma* spp. require a host to survive and proliferate often by sequestering molecules such as cholesterol from the host. However, there is, as of yet, no obvious correlation between differences in growth of the oyster host and the relative levels of

Mycoplasma spp. in the microbiomes. Instead, *Mycoplasma* spp. may act as a native commensal organism in their host animals, possibly providing some benefits when closely associated with the host epithelium such as the production of antimicrobials or host-available nutrients. Regardless, the mechanism by which this organism associates with its aquatic host needs to be further elucidated. The fact that the native microbiome also affords some protection against exogenous pathogen challenge was clearly demonstrated by the work in Chapter Five. Therefore, future work aimed at elucidating the core microbiome and its functionality within oysters should be a primary goal for this field. This is especially important as increasing global temperatures are leading to higher levels of pathogenic bacteria, including vibrios, in coastal waters.

Intrinsic host factors such as age and genetics can also impact the structure of the microbiome. In addition to studying the dietary use of probiotics, the impact of fish age on the microbial communities within the intestinal tract was investigated through the studies described in Chapter Four. It was found that the microbial diversity quickly increased from the egg stage to the feeding stage of fish. Following this point, the diversity gradually decreased as the microbial constituents best suited to the environment in the host intestinal tract environment took up permanent residence and became more dominant in the microbial community, while less suited microbial taxa became less prevalent. As the microbial biomass during extraction increased as the fish grew, this might have created additional bias against the rarer taxa. These are important caveats to consider when analyzing long-term microbiome studies. This steelhead trout study also provided evidence for a host age-influenced microbiome; an understudied topic in the aquaculture literature. Appendix E directly

investigated the impact of fish genetics on the microbiome, utilizing proprietary Nile tilapia that were differentiated by a single genetic trait. This groundbreaking study indicated a dramatic change to the dominant families within the intestinal microbiome to genetic differences in the host DNA. The difference in microbial structure was also correlated to a significantly increased rate of growth of one line of fish. It's possible the genetic alteration leading to the higher growth rates is also responsible for the retention of specific bacteria, such as the *Mycoplasma* spp. and *Fusobacteriaceae* identified in Appendix E. The altered physiology of the fish may even be selecting for microorganisms responsible for the increased fish growth rates. These findings are an important step toward trying to establish some direct links between host genetics and the microbiome, and they also suggest that further development of genetically modified fish may have benefits for animal health and production in the future. However, there are also going to be continuing challenges to further improving aquaculture practices.

Emerging pathogens, such as *Vibrio parahaemolyticus* (VP), are going to be one of these major challenges. VP is the number one causative agent of seafood-borne acute gastroenteritis worldwide, and the most common route of infection is through the ingestion of raw or undercooked shellfish (e.g., oysters). Though mitigation efforts have been developed, cases of VP infections continue to increase. Therefore, an oyster inoculation system was developed (Chapter Five) to test an understudied aspect of the disease cycle, the relationship between VP and their Eastern oyster reservoir hosts. The season in which the oysters were harvested and whether or not the existing microbiome had been exposed to an antibiotic were parameters that influenced VP inoculation levels. Important virulence factors such as the type III secretion systems (T3SS1 and T3SS2) and thermostabile direct hemolysin (TDH) were tested for their role

in oyster inoculation (Appendix F). Thus, this oyster inoculation system can be used in the future to examine the impact of a variety of different factors that might impact the host-microbe relationship (e.g., extended depurations, salinity levels, probiotic applications etc.) and suggest changes to aquaculture practices.

Ongoing work within the oyster system is being conducted to determine the localization pattern of VP within oysters. Because VP is capable of invading mammalian cells, it was hypothesized that it may be capable of invading oyster cells as well. Therefore, VP inoculated and uninoculated oyster tissues were fixed in formalin for immunohistochemistry analysis using antibodies that specifically recognize VP and methods to develop primary cell lines of oysters were completed as additional work not included as part of this dissertation.

This dissertation outlines various aquatic host-microbe relationships to broaden the knowledge of the microbial communities present within the host organisms and how they're affected by varying conditions. In order to fully understand their roles within the microbiomes and their impacts on host health, further studies focused on the mechanisms by which these microorganisms interact with their hosts will need to be conducted. By identifying the molecular basis of these mechanisms, targeted treatments to promote the growth of beneficial bacteria and inhibit potential pathogens can be developed. Overall, the work described in this dissertation has provided important findings that will permit for evidence-based improvements to the production of animals through aquaculture. Improving animal health and growth conditions will enable greater economic returns for the producer. Additionally, the work described here will help develop safer and more nutritious foods for human consumption.

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Appendix A

Chapter Two supplemental material

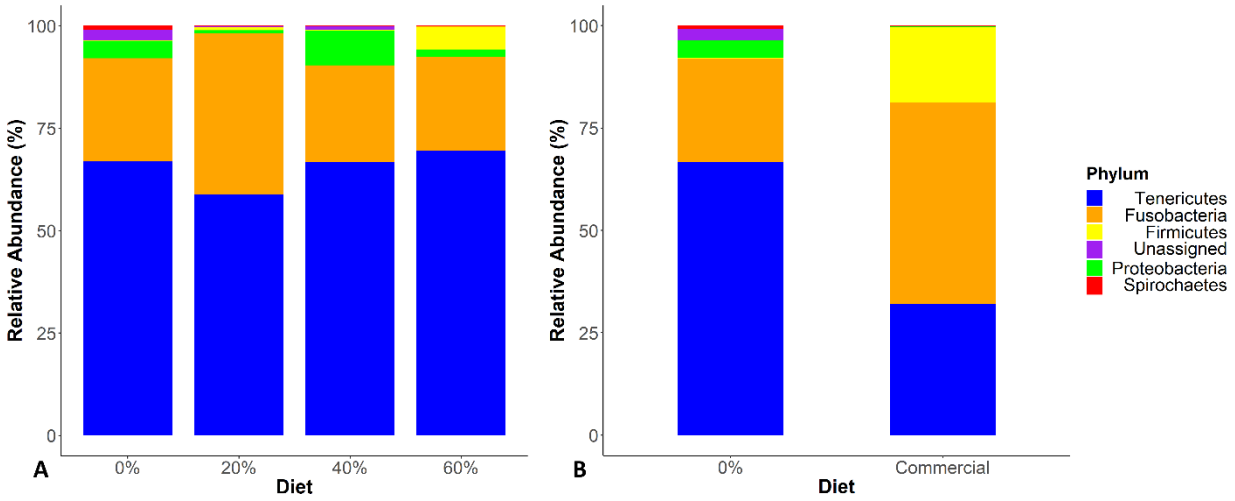

Figure A.1. Trout intestinal epithelial-associated phyla-level microbiome. The average reads generated from sequencing the 16S rRNA genes for each treatment group of four fish are plotted to illustrate the relative diversity of the most abundant bacterial phyla for the (A) 0% to 60% yeast supplement diets and (B) the 0% supplement and commercial diet groups. Diet treatment groups include: 0% supplement (0%), 20% supplement (20%), 40% supplement (40%), 60% supplement (60%), commercial diet (Commercial).

Appendix B

Chapter Three supplemental material

Table B.1. Polymerase and template gDNA variable concentration based upon tissue type.

Timepoint	Tissue type	gDNA amount (ng)	Polymerase
T-1	Eggs and water column	325	Quantabio 5 Prime HotStart
T0	Headless/gill-less	120	NEB Q5
T1	Headless/gill-less	120	NEB Q5
T2	Intestinal segment	30	NEB Q5
T3	Intestinal segment	30	NEB Q5
T0-T3	Water column	30	NEB Q5

Table B.2. Breakdown of the number of sequenced samples.

Timepoints	n (per treatment group)
T-1	6[1]
T0	10
T1	10
T2	8
T3 (homogenate)	12
T3 (swabs)	10[2]

[1] One sample removed from the non-disinfected egg group due to a low read count following filtering (n=5)

[2] One sample removed from diet C group and one from diet A group (for diversity analyses only) due to a low read count following filtering (n=9)

Table B.3. ASV relative abundance table.

	Relative abundance (%)[1]																
Timepoint	**T-1**		**T0**	**T1**		**T2**				**T3**							
Tissue type	**Egg**		**Whole Fish**	**Whole Fish**		**Intestinal Homogenates**				**Intestinal Homogenates**				**Intestinal Swabs**			
Diet/treatment	**Disinfected**	**Non-disinfected**	**N/A**	**A**	**B**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
Organism name																	
***Bacillus* sp.**	0.100 ± 0.010	0.044 ± 0.009	0.293 ± 0.010	0.517 ± 0.010	17.996 ± 0.385	1.143 ± 0.061	41.263 ± 0.955	45.77 ± 1.588	0.943 ± 0.039	10.822 ± 0.657	74.632 ± 0.281	71.671 ± 0.217	3.384 ± 0.080	44.330 ± 1.199	56.468 ± 0.964	60.180 ± 1.083	70.284 ± 1.272
***Methylotenera* sp.**	41.528 ± 0.875	59.549 ± 1.548	N/A	N/A	N/A	0.265 ± 0.007	5.209 ± 0.193	2.363 ± 0.090	6.614 ± 0.242	0.906 ± 0.013	0.359 ± 0.004	0.514 ± 0.012	0.537 ± 0.009	0.327 ± 0.009	0.101 ± 0.002	0.105 ± 0.003	N/A
***Acinetobacter* sp.**	0.466 ± 0.052	0.054 ± 0.007	35.435 ± 0.437	9.809 ± 0.175	4.680 ± 0.104	1.733 ± 0.084	2.303 ± 0.085	1.090 ± 0.047	2.644 ± 0.100	5.366 ± 0.126	1.849 ± 0.061	1.132 ± 0.018	3.343 ± 0.043	3.492 ± 0.172	2.589 ± 0.122	0.487 ± 0.023	2.317 ± 0.165
***Bacteroides* sp.**	N/A	N/A	N/A	8.377 ± 0.085	6.029 ± 0.065	17.763 ± 0.260	6.042 ± 0.108	3.277 ± 0.067	15.521 ± 0.250	1.193 ± 0.009	0.440 ± 0.004	0.817 ± 0.011	1.893 ± 0.017	N/A	N/A	N/A	N/A
***Aeromonas* sp.**	N/A	0.386 ± 0.076	N/A	8.769 ± 0.109	3.350 ± 0.064	1.716 ± 0.084	3.031 ± 0.303	3.378 ± 0.363	3.134 ± 0.151	0.959 ± 0.034	0.376 ± 0.012	1.070 ± 0.064	2.984 ± 0.177	5.975 ± 0.517	6.973 ± 0.506	10.532 ± 0.620	1.016 ± 0.073
***Undibacterium* sp.**	20.185 ± 0.913	19.212 ± 0.548	N/A	0.263 ± 0.008	N/A	N/A	2.482 ± 0.275	1.265 ± 0.141	2.800 ± 0.305	0.859 ± 0.033	0.127 ± 0.005	0.240 ± 0.018	N/A	N/A	N/A	N/A	0.272 ± 0.027
Enterobacteriaceae	N/A	0.022 ± 0.004	N/A	0.761 ± 0.017	0.495 ± 0.008	0.298 ± 0.023	0.505 ± 0.042	N/A	1.295 ± 0.117	1.184 ± 0.025	1.623 ± 0.086	1.633 ± 0.101	1.627 ± 0.071	5.492 ± 0.334	10.381 ± 0.471	9.995 ± 0.845	11.123 ± 0.967
***Vibrio* sp.**	1.282 ± 0.203	0.393 ± 0.074	2.609 ± 0.115	0.401 ± 0.015	0.204 ± 0.007	6.938 ± 0.579	7.586 ± 0.928	1.530 ± 0.127	3.799 ± 0.205	3.942 ± 0.213	1.273 ± 0.090	1.353 ± 0.098	1.659 ± 0.079	3.871 ± 0.233	1.022 ± 0.062	0.195 ± 0.012	3.816 ± 0.376
Yersiniaceae	N/A	N/A	N/A	2.407 ± 0.038	30.215 ± 0.955	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.175 ± 0.013	N/A	N/A

***Pseudomonas* sp.**	1.631 ± 0.067	0.732 ± 0.032	6.679 ± 0.158	8.410 ± 0.146	1.780 ± 0.041	2.299 ± 0.087	1.288 ± 0.049	0.137 ± 0.004	2.553 ± 0.088	1.429 ± 0.019	0.460 ± 0.007	0.516 ± 0.006	1.804 ± 0.029	0.987 ± 0.031	0.284 ± 0.010	0.185 ± 0.007	0.162 ± 0.006
Falsiporphyromonas endometrii	N/A	N/A	N/A	4.338 ± 0.069	3.307 ± 0.066	6.839 ± 0.200	3.165 ± 0.140	1.860 ± 0.090	6.885 ± 0.226	N/A	N/A	0.196 ± 0.009	0.621 ± 0.014	N/A	N/A	N/A	N/A
***Pantoea* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	7.407 ± 0.144	3.315 ± 0.132	3.014 ± 0.051	12.339 ± 0.312	N/A	N/A	N/A	N/A
Rhodobacteraceae	N/A	N/A	N/A	1.718 ± 0.027	1.684 ± 0.106	5.262 ± 0.381	3.213 ± 0.351	3.617 ± 0.289	3.777 ± 0.273	1.892 ± 0.042	0.219 ± 0.005	0.223 ± 0.008	0.437 ± 0.010	N/A	N/A	0.391 ± 0.039	0.350 ± 0.035
Lactobacillus amylovorus	N/A	N/A	N/A	N/A	N/A	N/A	0.593 ± 0.021	0.371 ± 0.016	N/A	7.862 ± 0.100	1.765 ± 0.025	2.448 ± 0.024	9.621 ± 0.088	N/A	N/A	N/A	0.045 ± 0.004
***Aquabacterium* sp.**	0.844 ± 0.124	N/A	7.547 ± 0.099	N/A	N/A	N/A	0.226 ± 0.019	0.146 ± 0.007	N/A	2.978 ± 0.131	1.955 ± 0.062	0.416 ± 0.007	2.777 ± 0.096	1.183 ± 0.055	2.920 ± 0.150	1.358 ± 0.121	0.259 ± 0.022
***Bosea* sp.**	N/A	N/A	N/A	N/A	0.166 ± 0.014	5.785 ± 0.373	2.067 ± 0.165	3.072 ± 0.253	4.906 ± 0.327	3.654 ± 0.150	0.148 ± 0.004	0.204 ± 0.007	0.674 ± 0.012	0.252 ± 0.024	N/A	0.089 ± 0.007	0.634 ± 0.063
***Flavobacterium* sp.**	7.498 ± 0.242	4.920 ± 0.184	0.965 ± 0.030	1.151 ± 0.017	0.556 ± 0.006	N/A	2.264 ± 0.106	0.693 ± 0.025	1.722 ± 0.051	N/A	N/A	N/A	N/A	N/A	0.129 ± 0.005	N/A	0.658 ± 0.033
Comamonadaceae	1.784 ± 0.076	2.598 ± 0.315	3.021 ± 0.075	1.681 ± 0.046	0.184 ± 0.004	N/A	0.290 ± 0.023	N/A	0.486 ± 0.024	1.304 ± 0.031	0.295 ± 0.003	0.314 ± 0.007	2.361 ± 0.065	0.640 ± 0.038	0.924 ± 0.040	0.204 ± 0.011	0.360 ± 0.027
***Peptococcus* sp.**	N/A	N/A	N/A	0.801 ± 0.016	0.485 ± 0.010	1.854 ± 0.058	0.839 ± 0.036	0.695 ± 0.024	2.184 ± 0.094	2.354 ± 0.043	0.772 ± 0.019	0.778 ± 0.010	3.126 ± 0.034	N/A	N/A	N/A	N/A
***Comamonas* sp.**	N/A	N/A	11.716 ± 0.331	N/A	N/A	N/A	N/A	N/A	N/A	0.684 ± 0.022	0.134 ± 0.005	0.205 ± 0.006	0.480 ± 0.021	0.415 ± 0.032	N/A	N/A	N/A
Class Gammaproteobacteria	N/A	0.059 ± 0.012	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1.540 ± 0.027	0.323 ± 0.007	0.457 ± 0.010	1.737 ± 0.032	3.099 ± 0.162	1.621 ± 0.064	3.209 ± 0.219	1.154 ± 0.074
***Sphingomonas* sp.**	3.771 ± 0.336	3.279 ± 0.490	1.245 ± 0.109	N/A	N/A	2.493 ± 0.223	N/A	N/A	N/A	N/A	N/A	0.162 ± 0.008	N/A	0.175 ± 0.017	0.114 ± 0.008	N/A	N/A

***Oribacterium* sp.**	N/A	N/A	N/A	1.695 ± 0.026	1.277 ± 0.029	2.549 ± 0.094	1.038 ± 0.040	0.643 ± 0.028	2.901 ± 0.099	0.392 ± 0.010	0.079 ± 0.003	N/A	0.579 ± 0.014	N/A	N/A	N/A	N/A
Simkaniaceae	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1.437 ± 0.026	0.557 ± 0.016	0.352 ± 0.005	2.283 ± 0.032	3.887 ± 0.306	0.609 ± 0.052	1.237 ± 0.107	0.422 ± 0.036
***Reyranella* sp.**	N/A	N/A	N/A	N/A	N/A	3.409 ± 0.251	0.879 ± 0.066	1.980 ± 0.157	2.226 ± 0.108	0.681 ± 0.018	0.081 ± 0.002	N/A	0.467 ± 0.016	0.460 ± 0.046	N/A	0.258 ± 0.016	N/A
***Lactobacillus* sp.**	N/A	N/A	0.563 ± 0.028	0.310 ± 0.005	0.165 ± 0.003	N/A	0.315 ± 0.008	0.236 ± 0.005	N/A	2.897 ± 0.030	0.750 ± 0.008	0.997 ± 0.010	3.459 ± 0.036	N/A	N/A	0.110 ± 0.005	N/A
***Escherichia-Shigella* sp.**	4.191 ± 0.625	0.174 ± 0.014	3.629 ± 0.204	0.250 ± 0.004	0.277 ± 0.010	0.321 ± 0.014	0.276 ± 0.013	N/A	0.345 ± 0.020	N/A	0.092 ± 0.006	N/A	N/A	N/A	N/A	N/A	N/A
***Pseudarcobacter* sp.**	3.006 ± 0.282	0.573 ± 0.060	5.053 ± 0.172	N/A	N/A	N/A	0.129 ± 0.012	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Bacteroides pyogenes	N/A	N/A	N/A	1.464 ± 0.026	1.116 ± 0.024	2.324 ± 0.086	0.672 ± 0.026	0.492 ± 0.027	2.574 ± 0.119	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Succiniclasticum* sp.**	N/A	N/A	N/A	1.216 ± 0.020	0.887 ± 0.021	2.143 ± 0.077	0.867 ± 0.041	0.497 ± 0.025	2.004 ± 0.071	N/A	N/A	0.164 ± 0.007	0.338 ± 0.011	N/A	N/A	N/A	N/A
***Candidatus Berkiella* sp.**	N/A	N/A	N/A	N/A	N/A	2.368 ± 0.149	0.901 ± 0.061	2.626 ± 0.168	1.973 ± 0.079	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Mesorhizobium* sp.**	N/A	N/A	N/A	0.391 ± 0.007	2.025 ± 0.192	1.517 ± 0.092	0.419 ± 0.033	1.475 ± 0.117	1.471 ± 0.087	N/A	N/A	N/A	N/A	N/A	N/A	0.058 ± 0.005	N/A
***Veillonella* sp.**	N/A	N/A	N/A	0.708 ± 0.007	0.612 ± 0.005	0.878 ± 0.016	0.314 ± 0.008	0.274 ± 0.006	1.015 ± 0.021	0.807 ± 0.011	0.326 ± 0.004	0.172 ± 0.002	1.272 ± 0.013	N/A	0.713 ± 0.036	N/A	N/A
Diplorickettsiaceae	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1.048 ± 0.028	0.256 ± 0.008	0.166 ± 0.004	1.228 ± 0.019	1.391 ± 0.079	0.692 ± 0.038	1.442 ± 0.098	0.599 ± 0.047
***Legionella* sp.**	N/A	N/A	0.176 ± 0.011	N/A	N/A	0.880 ± 0.051	0.356 ± 0.022	1.056 ± 0.053	0.684 ± 0.033	N/A	0.103 ± 0.003	0.184 ± 0.005	0.437 ± 0.022	0.627 ± 0.041	0.682 ± 0.035	0.536 ± 0.034	0.366 ± 0.027

***Lacihabitans* sp.**	N/A	N/A	N/A	4.873 ± 0.134	1.144 ± 0.039	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Lachnospiraceae	0.214 ± 0.012	N/A	N/A	0.745 ± 0.007	0.519 ± 0.006	1.014 ± 0.017	0.499 ± 0.009	0.362 ± 0.007	1.458 ± 0.027	0.400 ± 0.004	N/A	0.147 ± 0.002	0.653 ± 0.005	N/A	N/A	N/A	N/A
Unassigned	3.049 ± 0.249	2.495 ± 0.245	N/A	0.406 ± 0.018	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Methylobacterium-Methylorubrum* sp.**	2.512 ± 0.192	1.928 ± 0.270	0.869 ± 0.072	N/A	N/A	0.468 ± 0.052	0.134 ± 0.011	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Psychrobacter* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	5.752 ± 0.718	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Psychrobacter alimentarius	N/A	N/A	N/A	N/A	N/A	2.882 ± 0.183	1.834 ± 0.218	N/A	0.792 ± 0.063	N/A	N/A	N/A	N/A	0.205 ± 0.020	N/A	N/A	N/A
Pirellulaceae	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1.906 ± 0.031	0.281 ± 0.004	0.435 ± 0.007	1.068 ± 0.019	0.680 ± 0.015	0.480 ± 0.015	0.341 ± 0.009	0.450 ± 0.018
Order Oscillospirales	N/A	N/A	N/A	0.823 ± 0.012	0.607 ± 0.014	1.334 ± 0.052	0.638 ± 0.028	0.447 ± 0.024	1.437 ± 0.059	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Massilia* sp.**	0.158 ± 0.019	N/A	N/A	2.534 ± 0.067	1.089 ± 0.034	N/A	N/A	N/A	N/A	0.455 ± 0.012	0.107 ± 0.003	0.151 ± 0.004	0.547 ± 0.014	N/A	N/A	N/A	N/A
***Catenibacillus* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1.682 ± 0.032	0.480 ± 0.011	0.572 ± 0.008	2.141 ± 0.031	N/A	N/A	N/A	N/A
Halieaceae	N/A	N/A	N/A	1.283 ± 0.022	0.295 ± 0.004	0.456 ± 0.014	0.174 ± 0.004	0.211 ± 0.005	0.330 ± 0.010	0.973 ± 0.018	0.082 ± 0.001	0.137 ± 0.002	0.674 ± 0.008	N/A	N/A	0.060 ± 0.003	0.060 ± 0.003
Polyangiaceae	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.900 ± 0.046	N/A	0.667 ± 0.056	N/A	2.280 ± 0.207	N/A	0.736 ± 0.082	0.059 ± 0.006
Order Lactobacillales	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1.764 ± 0.040	0.576 ± 0.014	0.504 ± 0.012	1.776 ± 0.018	N/A	N/A	N/A	N/A

***Photobacterium* sp.**	N/A	N/A	N/A	1.073 ± 0.024	0.480 ± 0.013	0.973 ± 0.066	N/A	N/A	0.355 ± 0.019	N/A	0.089 ± 0.004	N/A	1.488 ± 0.094	N/A	N/A	N/A	N/A
Order Chitinophagales	N/A	N/A	N/A	3.476 ± 0.050	0.763 ± 0.014	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Enhydrobacter* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	0.149 ± 0.016	N/A	N/A	N/A	N/A	N/A	3.401 ± 0.270	0.532 ± 0.053	N/A	N/A	N/A
***Shinella* sp.**	N/A	N/A	N/A	N/A	N/A	0.374 ± 0.024	N/A	0.137 ± 0.009	0.737 ± 0.072	0.563 ± 0.042	N/A	N/A	N/A	N/A	N/A	1.817 ± 0.201	0.413 ± 0.041
***Curtobacterium* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1.779 ± 0.026	0.395 ± 0.007	0.437 ± 0.007	1.392 ± 0.022	N/A	N/A	N/A	N/A
***Shewanella* sp.**	0.067 ± 0.008	0.029 ± 0.006	0.188 ± 0.019	2.159 ± 0.043	1.300 ± 0.037	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.237 ± 0.024	N/A	N/A
***Aquicella* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.197 ± 0.003	N/A	1.268 ± 0.029	0.471 ± 0.021	0.408 ± 0.018	0.914 ± 0.057	0.415 ± 0.031
Verrucomicrobiaceae	N/A	N/A	0.299 ± 0.010	2.017 ± 0.020	1.341 ± 0.019	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Devosia* sp.**	N/A	N/A	N/A	0.253 ± 0.006	0.210 ± 0.018	1.030 ± 0.066	0.277 ± 0.025	0.692 ± 0.056	0.776 ± 0.045	N/A	N/A	N/A	N/A	0.325 ± 0.023	N/A	N/A	N/A
Moraxellaceae	N/A	N/A	N/A	2.460 ± 0.018	1.082 ± 0.013	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Sutterella* sp.**	N/A	N/A	N/A	0.412 ± 0.008	0.288 ± 0.006	1.507 ± 0.061	0.198 ± 0.010	0.210 ± 0.009	0.864 ± 0.029	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Luteolibacter* sp.**	N/A	N/A	N/A	2.125 ± 0.043	1.294 ± 0.034	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Rhodoferax* sp.**	0.271 ± 0.014	0.307 ± 0.034	N/A	1.405 ± 0.028	1.322 ± 0.026	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

***Acidovorax* sp.**	0.140 ± 0.016	0.074 ± 0.010	1.106 ± 0.036	N/A	N/A	N/A	N/A	N/A	N/A	0.398 ± 0.014	0.198 ± 0.008	N/A	0.493 ± 0.013	0.308 ± 0.015	0.337 ± 0.023	0.231 ± 0.022	N/A
Lactobacillus aviarius	N/A	N/A	N/A	N/A	N/A	N/A	0.329 ± 0.014	0.170 ± 0.008	N/A	0.812 ± 0.016	0.296 ± 0.005	0.368 ± 0.005	1.221 ± 0.021	N/A	N/A	N/A	N/A
***Terrimicrobium* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.396 ± 0.009	0.313 ± 0.013	0.901 ± 0.014	N/A	0.157 ± 0.004	N/A	1.019 ± 0.035	N/A	0.168 ± 0.005	0.183 ± 0.007
***Staphylococcus* sp.**	0.128 ± 0.013	0.063 ± 0.006	0.218 ± 0.017	0.404 ± 0.009	0.173 ± 0.004	N/A	0.303 ± 0.010	0.153 ± 0.007	0.494 ± 0.017	0.626 ± 0.014	0.091 ± 0.002	N/A	0.369 ± 0.011	N/A	N/A	N/A	N/A
Thermus scotoductus	0.17 ± 0.018	0.251 ± 0.048	2.458 ± 0.109	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Rubinisphaeraceae	N/A	N/A	N/A	N/A	N/A	0.290 ± 0.009	0.130 ± 0.007	0.286 ± 0.013	0.351 ± 0.013	0.609 ± 0.022	N/A	N/A	N/A	0.300 ± 0.011	0.605 ± 0.030	0.269 ± 0.014	N/A
***Paracoccus* sp.**	N/A	N/A	N/A	N/A	N/A	0.813 ± 0.072	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1.986 ± 0.191	N/A	N/A	N/A
Order Rhizobiales	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.552 ± 0.009	N/A	0.113 ± 0.003	N/A	0.814 ± 0.022	0.323 ± 0.010	0.554 ± 0.015	0.438 ± 0.015
Ureibacillus thermosphaericus	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1.488 ± 0.145	0.937 ± 0.086	0.269 ± 0.027	N/A
Order Planctomycetales	N/A	N/A	N/A	N/A	N/A	0.336 ± 0.020	N/A	0.364 ± 0.033	0.280 ± 0.020	0.540 ± 0.019	0.153 ± 0.005	N/A	0.860 ± 0.022	N/A	N/A	0.072 ± 0.005	N/A
Rhizobiaceae	N/A	0.263 ± 0.025	N/A	N/A	0.209 ± 0.009	0.662 ± 0.015	0.163 ± 0.004	0.342 ± 0.009	0.467 ± 0.012	N/A	N/A	N/A	N/A	0.255 ± 0.007	N/A	0.182 ± 0.007	0.051 ± 0.002
***Pseudochrobactrum* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	2.519 ± 0.315	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Alloprevotella* sp.**	N/A	N/A	N/A	0.472 ± 0.007	0.317 ± 0.005	0.652 ± 0.017	0.252 ± 0.006	0.150 ± 0.004	0.626 ± 0.016	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

***Geobacillus* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1.179 ± 0.018	0.273 ± 0.005	0.291 ± 0.003	0.700 ± 0.014	N/A	N/A	N/A	N/A
Clostridium cochlearium	N/A	N/A	N/A	1.239 ± 0.026	0.529 ± 0.012	0.388 ± 0.025	0.214 ± 0.011	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Phreatobacter* sp.**	N/A	N/A	N/A	N/A	N/A	0.740 ± 0.026	N/A	N/A	0.259 ± 0.009	0.945 ± 0.026	N/A	N/A	N/A	N/A	N/A	0.093 ± 0.005	0.194 ± 0.010
***Rhodobacter* sp.**	N/A	N/A	N/A	N/A	N/A	0.465 ± 0.041	0.592 ± 0.074	0.484 ± 0.041	0.585 ± 0.048	N/A	N/A	N/A	N/A	N/A	N/A	0.081 ± 0.008	N/A
***Alkanindiges* sp.**	N/A	N/A	N/A	0.931 ± 0.016	1.256 ± 0.032	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Kurthia* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.999 ± 0.023	0.107 ± 0.002	0.270 ± 0.003	0.633 ± 0.015	N/A	N/A	N/A	N/A
Clostridium perfringens	N/A	N/A	N/A	0.246 ± 0.006	N/A	N/A	N/A	N/A	N/A	0.699 ± 0.018	0.118 ± 0.002	0.159 ± 0.003	0.727 ± 0.014	N/A	N/A	N/A	N/A
Bacillus smithii	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.634 ± 0.016	0.100 ± 0.003	0.172 ± 0.006	0.998 ± 0.030	N/A	N/A	N/A	N/A
Pediococcus acidilactici	N/A	N/A	N/A	0.382 ± 0.007	0.198 ± 0.004	0.256 ± 0.011	0.303 ± 0.013	0.221 ± 0.008	0.421 ± 0.021	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Candidatus Ovatusbacter* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.862 ± 0.067	0.276 ± 0.019	0.377 ± 0.022	0.200 ± 0.016
Photobacterium damselae	N/A	N/A	N/A	1.101 ± 0.022	0.565 ± 0.014	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Cloacibacterium* sp.**	0.083 ± 0.008	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.094 ± 0.003	N/A	N/A	N/A	1.335 ± 0.115	N/A	N/A
***Weissella* sp.**	N/A	N/A	N/A	0.329 ± 0.007	0.184 ± 0.004	0.582 ± 0.040	N/A	N/A	0.398 ± 0.025	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

***Leucobacter* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1.461 ± 0.179	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Fibrobacteraceae	0.066 ± 0.010	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.196 ± 0.006	N/A	0.566 ± 0.018	N/A	0.243 ± 0.016	N/A	0.345 ± 0.034
***Haloimpatiens* sp.**	N/A	N/A	N/A	N/A	N/A	0.822 ± 0.047	0.142 ± 0.008	N/A	0.388 ± 0.017	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Corynebacterium* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.476 ± 0.059	0.274 ± 0.027	N/A	N/A	N/A	N/A	N/A	0.546 ± 0.055	0.050 ± 0.006	N/A
***Neochlamydia* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.571 ± 0.046	0.636 ± 0.040	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.087 ± 0.007
Thermobacillus composti	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.886 ± 0.068	0.208 ± 0.017	0.192 ± 0.016	N/A
Rikenellaceae	N/A	N/A	N/A	N/A	0.156 ± 0.004	0.442 ± 0.016	0.186 ± 0.010	N/A	0.495 ± 0.019	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Clostridium chauvoei	N/A	N/A	N/A	N/A	N/A	0.436 ± 0.020	0.218 ± 0.013	0.162 ± 0.008	0.457 ± 0.026	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Sphingobium* sp.**	N/A	0.061 ± 0.012	1.118 ± 0.048	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Vagococcus teuberi	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.489 ± 0.017	0.100 ± 0.002	N/A	0.574 ± 0.014	N/A	N/A	N/A	N/A
Microbacteriaceae	N/A	N/A	0.333 ± 0.023	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.469 ± 0.024	0.317 ± 0.032	N/A	N/A
Spirochaetaceae	0.488 ± 0.081	0.078 ± 0.010	0.508 ± 0.031	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Vermiphilaceae* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.321 ± 0.022	0.466 ± 0.020	0.115 ± 0.010	0.156 ± 0.011

***Bergeyella* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1.048 ± 0.105	N/A	N/A
Sphingomonas faeni	0.381 ± 0.040	0.214 ± 0.031	0.364 ± 0.014	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Cytophaga hutchinsonii	N/A	0.034 ± 0.007	0.820 ± 0.052	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.089 ± 0.009	N/A	N/A
***Mycobacterium* sp.**	N/A	N/A	0.565 ± 0.046	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.358 ± 0.021	N/A	N/A	N/A
***Coxiella* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.460 ± 0.026	N/A	N/A	0.388 ± 0.037	N/A	N/A	0.047 ± 0.003
***Thermobacillus* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.675 ± 0.033	0.198 ± 0.008	N/A
***Thermoactinomyces* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.153 ± 0.013	0.615 ± 0.061	0.081 ± 0.009	N/A
***Zoogloea* sp.**	N/A	N/A	0.719 ± 0.019	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.094 ± 0.010	N/A
***Paenarthrobacter* sp.**	0.515 ± 0.036	0.288 ± 0.029	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Lachnoclostridium* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.112 ± 0.001	0.144 ± 0.002	0.511 ± 0.007	N/A	N/A	N/A	N/A
***Enterococcus* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.193 ± 0.013	N/A	N/A	N/A	N/A	0.568 ± 0.011	N/A	N/A	N/A	N/A
***Ralstonia* sp.**	N/A	N/A	N/A	N/A	N/A	0.568 ± 0.071	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.164 ± 0.016	N/A	N/A
Weeksellaceae	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.713 ± 0.071	N/A	N/A

***Janthinobacterium* sp.**	N/A	0.042 ± 0.006	N/A	0.500 ± 0.011	0.167 ± 0.010	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Herminiimonas* sp.**	0.297 ± 0.033	0.147 ± 0.011	0.261 ± 0.011	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Gracilibacteria* sp.**	N/A	N/A	N/A	0.503 ± 0.005	0.164 ± 0.001	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Methylobacterium hispanicum	0.443 ± 0.037	0.200 ± 0.036	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Exiguobacterium* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.510 ± 0.039	N/A	N/A	0.111 ± 0.005	N/A	N/A	N/A	N/A	N/A
Enterococcus cecorum	N/A	N/A	N/A	N/A	N/A	0.343 ± 0.021	N/A	N/A	0.272 ± 0.019	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Phenylobacterium* sp.**	N/A	N/A	0.600 ± 0.046	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Rhodococcus* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	0.152 ± 0.012	N/A	N/A	N/A	N/A	N/A	N/A	0.388 ± 0.036	N/A	0.056 ± 0.005	N/A
***Frisingicoccus* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.388 ± 0.008	0.082 ± 0.002	0.119 ± 0.003	N/A	N/A	N/A	N/A	N/A
***Aureimonas* sp.**	N/A	N/A	0.580 ± 0.058	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Rhizobacter* sp.**	0.343 ± 0.042	0.234 ± 0.023	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Bauldia* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.159 ± 0.011	N/A	0.309 ± 0.026	0.098 ± 0.006
Microtrichaceae	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.549 ± 0.025	N/A	N/A	N/A

***Savagea* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.426 ± 0.013	N/A	0.115 ± 0.004	N/A	N/A	N/A	N/A	N/A
***Lentimonas* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.227 ± 0.012	0.198 ± 0.013	0.112 ± 0.008	N/A
***Cellvibrio* sp.**	N/A	0.043 ± 0.003	0.264 ± 0.009	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.137 ± 0.002	N/A	N/A	0.088 ± 0.003	N/A	N/A
***Sporosarcina* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.373 ± 0.008	N/A	0.156 ± 0.006	N/A	N/A	N/A	N/A	N/A
***Hydrogenophaga* sp.**	0.054 ± 0.009	N/A	0.391 ± 0.027	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.050 ± 0.005
***Sinobaca* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.487 ± 0.049	N/A	N/A
***Rudanella* sp.**	N/A	N/A	N/A	0.480 ± 0.009	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Pedobacter* sp.**	N/A	N/A	N/A	0.319 ± 0.006	0.143 ± 0.002	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Hyphomicrobium* sp.**	0.403 ± 0.032	0.030 ± 0.004	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Siphonobacter* sp.**	N/A	N/A	0.421 ± 0.042	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Galbitalea* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.300 ± 0.023	N/A	0.071 ± 0.005	0.047 ± 0.004
***Rhizorhapis* sp.**	0.417 ± 0.069	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Pyramidobacter* sp.**	N/A	N/A	N/A	N/A	N/A	0.414 ± 0.016	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

***Pediococcus* sp.**	N/A	N/A	N/A	N/A	N/A	0.413 ± 0.052	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Clostridium novyi	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.405 ± 0.012	N/A	N/A	N/A	N/A
***Bdellovibrio* sp.**	0.061 ± 0.003	N/A	N/A	N/A	N/A	N/A	N/A	0.167 ± 0.004	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.176 ± 0.006
Clostridiaceae	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.389 ± 0.002	N/A	N/A	N/A	N/A
***Dokdonella* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.379 ± 0.038	N/A	N/A	N/A
***Sandaracinus* sp.**	N/A	N/A	N/A	0.372 ± 0.008	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Aminobacter* sp.**	N/A	N/A	N/A	N/A	0.367 ± 0.036	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Arthrobacter agilis	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.362 ± 0.045	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Lactobacillus agilis	N/A	N/A	N/A	N/A	N/A	0.355 ± 0.040	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Asticcacaulis* sp.**	N/A	N/A	0.350 ± 0.020	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Order Cytophagales	N/A	N/A	0.331 ± 0.012	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Sediminibacterium* sp.**	N/A	N/A	0.263 ± 0.016	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.068 ± 0.007
Desulfovibrio desulfuricans	N/A	N/A	N/A	N/A	N/A	0.328 ± 0.036	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Shewanella baltica	0.079 ± 0.013	0.062 ± 0.007	0.185 ± 0.019	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Oceanobacillus caeni	N/A	N/A	N/A	N/A	N/A	N/A	0.174 ± 0.006	0.151 ± 0.005	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Tepidimonas* sp.**	0.136 ± 0.009	0.034 ± 0.004	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.144 ± 0.008	N/A	N/A
***Dietzia* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.313 ± 0.031	N/A	N/A
Bacillaceae	N/A	N/A	0.305 ± 0.031	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Brevundimonas* sp.**	0.077 ± 0.011	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.222 ± 0.015	N/A	N/A	N/A
Order Sphingobacteriales	N/A	0.027 ± 0.001	0.270 ± 0.007	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Corynebacterium tuberculostearicum	0.071 ± 0.010	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.224 ± 0.022	N/A	N/A	N/A
***Vulcaniibacterium* sp.**	N/A	N/A	0.291 ± 0.020	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Leuconostoc* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.290 ± 0.032	N/A
Class Polyangia	0.162 ± 0.011	0.122 ± 0.009	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Marivita* sp.**	N/A	N/A	0.275 ± 0.028	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Cellulomonas* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.128 ± 0.013	N/A	0.142 ± 0.012

***Gemmata* sp.**	N/A	N/A	0.269 ± 0.009	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Stenotrophomonas rhizophila	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.269 ± 0.025	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Porphyrobacter* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.264 ± 0.024	N/A	N/A	N/A	N/A
***Altererythrobacter* sp.**	N/A	N/A	0.253 ± 0.025	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Jeotgalicoccus* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	0.252 ± 0.014	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Fluviicola* sp.**	N/A	N/A	N/A	0.248 ± 0.005	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Bradyrhizobium* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.247 ± 0.020	N/A	N/A	N/A	N/A
Xanthobacteraceae	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.124 ± 0.008	0.116 ± 0.006	
Sphingobium yanoikuyae	N/A	0.028 ± 0.006	0.208 ± 0.010	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Psychromonas* sp.**	0.189 ± 0.032	0.046 ± 0.006	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Order Thermomicrobiales	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.186 ± 0.009	N/A	0.049 ± 0.002	N/A	
***Denitratisoma* sp.**	N/A	N/A	0.234 ± 0.017	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Novosphingobium* sp.**	N/A	N/A	0.219 ± 0.016	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Shewanella denitrificans	N/A	N/A	0.214 ± 0.021	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Nitrosomonadaceae	N/A	N/A	0.201 ± 0.020	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Bacillus thermolactis	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.201 ± 0.019	N/A	N/A	N/A
Alcaligenaceae	N/A	N/A	0.200 ± 0.011	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Meiothermus* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.200 ± 0.020	N/A	N/A	N/A	N/A
Beutenbergiaceae	N/A	N/A	0.199 ± 0.020	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Delftia* sp.**	N/A	N/A	0.194 ± 0.010	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Acinetobacter baumannii	N/A	0.193 ± 0.039	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Holosporaceae	N/A	0.027 ± 0.005	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.164 ± 0.017	N/A	N/A
***Candidatus Methylomirabilis* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.187 ± 0.019	N/A
***Cerasibacillus* sp.**	N/A	N/A	N/A	N/A	0.179 ± 0.014	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Order Gaiellales	0.151 ± 0.013	0.025 ± 0.002	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Anoxybacillus* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.176 ± 0.018	N/A

***Carnobacterium* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.165 ± 0.008	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Sphingomonas changbaiensis	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.163 ± 0.018	N/A
Cardiobacteriaceae	N/A	N/A	N/A	N/A	0.161 ± 0.004	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Proteus* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.159 ± 0.003	N/A	N/A	N/A	N/A	N/A
***Nocardioides* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.158 ± 0.015	N/A	N/A	N/A
Savagea faecisuis	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.149 ± 0.006	N/A	N/A	N/A	N/A	N/A
***Starkeya* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.142 ± 0.014
***Macrococcus* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.134 ± 0.013	N/A	N/A
Lactobacillus murinus	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.124 ± 0.012
***Pirellula* sp.**	0.123 ± 0.009	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Afipia* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.122 ± 0.012
***Aliivibrio* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.121 ± 0.010	N/A	N/A	N/A	N/A	N/A
***Roseomonas* sp.**	0.087 ± 0.005	0.030 ± 0.002	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

***Candidatus Protochlamydia* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.114 ± 0.011
***Candidatus Vogelbacteria* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.110 ± 0.011	N/A	N/A
***Sulfurospirillum* sp.**	0.104 ± 0.014	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Labrys* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.102 ± 0.010	N/A	N/A
Order Bacillales	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.101 ± 0.010
***Schlegelella* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.100 ± 0.006	N/A	N/A
***Marmoricola* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.100 ± 0.010
***Kocuria* sp.**	N/A	0.098 ± 0.020	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Hydrogenophilaceae	0.096 ± 0.006	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Thermomonas* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.093 ± 0.009	N/A	N/A
***Curvibacter* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.093 ± 0.009
***Virgibacillus* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.093 ± 0.009
***Cnuella* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.090 ± 0.009	N/A	N/A
***Actinomyces* sp.**	0.089 ± 0.012	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Sphingomonas phyllosphaerae	N/A	0.085 ± 0.016	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Mobilicoccus pelagius	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.082 ± 0.007	N/A	N/A	N/A	N/A	N/A	N/A
Megasphaera elsdenii	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.082 ± 0.003	N/A	N/A	N/A	N/A	N/A	N/A
***Microvirga* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.081 ± 0.007	N/A	N/A	N/A	N/A	N/A	N/A
***Azospira* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.075 ± 0.008	N/A
***Oerskovia* sp.**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.068 ± 0.007
***Chryseobacterium* sp.**	0.061 ± 0.010	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Deep_Sea_Euryarchaeotic_Group(DSEG)	0.059 ± 0.010	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Muricoccus* sp.**	N/A	0.059 ± 0.012	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Fimbriimonadales* sp.**	0.057 ± 0.009	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Shewanella livingstonensis	0.055 ± 0.006	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Asticcacaulis excentricus	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.055 ± 0.005
Order Solirubrobacterales	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.054 ± 0.003	N/A
Shewanella aestuarii	0.053 ± 0.009	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Meiothermus silvanus	N/A	0.052 ± 0.010	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Agrobacterium agile	N/A	0.021 ± 0.004	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Polaromonas* sp.**	N/A	0.021 ± 0.004	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
***Streptomyces* sp.**	N/A	0.020 ± 0.004	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

[1] Error is represented by the mean relative abundance ± standard error of the mean (SEM) of the number of fish specified in Table B.2

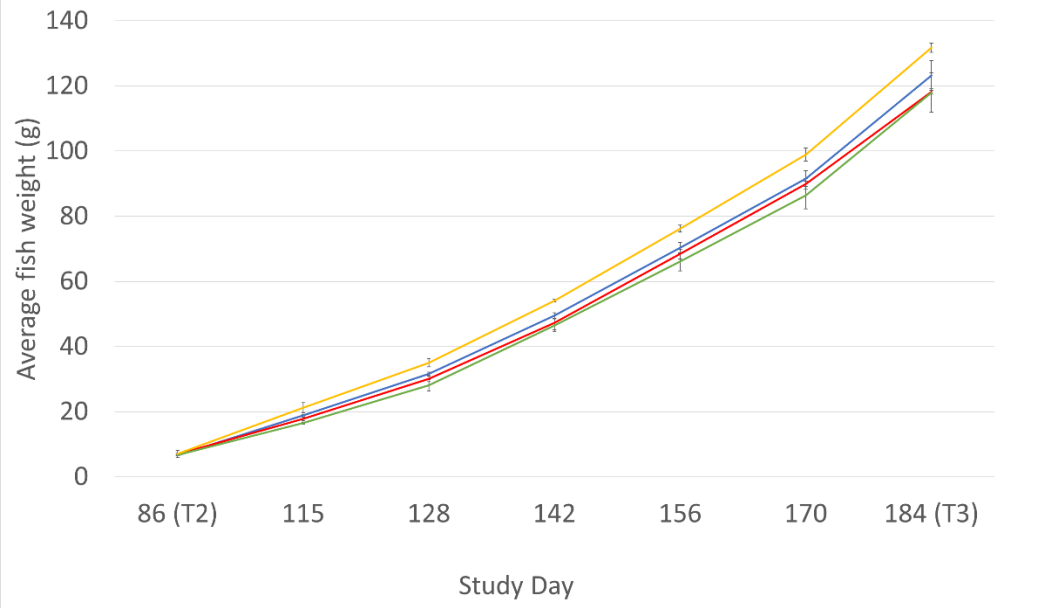

Figure B.1. Production period growth curve based on bi-weekly weights of steelhead trout on a per tank basis. Each diet is represented as follows: A (blue), B (red), C (green), and D (yellow). Error bars represent the mean ± standard error mean of two tanks (days 86 and 115), three tanks (day 128), or four tanks (days 142-184).

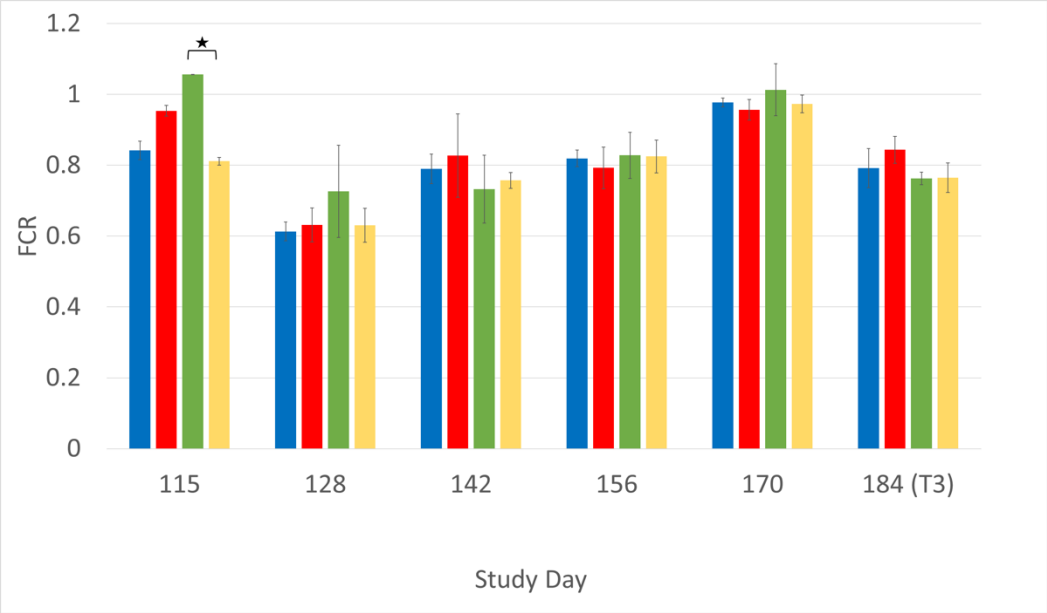

Figure B.2. Feed conversion ratio (FCR) during the production period of steelhead trout on a per tank basis. Each diet is represented as follows A (blue), B (red), C (green), D (yellow). Error bars represent the mean ± standard error mean of two tanks (day 115), three tanks (day 128), or four tanks (days 142-184). One star represents $P<0.05$ following one-way ANOVA with Tukey's post-hoc test.

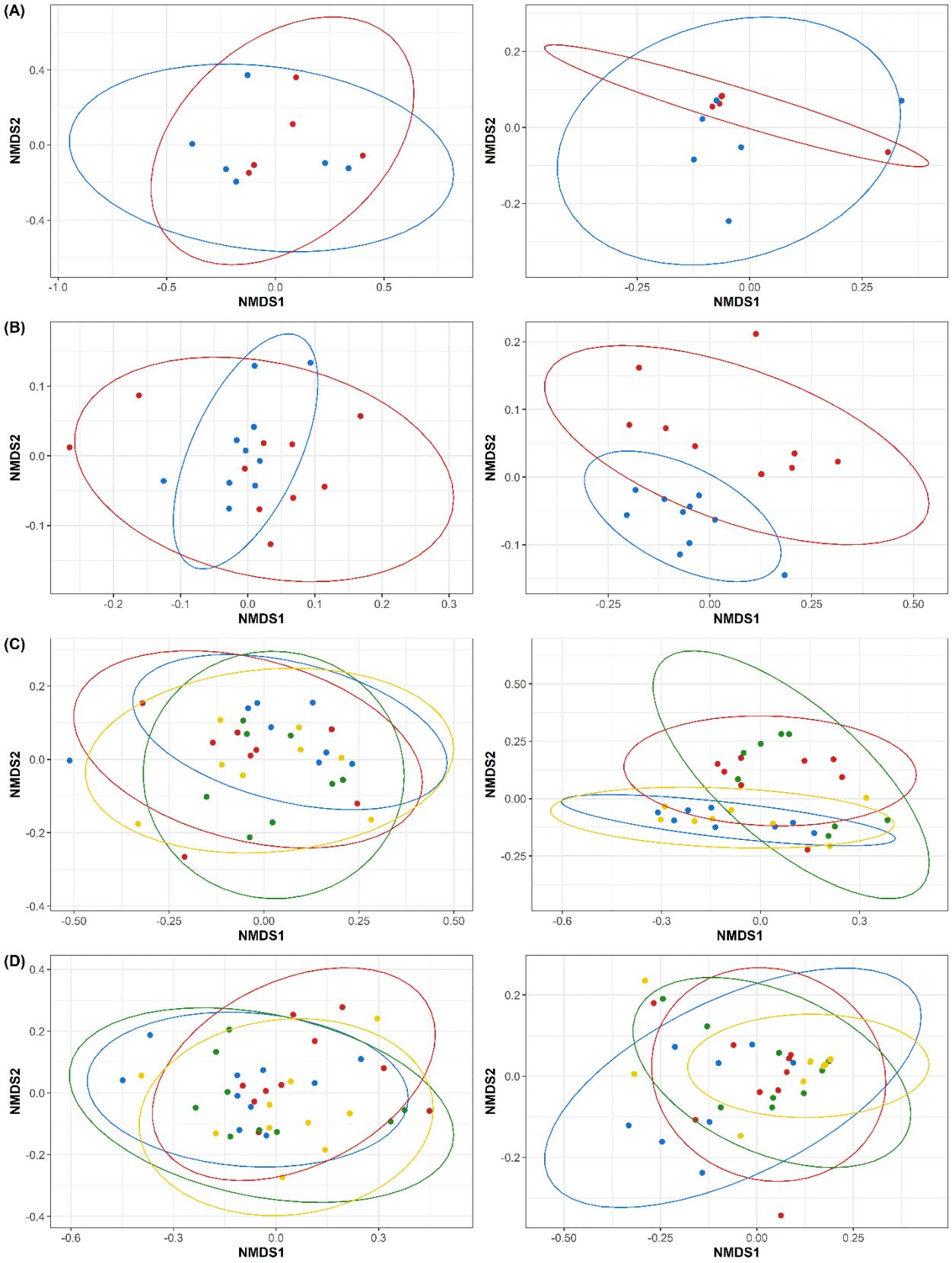
(A)
NMDS2
NMDS1
(B)
NMDS2
NMDS1
(C)
NMDS2
NMDS1
(D)
NMDS2
NMDS1

Figure B.3. Non-metric Multidimensional Scaling (NMDS) plots representing the unweighted and weighted beta diversities of steelhead trout microbiomes for the duration of a probiotic feeding regime. Distances were calculated by (left column) unweighted and (right column) weighted UniFrac between the four diets for fish sampled at timepoints (A) T-1, (B) T1, (C) T2, and (D) T3 intestinal swabs. Colors represent the following diets/treatments: A/disinfected eggs (blue), B/non-disinfected eggs (red), C (green), and D (yellow). Ellipses represent the 95% confidence intervals calculated via Student's T test for six disinfected eggs, five non-disinfected eggs, 10 T1 internal homogenates, eight T2 intestinal homogenates, and 10 T3 intestinal swabs (nine for diet C).

Appendix C

Chapter Five supplemental material

Table C.1. Buoy information.

Purchase date	Buoy location[1]	Temperature[1] (°C)	Salinity[1] (PSU[2])	Trial type
7/16/2020	38.55635, -76.41456	28.0	12.7	Summer
7/30/2020	38.55628, -76.41455	29.4	12.7	Summer
8/14/2020	38.55635, -76.41456	28.2	13.1	Summer
1/14/2021	38.5563, -76.4147	6.47	14.0	Winter
1/21/2021	38.5563, -76.4147	4.88	9.19	Winter

[1] Buoy locations were determined to be the closest available data-collecting sources to the oyster harvest sites; information was retrieved from NOAA's Chesapeake Bay Interpretive Buoy System (NOAA)

[2] Practical Salinity Unit (PSU)

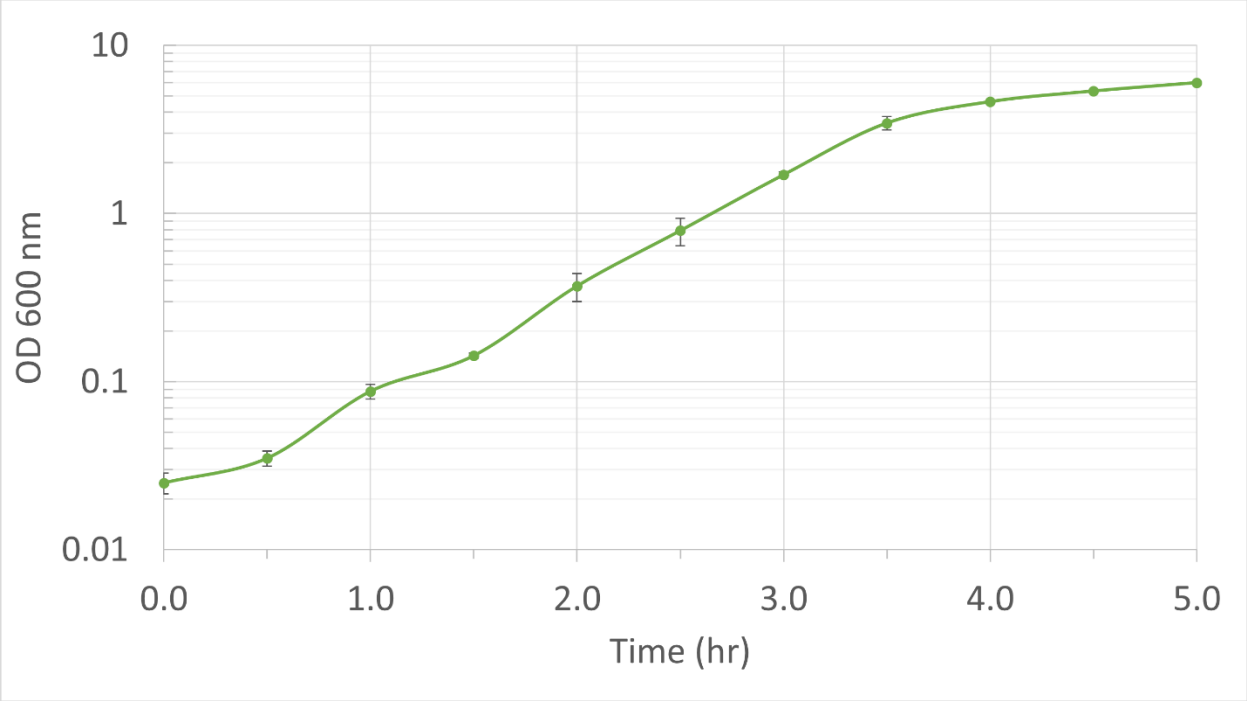

Figure C.1. Growth curve for VP RIMDmC. Cultures were grown in TSB + 2% NaCl + Cm10 and the OD_{600} was measured every 30 minutes. Error bars represent the mean ± standard deviation of two independently grown cultures.

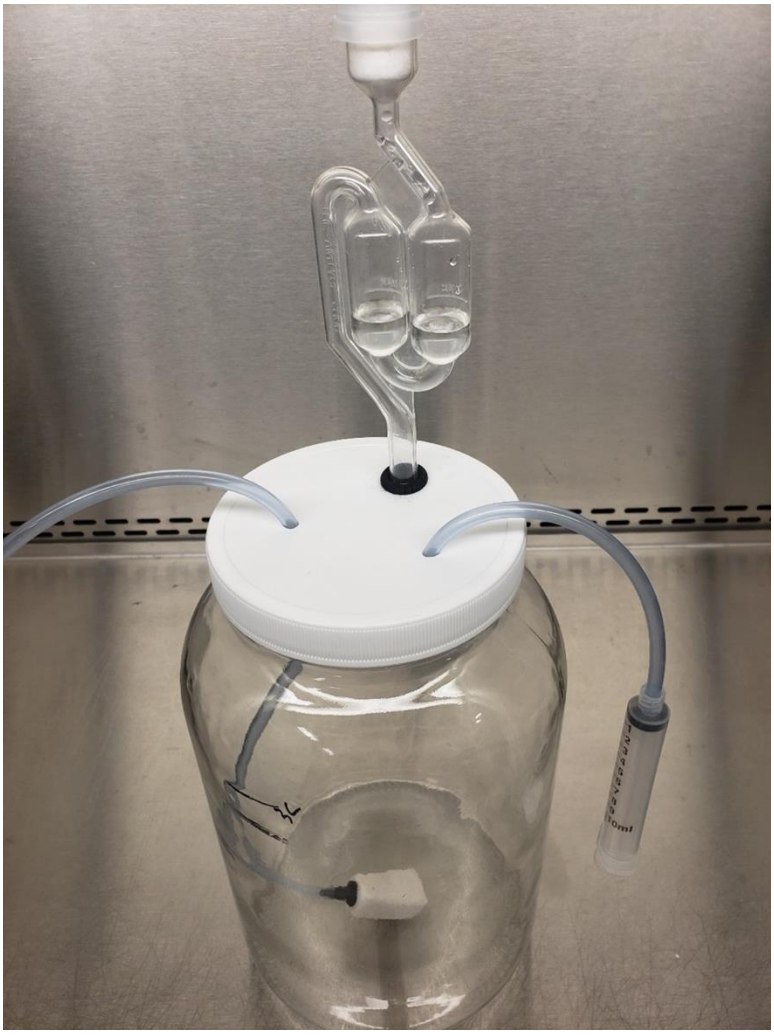

Figure C.2. Modification of fermentation jar lid. Lids associated with one-gallon glass fermentation jars (www.woodyshomebrew.com) were altered as follows. The CO_2 trap was modified by adding dH_2O and a cotton ball to reduce aerosolization. Air exchange was accomplished using a typical air stone connected by tubing passing through a drilled hole to an air pump (not shown). A 10 mL syringe was connected to the jar via tubing passing through a drilled hole to permit for liquid sample removal or addition without an interruption to the air flow. Jars used for exposure trials were maintained in a BSL-2 biosafety cabinet and two or three animals were added to each jar with one liter of artificial sea water per animal.

Appendix D

Chapter Six supplemental Material

Table D.1. ASV relative abundance table.

Phylogeny			**Relative abundance (%)[1]**					
Phylum	**Family**	**Organism name**	**Winter 2020**	**Spring**	**Summer**	**Fall**	**Winter 2021**	**Average**
Spirochaetota	*Spirochaetaceae*	***Spirochaetaceae***	18.339 ± 0.088	24.742 ± 0.102	7.979 ± 0.039	9.125 ± 0.050	30.003 ± 0.121	18.038
Firmicutes	*Mycoplasmataceae*	***Mycoplasma* sp.**	10.373 ± 0.091	23.920 ± 0.153	11.116 ± 0.099	12.331 ± 0.097	19.090 ± 0.128	15.366
Cyanobacteria	*Cyanobiaceae*	**Cyanobium_PCC-6307**	0.089 ± 0.000	14.251 ± 0.061	39.849 ± 0.147	1.708 ± 0.008	N/A	11.179
Proteobacteria	*Pseudoalteromonadaceae*	***Pseudoalteromonas* sp.**	3.756 ± 0.031	N/A	N/A	30.606 ± 0.308	1.795 ± 0.020	7.231
Campilobacterota	*Arcobacteraceae*	***Pseudarcobacter* sp.**	6.241 ± 0.055	N/A	N/A	0.715 ± 0.010	17.008 ± 0.127	4.793
Proteobacteria	*Psychromonadaceae*	***Psychromonas* sp.**	17.058 ± 0.110	N/A	0.124 ± 0.001	1.259 ± 0.018	4.833 ± 0.041	4.655

Firmicutes	*Mycoplasmataceae*	***Mycoplasmataceae***	N/A	3.671 ± 0.048	10.239 ± 0.155	1.168 ± 0.033	N/A	3.016
Proteobacteria	*Moraxellaceae*	***Psychrobacter* sp.**	5.115 ± 0.052	N/A	0.144 ± 0.002	5.695 ± 0.091	2.101 ± 0.028	2.611
Proteobacteria	*Vibrionaceae*	***Aliivibrio* sp.**	6.752 ± 0.100	N/A	N/A	0.535 ± 0.013	3.779 ± 0.096	2.213
Campilobacterota	*Arcobacteraceae*	***Arcobacteraceae***	1.981 ± 0.021	N/A	N/A	6.261 ± 0.125	1.773 ± 0.019	2.003
Verrucomicrobiota	Order Chlamydiales	**Order Chlamydiales**	1.510 ± 0.041	5.525 ± 0.151	0.775 ± 0.018	0.652 ± 0.022	0.271 ± 0.006	1.747
Proteobacteria	*Shewanellaceae*	***Shewanella frigidimarina***	5.481 ± 0.058	N/A	N/A	N/A	2.870 ± 0.021	1.670
Proteobacteria	*Endozoicomonadaceae*	***Endozoicomonas* sp.**	3.720 ± 0.089	N/A	N/A	0.304 ± 0.007	0.968 ± 0.016	0.998
Proteobacteria	*Shewanellaceae*	***Shewanella baltica***	0.713 ± 0.012	N/A	0.302 ± 0.004	2.782 ± 0.032	1.157 ± 0.007	0.991

Spirochaetota	*Spirochaetaceae*	**Spirochaeta_2**	1.453 ± 0.025	1.084 ± 0.018	0.547 ± 0.007	0.280 ± 0.007	1.430 ± 0.030	0.959
Verrucomicrobiota	*Chthoniobacteraceae*	**LD29**	0.082 ± 0.001	1.486 ± 0.010	2.732 ± 0.014	0.279 ± 0.003	N/A	0.916
Proteobacteria	*Marinomonadaceae*	***Marinomonas primoryensis***	0.540 ± 0.004	N/A	N/A	3.206 ± 0.054	0.512 ± 0.004	0.852
Planctomycetota	*Pirellulaceae*	**marine metagenome**	N/A	0.940 ± 0.005	1.740 ± 0.007	1.503 ± 0.008	0.057 ± 0.000	0.848
Proteobacteria	Class Alphaproteobacteria	**Class Alphaproteobacteria**	1.147 ± 0.028	0.602 ± 0.005	0.452 ± 0.005	0.379 ± 0.004	1.426 ± 0.026	0.801
Planctomycetota	*Phycisphaeraceae*	**CL500-3**	N/A	1.414 ± 0.007	1.710 ± 0.005	0.672 ± 0.004	N/A	0.759
Proteobacteria	*Shewanellaceae*	***Shewanella denitrificans***	1.114 ± 0.012	N/A	N/A	1.534 ± 0.013	0.868 ± 0.007	0.703
Proteobacteria	*Vibrionaceae*	***Vibrio mediterranei***	N/A	0.809 ± 0.015	1.788 ± 0.023	0.708 ± 0.008	N/A	0.661

Proteobacteria	*Shewanellaceae*	***Shewanella aestuarii***	1.629 ± 0.014	N/A	0.187 ± 0.005	0.303 ± 0.004	0.663 ± 0.005	0.556
Unassigned Phyla	Unassigned	**Unassigned**	1.050 ± 0.019	0.241 ± 0.001	0.661 ± 0.003	0.178 ± 0.001	0.375 ± 0.005	0.501
Cyanobacteria	*Cyanobiaceae*	***Synechococcus* sp.**	N/A	1.238 ± 0.011	1.039 ± 0.005	N/A	N/A	0.455
Proteobacteria	*Nitrincolaceae*	***Neptunomonas* sp.**	1.174 ± 0.017	N/A	N/A	N/A	0.792 ± 0.009	0.393
Cyanobacteria	*Microcystaceae*	***Merismopedia* sp.**	N/A	1.047 ± 0.011	0.876 ± 0.005	N/A	N/A	0.385
Cyanobacteria	*Cyanobiaceae*	**Synechococcus_CC9902**	N/A	0.910 ± 0.008	0.247 ± 0.002	0.665 ± 0.011	N/A	0.364
Proteobacteria	*Pseudomonadaceae*	***Pseudomonas* sp.**	0.485 ± 0.004	N/A	N/A	0.685 ± 0.014	0.600 ± 0.006	0.354
Proteobacteria	*Comamonadaceae*	***Aquabacterium* sp.**	N/A	N/A	N/A	1.385 ± 0.066	0.262 ± 0.004	0.329

Planctomycetota	*Pirellulaceae*	***Pirellula* sp.**	0.068 ± 0.000	0.351 ± 0.001	1.179 ± 0.003	N/A	N/A	0.320
Planctomycetota	*Pirellulaceae*	***Pirellulaceae***	N/A	0.288 ± 0.002	1.057 ± 0.006	0.167 ± 0.001	N/A	0.302
Proteobacteria	*Moraxellaceae*	***Acinetobacter* sp.**	N/A	N/A	N/A	1.430 ± 0.061	0.077 ± 0.001	0.301
SAR324_clade (Marine_group_B)	SAR324_clade (Marine_group_B)	**SAR324_clade (Marine_group_B)**	N/A	1.080 ± 0.004	0.416 ± 0.001	N/A	N/A	0.299
Planctomycetota	*Pirellulaceae*	***Rhodopirellula* sp.**	N/A	0.413 ± 0.003	1.054 ± 0.005	N/A	N/A	0.293
Actinobacteriota	PeM15	**PeM15**	0.090 ± 0.000	0.542 ± 0.001	0.408 ± 0.001	0.362 ± 0.001	N/A	0.280
Proteobacteria	*Halieaceae*	***Halioglobus* sp.**	0.189 ± 0.003	0.583 ± 0.007	0.277 ± 0.002	0.244 ± 0.002	0.104 ± 0.001	0.279
Cyanobacteria	*Cyanobiaceae*	***Cyanobium* sp.**	N/A	0.442 ± 0.004	0.322 ± 0.001	0.623 ± 0.010	N/A	0.277

Proteobacteria	*Vibrionaceae*	***Vibrio* sp.**	N/A	0.173 ± 0.004	0.169 ± 0.002	0.959 ± 0.020	N/A	0.260
Fusobacteriota	*Fusobacteriaceae*	***Psychrilyobacter* sp.**	0.162 ± 0.003	N/A	N/A	0.971 ± 0.03	0.165 ± 0.004	0.260
Proteobacteria	Class Gammaproteobacteria	**Class Gammaproteobacteria**	0.187 ± 0.001	0.364 ± 0.001	0.274 ± 0.001	0.152 ± 0.000	0.282 ± 0.002	0.252
Proteobacteria	*Sphingomonadaceae*	***Sphingoaurantiacus* sp.**	0.143 ± 0.008	0.799 ± 0.041	0.212 ± 0.004	N/A	0.076 ± 0.004	0.246
Firmicutes	*Spiroplasmataceae*	***Spiroplasma* sp.**	N/A	0.390 ± 0.009	0.611 ± 0.012	0.155 ± 0.005	N/A	0.231
Bacteroidota	*Flavobacteriaceae*	***Flavobacterium* sp.**	0.796 ± 0.007	N/A	N/A	N/A	0.293 ± 0.004	0.218
Proteobacteria	*Marinomonadaceae*	***Marinomonas* sp.**	N/A	N/A	N/A	1.074 ± 0.004	N/A	0.215

Proteobacteria	*Rhodobacteraceae*	***Rhodobacteraceae***	0.363 ± 0.004	0.389 ± 0.006	N/A	N/A	0.191 ± 0.003	0.189
Planctomycetota	*Isosphaeraceae*	***Isosphaeraceae***	N/A	0.496 ± 0.004	0.335 ± 0.002	N/A	N/A	0.166
Proteobacteria	*Shewanellaceae*	***Shewanella amazonensis***	N/A	0.574 ± 0.029	0.204 ± 0.006	N/A	N/A	0.156
Planctomycetota	*Rubinisphaeraceae*	***Planctomicrobium* sp.**	N/A	0.617 ± 0.004	0.137 ± 0.000	N/A	N/A	0.151
Verrucomicrobiota	WCHB1-41	**WCHB1-41**	N/A	0.253 ± 0.002	0.499 ± 0.002	N/A	N/A	0.150
Proteobacteria	*Aeromonadaceae*	***Oceanisphaera* sp.**	0.092 ± 0.001	N/A	N/A	0.595 ± 0.004	0.056 ± 0.000	0.149
Planctomycetota	*Gimesiaceae*	***Gimesiaceae***	N/A	N/A	0.529 ± 0.001	0.200 ± 0.002	N/A	0.146
Chloroflexi	*Anaerolineaceae*	***Anaerolineaceae***	N/A	0.382 ± 0.001	0.302 ± 0.000	N/A	N/A	0.137

Proteobacteria	*Shewanellaceae*	***Shewanella* sp.**	0.221 ± 0.004	N/A	N/A	0.449 ± 0.004	N/A	0.134
Desulfobacterota	*Desulfosarcinaceae*	**Sva0081_sediment_group**	N/A	0.275 ± 0.001	0.212 ± 0.001	0.163 ± 0.001	N/A	0.130
Proteobacteria	*Colwelliaceae*	***Colwellia* sp.**	0.461 ± 0.007	N/A	N/A	N/A	0.176 ± 0.002	0.127
Campilobacterota	*Sulfurospirillaceae*	***Sulfurospirillum* sp.**	0.352 ± 0.006	N/A	N/A	N/A	0.284 ± 0.004	0.127
Proteobacteria	*Colwelliaceae*	***Colwellia beringensis***	N/A	N/A	N/A	0.621 ± 0.022	N/A	0.124
Planctomycetota	*Rubinisphaeraceae*	***Rubinisphaeraceae***	N/A	N/A	0.593 ± 0.003	N/A	N/A	0.119
Proteobacteria	*Shewanellaceae*	***Shewanella colwelliana***	0.102 ± 0.002	N/A	N/A	0.436 ± 0.006	N/A	0.108
Bacteroidota	*Flavobacteriaceae*	***Flavobacterium tegetincola***	0.218 ± 0.003	N/A	N/A	N/A	0.313 ± 0.003	0.106

Proteobacteria	*Shewanellaceae*	***Shewanella livingstonensis***	0.194 ± 0.001	N/A	N/A	0.324 ± 0.005	N/A	0.104
Firmicutes	*Fusibacteraceae*	***Fusibacter* sp.**	0.340 ± 0.006	N/A	N/A	N/A	0.143 ± 0.001	0.097
Fusobacteriota	*Fusobacteriaceae*	***Fusobacterium* sp.**	N/A	N/A	0.349 ± 0.013	N/A	0.089 ± 0.003	0.088
Proteobacteria	*Vibrionaceae*	***Photobacterium* sp.**	0.223 ± 0.002	N/A	N/A	0.152 ± 0.004	0.058 ± 0.002	0.087
Proteobacteria	Order Rhizobiales	**Order Rhizobiales**	N/A	N/A	N/A	0.226 ± 0.012	0.206 ± 0.010	0.086
Proteobacteria	*Moritellaceae*	***Moritella* sp.**	0.271 ± 0.002	N/A	N/A	N/A	0.141 ± 0.001	0.082
Proteobacteria	*Rhodobacteraceae*	***Marivita* sp.**	0.182 ± 0.002	0.228 ± 0.002	N/A	N/A	N/A	0.082
Desulfobacterota	*Desulfocapsaceae*	***Desulfocapsaceae***	0.069 ± 0.001	0.185 ± 0.001	N/A	N/A	0.142 ± 0.001	0.079

Chloroflexi	*Caldilineaceae*	***Caldilineaceae***	N/A	N/A	0.375 ± 0.002	N/A	N/A	0.075
Firmicutes	*Planococcaceae*	***Planococcaceae***	N/A	N/A	N/A	0.365 ± 0.015	N/A	0.073
Proteobacteria	*Sphingomonadaceae*	***Sphingomonas* sp.**	N/A	0.159 ± 0.006	N/A	N/A	0.203 ± 0.007	0.072
Bdellovibrionota	*Silvanigrellaceae*	***Silvanigrellaceae***	N/A	0.147 ± 0.002	0.213 ± 0.002	N/A	N/A	0.072
Bacteroidota	*Flavobacteriaceae*	***Flavobacterium jumunjinense***	0.297 ± 0.007	N/A	N/A	N/A	0.060 ± 0.001	0.071
Planctomycetota	*Pirellulaceae*	***Blastopirellula* sp.**	N/A	0.348 ± 0.002	N/A	N/A	N/A	0.070
Verrucomicrobiota	DEV007	**DEV007**	N/A	0.192 ± 0.000	0.132 ± 0.000	N/A	N/A	0.065
Proteobacteria	*Saccharospirillaceae*	***Oleispira* sp.**	0.069 ± 0.001	N/A	N/A	0.247 ± 0.003	N/A	0.063

Verrucomicrobiota	*Rubritaleaceae*	***Luteolibacter* sp.**	0.125 ± 0.001	N/A	0.187 ± 0.001	N/A	N/A	0.062
Planctomycetota	*Pirellulaceae*	**bacterium enrichment**	N/A	N/A	0.308 ± 0.000	N/A	N/A	0.062
Proteobacteria	B2M28	**B2M28**	N/A	0.132 ± 0.001	0.138 ± 0.001	N/A	N/A	0.054
Proteobacteria	Run-SP154	**Run-SP154**	N/A	0.123 ± 0.001	0.125 ± 0.001	N/A	N/A	0.050
Proteobacteria	*Holosporaceae*	***Holosporaceae***	N/A	N/A	0.203 ± 0.001	N/A	N/A	0.041
Desulfobacterota	uncultured bacterium	**uncultured bacterium**	N/A	N/A	0.202 ± 0.001	N/A	N/A	0.040
Cyanobacteria	*Microcystaceae*	**Synechocystis_PCC-6803 sp.**	N/A	0.199 ± 0.002	N/A	N/A	N/A	0.040
Bacteroidota	*Flavobacteriaceae*	***Flavobacteriaceae***	0.109 ± 0.001	N/A	N/A	N/A	0.084 ± 0.001	0.039

Proteobacteria	*Rickettsiaceae*	**Candidatus_Megaira sp.**	N/A	N/A	0.178 ± 0.001	N/A	N/A	0.036
Proteobacteria	*Comamonadaceae*	***Acidovorax* sp.**	N/A	N/A	N/A	0.173 ± 0.008	N/A	0.035
Margulisbacteria	*Margulisbacteria*	***Margulisbacteria* sp.**	N/A	N/A	N/A	N/A	0.170 ± 0.002	0.034
Modulibacteria	*Moduliflexaceae*	***Moduliflexaceae* sp.**	N/A	N/A	N/A	N/A	0.166 ± 0.005	0.033
Proteobacteria	*Halieaceae*	***Luminiphilus* sp.**	N/A	0.163 ± 0.002	N/A	N/A	N/A	0.033
Proteobacteria	*Spongiibacteraceae*	**BD1-7_clade**	N/A	0.160 ± 0.001	N/A	N/A	N/A	0.032
Proteobacteria	*Chromatiaceae*	***Chromatiaceae***	N/A	0.156 ± 0.001	N/A	N/A	N/A	0.031
Campilobacterota	*Arcobacteraceae*	***Arcobacter nitrofigilis***	N/A	N/A	N/A	N/A	0.153 ± 0.004	0.031

Proteobacteria	*Steroidobacteraceae*	***Steroidobacteraceae***	N/A	0.136 ± 0.001	N/A	N/A	N/A	0.027
Proteobacteria	*Aeromonadaceae*	***Aeromonas* sp.**	N/A	N/A	N/A	0.136 ± 0.005	N/A	0.027
Patescibacteria	*Saccharimonadales*	***Saccharimonadales* sp.**	N/A	N/A	0.135 ± 0.001	N/A	N/A	0.027
Proteobacteria	*Halieaceae*	***Halieaceae***	N/A	0.131 ± 0.001	N/A	N/A	N/A	0.026
Actinobacteriota	*Mycobacteriaceae*	***Mycobacterium* sp.**	N/A	N/A	N/A	0.130 ± 0.002	N/A	0.026
Proteobacteria	*Alcaligenaceae*	***Bordetella* sp.**	N/A	0.129 ± 0.001	N/A	N/A	N/A	0.026
Proteobacteria	*Shewanellaceae*	***Shewanella loihica***	N/A	0.126 ± 0.003	N/A	N/A	N/A	0.025
Desulfobacterota	*Desulfocapsaceae*	***Desulforhopalus* sp.**	N/A	0.124 ± 0.001	N/A	N/A	N/A	0.025

Bacteroidota	*Flavobacteriaceae*	***Lutibacter* sp.**	0.120 ± 0.001	N/A	N/A	N/A	N/A	0.024
Proteobacteria	*Moraxellaceae*	***Psychrobacter maritimus***	0.115 ± 0.001	N/A	N/A	N/A	N/A	0.023
Patescibacteria	JGI_0000069-P22	**JGI_0000069-P22**	N/A	N/A	N/A	N/A	0.112 ± 0.001	0.022
Crenarchaeota	*Nitrosopumilaceae*	**Candidatus_Nitrosopumilus sp.**	N/A	N/A	N/A	N/A	0.093 ± 0.001	0.019
Firmicutes	*Bacillaceae*	***Bacillus* sp.**	0.087 ± 0.002	N/A	N/A	N/A	N/A	0.017
Proteobacteria	*Beijerinckiaceae*	***Methylobacterium-Methylorubrum* sp.**	N/A	N/A	N/A	N/A	0.079 ± 0.003	0.016
Proteobacteria	*Hyphomonadaceae*	**Alphaproteobacterium**	N/A	N/A	N/A	N/A	0.070 ± 0.000	0.014
Verrucomicrobiota	*Rubritaleaceae*	***Persicirhabdus* sp.**	0.069 ± 0.001	N/A	N/A	N/A	N/A	0.014

Bacteroidota	*Marinifilaceae*	***Marinifilaceae***	N/A	N/A	N/A	N/A	0.064 ± 0.001	0.013

[1] Error is represented by the mean relative abundance ± standard error of the mean (SEM) of 18 oysters

Table D.2. Oyster harvest location buoy data.

Purchase date	Location[1]	Temperature (°C)	Salinity (PSU[2])	Dissolved Oxygen (mL O_2/L)	Sampling Period
2/28/2020	38.03326, -76.33733	6.00	14.7	17.4	Winter 2020
6/16/2020	38.55635, -76.41456	22.7	10.4	N/A	Spring
8/26/2020	38.55607, -76.41454	26.8	13.3	10.83	Summer
11/23/2020	38.5563, -76.4147	16.4	16.1	9.49	Fall
2/23/2021	38.03327, -76.33723	3.33	12.3	7.73	Winter 2021

[1] Information retrieved from NOAA's Chesapeake Bay Interpretive Buoy System (NOAA); available data taken from closest buoy to harvest location

[2] Practical salinity unit (PSU)

Appendix E

Influence of host genotype on the physiology and microbiomes of three related tilapia lines (*Oreochromis niloticus*)

Experimental design

The genetics of the host has been implicated as a factor in the selection of microorganisms that make up the microbiome. The intestinal epithelial-associated and scale-associated microbiomes were analyzed from three genetically distinct lines (i.e., A, B and C) of proprietary Nile tilapia. These three lines are differentiated by a single genetic trait. Samples for each line consisted of tissues harvested from nine fish. Findings from the laboratory of David Kuhn (Food Science and Technology, Virginia Tech) showed that line A had a significantly higher growth rate (Figure E.1). This calculation measures the amount of feed (kg) necessary to produce fish of a certain weight (kg). Sequencing and analysis are as described in Chapter Two.

Results

Scale-associated microbiome

Fish physiology data (Figure E.1) indicated line A grew significantly faster than lines B and C (control). For the first time point, no correlation between this increase in growth rate and the scales-associated microbiome was found. A high diversity of bacterial families within the scale-associated microbiome was attributed to each line (Figure E.2). The scale of this diversity was not significantly different between samples (Table E.1). Thus, there is no evidence for a shift in the scale-associated microbial communities in response to host genotype. In addition, there is a clustering of the samples from all three genetic lines (Figure E.3). Figure E.2 represents six fish though seven fish were used for QIIME2 analyses. This was due to the ability to add one more sample without sacrificing quality in the reads per sample. Of note, a PERMANOVA indicates the microbial communities of lines B and C are significantly different when measured

using the Jaccard metric (Figure E.3A). However, this test does not take relative abundance into account unlike the weighted Unifrac (Figure E.3B), so these taxa that are different between the two lines are in too low of abundance to be considered significant. In summary, the diversity of the scales-associated microbiome does not appear to be affected by the change in the single genetic trait.

Intestinal epithelial-associated microbiome

Two dominant families were present in the intestinal microbiome for line A, *Mycoplasmataceae* and *Fusobacteriaceae*, compared to *Enterobacteraceae* as the most dominant family in lines B and C (Figure E.4), for the first time point. Though the overall diversity of each genotype is lower than their corresponding scales-associated diversity, there are no significant differences between the three genotypes based on the alpha diversities (Table E.2). According to the weighted Unifrac beta diversity result reported here (Figure E.5B), line A harbors an adherent intestinal microbiome significantly different than the other two lines ($P<0.05$ following PERMANOVA). In contrast to this, the Jaccard presence/absence metric (Figure E.5A) reports these groups are not statistically different. However, considering the clear dominance of the *Mycoplasmataceae* and *Fusobacteriaceae* within the line A community structure compared to lines B and C, the data provide evidence that the genotype of the line A host selects for different taxa. These microbes appear to facilitate increased growth rates, likely a result of better feed efficiency within the intestinal epithelial-associated microbiome of fish.

Table E.1. Scales-associated microbiome alpha diversity metrics.

Line	H[1]	ENS[2]	Richness[3]	Evenness
A	4.47	87.1	583	0.701
B	3.98	53.5	515	0.637
C	4.34	77.0	570	0.685

[1] Shannon diversity index

[2] Effective number of species

[3] Number of OTUs

Table E.2. Intestinal-associated microbiome alpha diversity metrics.

Line	H[1]	ENS[2]	Richness[3]	Evenness
A	1.37	3.92	77.0	0.315
B	0.823	2.28	58.0	0.203
C	0.630	1.88	117	0.132

[1] Shannon diversity index

[2] Effective number of species

[3] Number of OTUs

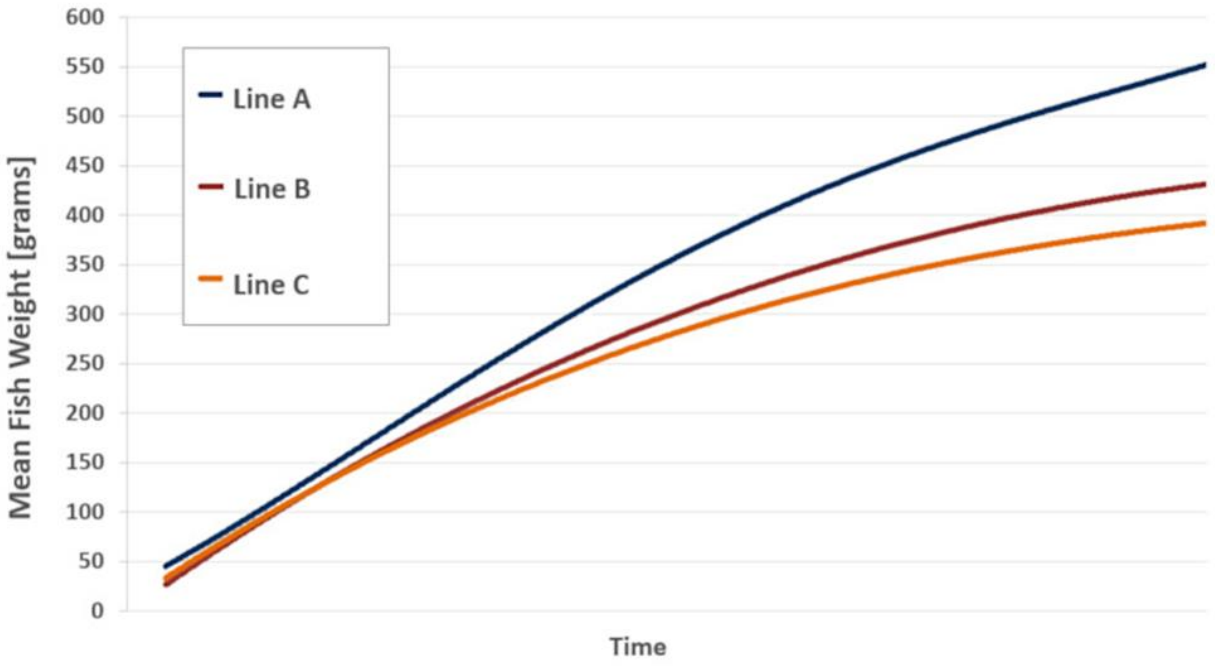

Figure E.1. Relative growth rate of three genetically distinct lines of Nile tilapia. Comparative weight gains of three related lines of Nile tilapia over time.

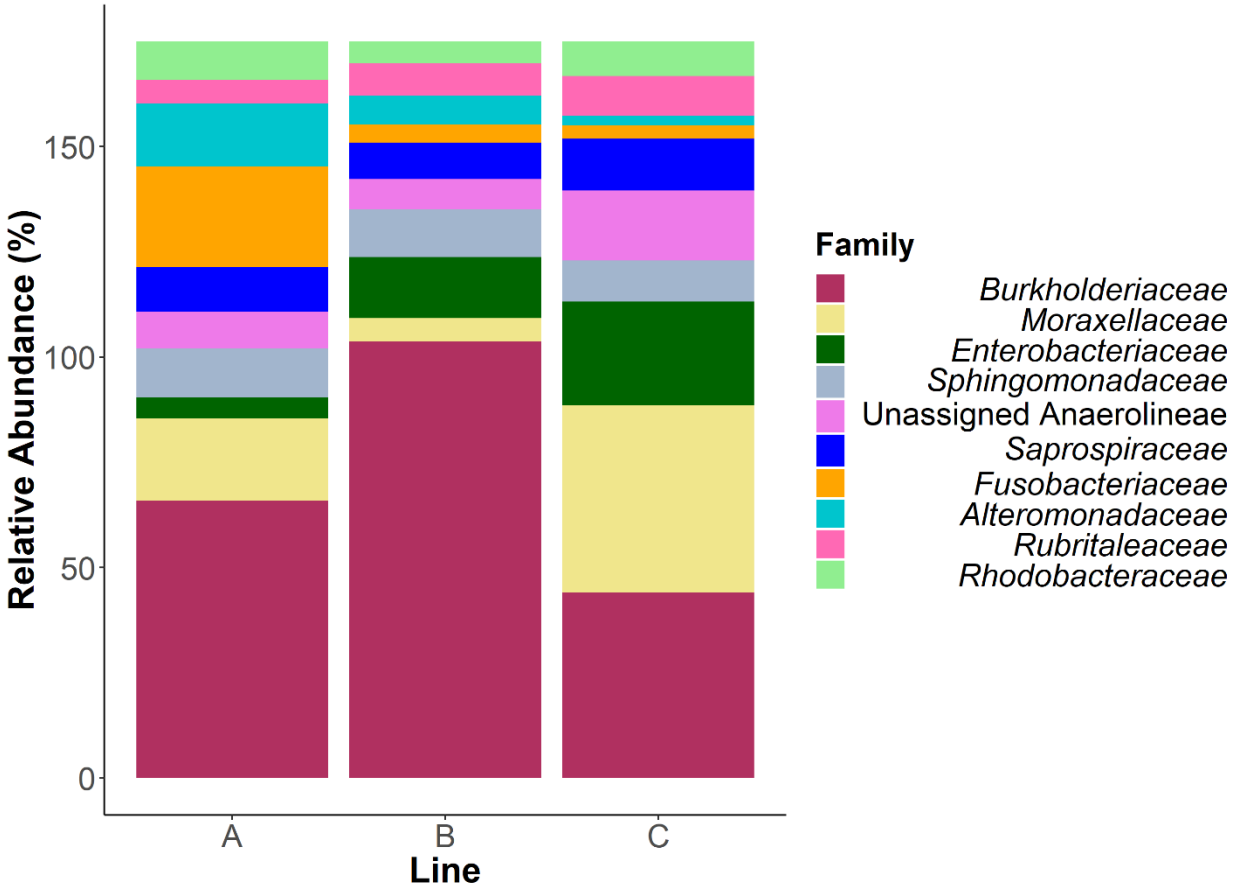

Figure E.2. Skin-associated microbiota of three genetically distinct lines of Nile tilapia. The relative diversity of the top 10 most abundant families within each sample is represented. Reads from six fish from each genotype group, line A (FCR-enhanced), line B, line C (control) are averaged above.

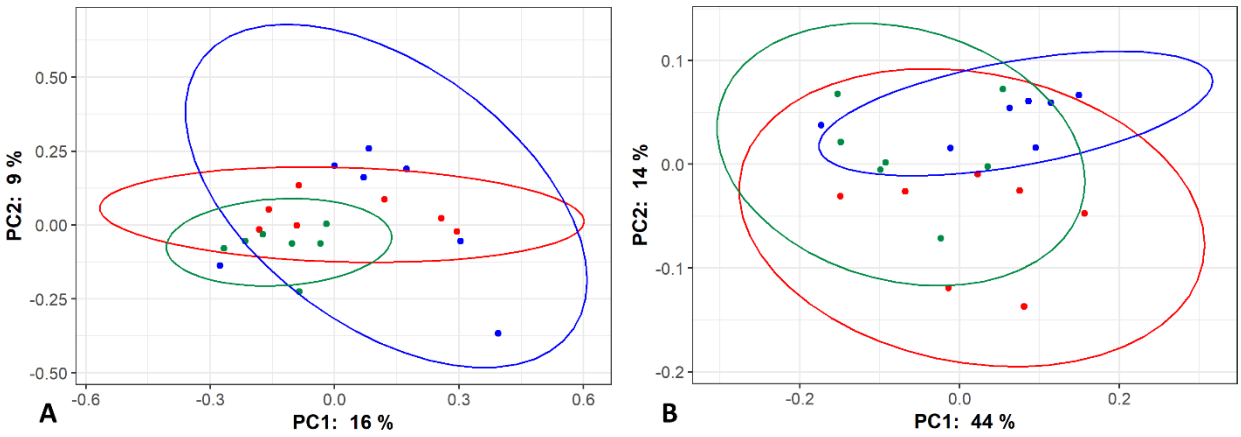

Figure E.3. Principal coordinates of analysis (PCoA) plots representing the beta diversity distances between the scales-associated microbiomes of three genetically distinct lines of Nile tilapia. Distances were calculated by (A) Jaccard shared OTU presence/absence or (B) relative abundance-weighted Unifrac. Genotypic lines are identified by the following colors: blue (line A) red (line B), green (line C). A PERMANOVA was used to test for a significant dissimilarity between genetic lines for each metric. This indicated lines B and C harbor significantly different microbial communities using the Jaccard metric ($p < 0.05$).

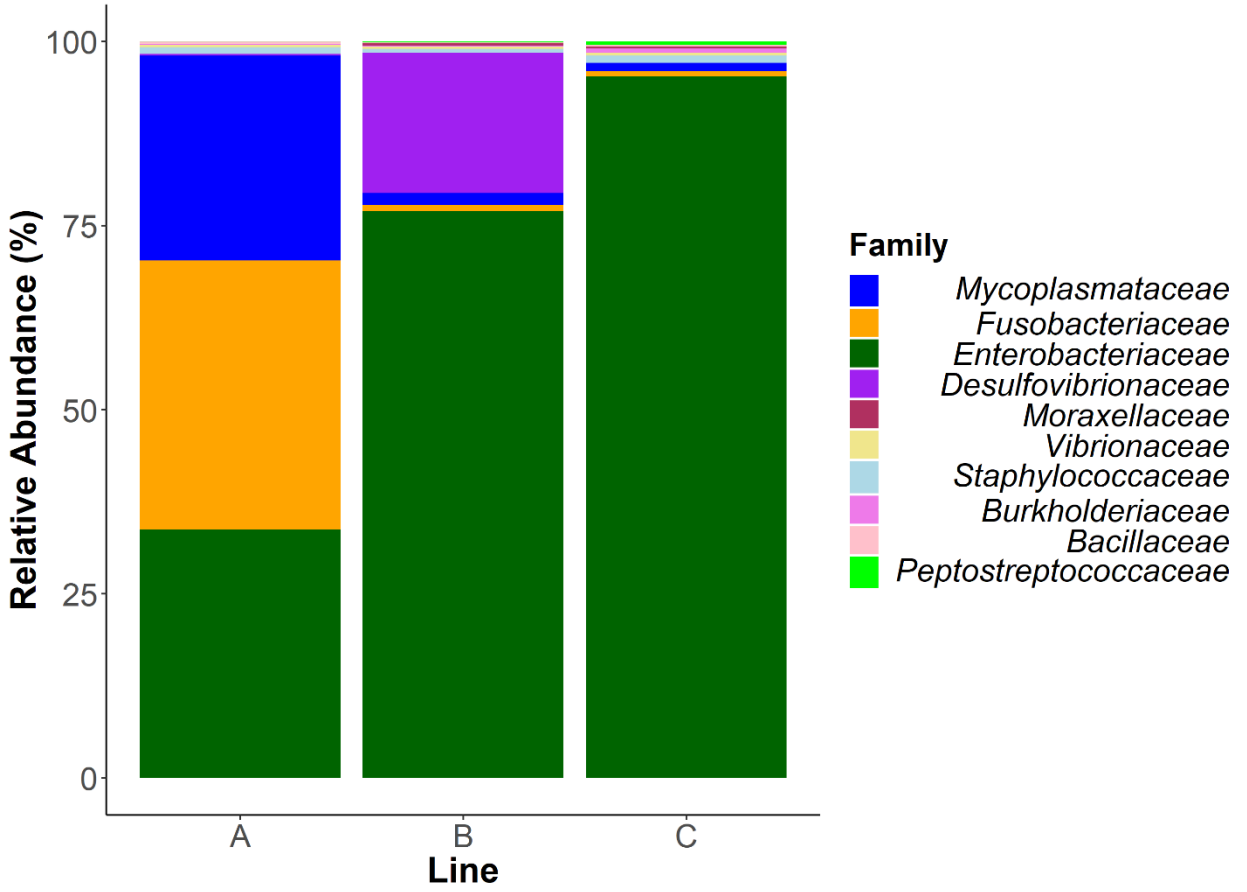

Figure E.4. Intestinal epithelial-associated microbiota of three genetically distinct lines of Nile tilapia. Intestinal epithelial-associated microbiota relative diversity of the top 10 most abundant OTUs within each sample. Reads for four fish from each genotype group, line A (FCR-enhanced), line B, line C (control), are averaged above.

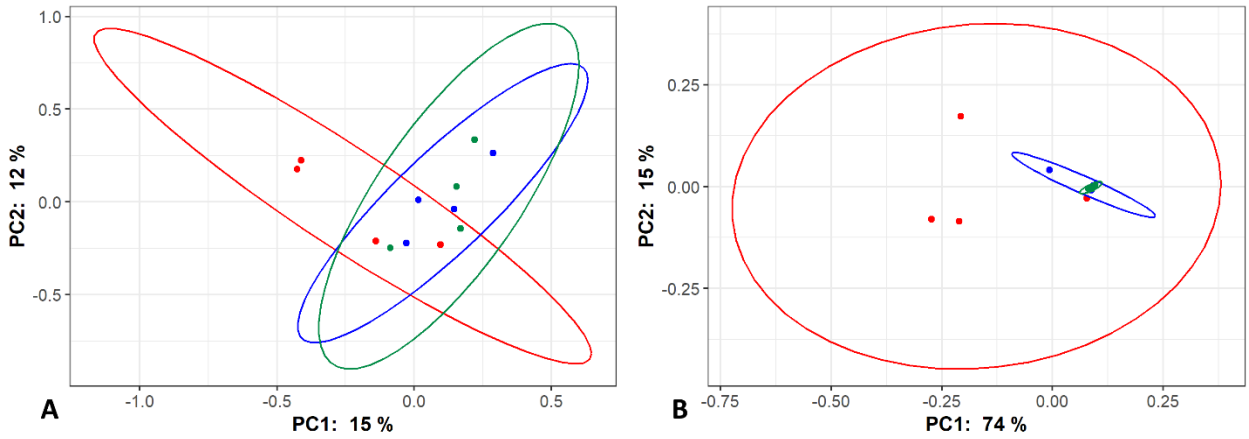

Figure E.5. Principal coordinates of analysis (PCoA) plots representing the beta diversity distances between the intestinal epithelial-associated microbiomes of three genetically distinct lines of Nile tilapia. Distances were calculated by (A) Jaccard shared OTU presence/absence or (B) relative abundance-weighted Unifrac. Genotypic lines are identified by the following colors: blue (line A) red (line B), green (line C). A PERMANOVA was used to test the statistical difference/similarity between groups, and it indicates that, using the weighted Unifrac metric, the microbial community of line A is significantly different from the communities of lines B and C ($p < 0.05$).

Appendix F

Testing the Ability of Isogenic *Vibrio parahaemolyticus* strains to be Inoculated into Oysters

Experimental design

Human infection and disease pathogenesis caused by *Vibrio parahaemolyticus* (VP) is attributed to expression of key virulence factors. These factors include a thermostable direct hemolysin (TDH) and two type III secretion systems (T3SS1 and T3SS2) (Broberg et al., 2011; Wang et al., 2015). Therefore, a set of isogenic mutant strains of VP RIMD 2210633, POR2 and POR3 lacking functional TDH and either T3SS1 or T3SS2 (35) were obtained for testing and transconjugated with the pRMJ3 plasmid in the same manner as for VP RIMDmC (see Chapter Five). The ability of these three strains to be inoculated into oysters was then tested.

Results and discussion

All three isogenic VP strains (WT, POR2, POR3) exhibited ~100% inoculation efficiency in oysters harvested in winter that were treated with Cm (Figure F.1), suggesting that neither TDH nor either of the two T3SS in VP RIMD plays a role in the ability of VP to associate with oysters. The POR3 strain displayed increased growth within the oyster tissues by ~1 log higher compared to the other two strains. However, the increased growth within tissues appears be correlated to a higher rate of proliferation within the water column (Figure F.2), as all three strains grew at similar rates *in vitro* in TSB rich medium (Figure F.3).

In mammalian systems, overexpression of the T3SS1 may decrease colonization efficacy and the T3SS2 is necessary for host cell invasion and partially responsible for colonization (48, 49). Contrary to mammalian colonization, neither of the nonfunctional T3SS mutants tested here led to a reduction nor increase in inoculation efficiency (VP RIMDmC/ total *Vibrio* population). This suggests that the host-microbe relationship may not be conserved between mammals and invertebrates or that the appropriate conditions for colonization were not

employed. Interestingly, the absence of the T3SS2 impacted bacterial growth rates in low nutrient conditions for unknown reasons. Future studies examining impacts of temperature and bile salt on the ability of exogenous VP to inoculate oysters should prove interesting. Oysters may not produce bile or a compound homologous enough to bile that can lead to upregulation of the VP RIMD T3SS2, but bile may be present in native waters from other animals that was absent in our lab-scale system.

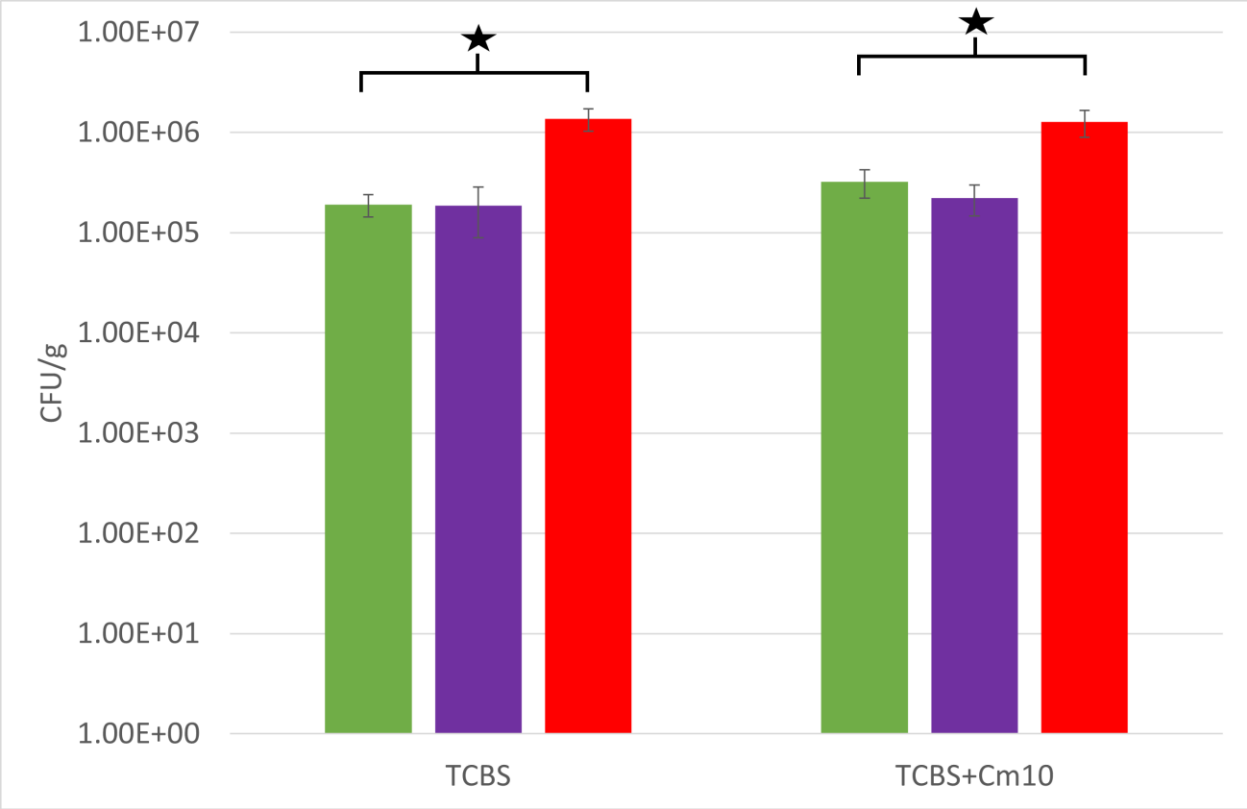

Figure F.1. *Vibrio* levels in whole oyster homogenates (isogenic mutants). Tank treatments included chloramphenicol 10 mg/ml (Cm10) with one of the following strains: wild type (WT) (green), POR2 (nonfunctional T3SS1, purple), and POR3 (nonfunctional T3SS2, red). Whole oyster homogenates were enumerated on TCBS without Cm10 and TCBS with Cm10 as indicated. One star represents $p < 0.05$ calculated via Wilcoxon signed-rank test. Error is represented by the mean weight-normalized CFU/g values for six animals per tank treatment ± SEM.

Figure F.2. VP RIMDmC levels in the water column with oysters used for isogenic strain experimentation over time. Tank treatments included chloramphenicol 10 mg/ml (Cm10) with one of the following strains: wild type (WT) (green), POR2 (nonfunctional T3SS1, purple), and POR3 (nonfunctional T3SS2, red). The green arrow represents the point at which the oysters were subjected to modified depuration with fresh 20 ppt artificial sea water. Error bars represent the mean CFU/mL ± SEM of three independent trials.

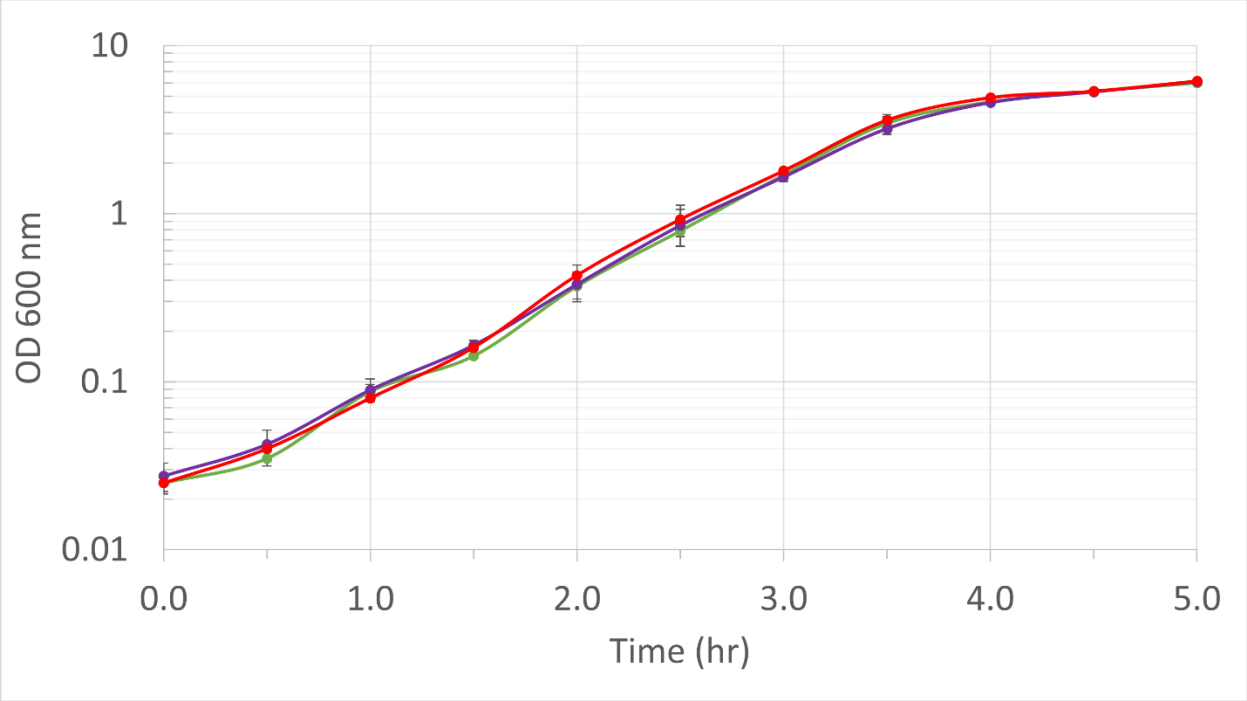

Figure F.3. Growth curves for isogenic mutant VP strains. All cultures were grown in TSB + 2% NaCl + chloramphenicol 10 mg/ml and the OD_{600} was measured via cuvettes. Cultures included: wild type (WT) (green), POR2 (purple), and POR3 (red). Error bars represent the mean ± standard deviation of two independently grown cultures.

References

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