Molecular Evaluations of Interaction Between Host and Mucosal Microbiota in Cultured Salmonids

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Jacob W. Bledsoe

Major Professor: Brian C. Small, Ph.D.

Committee Members: Brian C. Peterson, Ph.D; Kenneth E. Overturf, Ph.D;

Kenneth D. Cain, Ph.D

Department Administrator: Lisette P. Waits, Ph.D.

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Authorization to Submit Dissertation

This dissertation of Jacob W. Bledsoe, submitted for the degree of Doctor of Philosophy with a Major in Natural Resources and titled "Molecular Evaluations of Interaction Between Host and Mucosal Microbiota in Cultured Salmonids," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

Major Professor: _____ Date: _____
Brian C. Small, Ph.D.

Committee Members: _____ Date: _____
Brian C. Peterson, Ph.D

_____ Date: _____
Kenneth E. Overturf, Ph.D.

_____ Date: _____
Kenneth D. Cain, Ph.D.

Department
Administrator: _____ Date: _____
Lisette P. Waits, Ph.D.

Abstract

The biggest hurdles to the environmentally and economically sustainable aquaculture production of finfish involve issues related to disease and nutrition. Mucosal tissues and their associated commensal microbiota lie at the interface between animal and environment and are known to play an integral role in both nutrition and immunity, yet their interactions have been poorly studied in fish. As such, the aim of the work presented here was to utilize high-throughput molecular methods to provide a comprehensive and resolute characterization of salmonid mucosal tissues and their bacterial microbiota in response to multiple sources of variation, which are commonly encountered in an aquaculture setting (i.e. host genetics, mucosal tissue, developmental stage, diet, viral and bacterial disease, and stage of infection). In the first study, it was shown that Atlantic salmon differentially regulated their gut, gill, and skin microbiota, irrelevant of dietary functional feed treatments, and differences in key host regulatory immune genes across tissue showed high correlation with bacterial microbiota communities (Procrustes, correlation = 0.818, $p \leq 0.001$). The functional capacity of microbiota showed adaptive differences in bacterial metabolism by tissues with increased fermentation and nutrient metabolism pathways detected in the gut and denitrification pathways being more abundant in microbiota of the gill, the primary site of excretion of endogenous ammonia in fish. Bacterial gene ontology was correlated with bacterial phylogenetic composition, but pathway level comparisons showed many bacterial pathways to be highly conserved across phylogeny. A second study was conducted to compare intestinal transcription and gut microbiota at critical early life stages (40 and 65 days post hatch) in a commercial strain of rainbow trout and a strain selectively bred for growth performance on a sustainable all plant-protein diet. Selected trout showed superior growth at early life stages and hundreds of genes and gut bacteria were identified as biomarkers of the select strain. As the first study to conduct high-throughput differential transcript usage (DTU) analysis on RNA sequencing data in an aquaculture species, results also highlighted some 74 intestinal genes that were expressed by different mRNA isoforms, depending on trout genetics and developmental stage. As in the first research chapter, the dynamics of gut microbiota communities across trout strains and ontogeny showed high congruency to that of transcriptome-wide intestinal gene expression profiles (Procrustes $m^2 = 0.19$, correlation

=0.9, $p \leq 0.001$). In the final study, naïve individuals from these same strains of rainbow trout were challenged with virulent infectious hematopoietic necrosis virus (IHNV) or *Flavobacterium psychrophilum*, (*Fp*) viral and bacterial pathogens that cause severe epizootics in salmonids, often with high mortality rates. Samples were collected at early (4-5 days post challenge [dpc]) and late (20-21 dpc) timepoints to track the dynamics of serum immune response, intestinal transcription, and gut microbial ecology. The select stain showed superior resistance to the bacterial challenge compared to the commercial strain (70.4% vs 94.8% mortality), but not the viral challenge (51% vs. 52%). Serum lysozyme and alternative complement activity were generally higher in the select strain. Intestinal transcription data indicated that DTU is an advantageous molecular mechanism utilized by rainbow trout to cope with both viral and bacterial infections, as many of the isoforms involved in DTU were related to crucial functions involved in effective disease response. Gene expression data showed rather classical responses to infection, with some clear differences between trout genetics as well. The IHNV challenge led to an influx of opportunistic bacteria not traditionally observed in the fish gut, though the community was slightly stabilized by the late recovery stage of disease. In the *Fp* challenges, the pathogen was found to take over the gut bacterial communities and showed dominant abundance in the gut during early infections, despite not being thought of as a target tissue of the bacterium. However, by the late recovery stage, beneficial commensal bacteria began recolonizing the gut of challenged select strain survivors, as the communities began to more closely resemble uninfected controls. Together, these studies provide many novel data on host and microbiota responses in cultured salmonids. The insights presented here have valuable implications on continued efforts to improve mucosal vaccines, selective breeding of finfish, fish health management, pre- and probiotic development, as well as our basic understanding of host-microbiota-environment interactions, in general.

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Table of Contents

List of Tables

List of Figures

Chapter 1: Current Perspectives on Finfish Microbiota and Direct-Fed Microbial Applications in Aquaculture

Abstract

Globally, the production of food fish has continued to grow at a pace unrivaled by other animal production sectors, with over half of the worlds seafood now coming from aquaculture. As aquaculture continues to grow, managing finfish health and nutrition in an economically and environmentally sustainable manner is imperative but has become more difficult due to multifaceted pressures facing the industry. Mounting research evidence on the interplay between finfish and the microbes inhabiting their nares, skin, gills, and especially gut, suggests that microbiota play an important role in the physiological processes of fish. Here, we review the status of microbiota research in finfish and application of direct-fed-microbial (DFM) strategies to improve finfish production performance in aquaculture. While many DFMs, including probiotic and synbiotic supplements, have been tested in aquaculture with varied outcomes, current implementation remains largely relegated to the research setting. However, it is expected that the continued and increasing pressure to eliminate antibiotic use and increase reliance on alternative diet formulations will make DFMs an integral part of sustainable large-scale aquaculture moving forward. Successful implementation will clearly hinge on a range of key factors and considerations, as will be addressed in this chapter.

Introduction

Aquaculture continues to be the fastest growing global food production sector, with an average annual growth rate of 5.9% between 2000-2015 (Zhou 2017). Increased production has been achieved through intensification of production practices, which present new difficulties in managing fish health and nutrition. As rearing densities and feed costs continue to rise, the aquaculture industry searches for novel means to manage fish health and improve the economic and environmental sustainability of feeding more fish. Similar to other livestock industries, the use of antibiotics to meet production goals has led to the global aquaculture industry receiving criticism as a potential source of antibiotic resistance, both in the environment and the human food supply (Cabello et al. 2016). Such concerns, along with

consumer influence from developed markets, have placed pressure on the industry to reduce chemical therapeutant use for control pathogens and ectoparasites (de Bruijn et al. 2018). Concurrently, industry intensification has increased the intensity of stressors placed on aquaculture fish through handling, crowding, and transport. As a result, losses to fish disease remain a significant challenge in aquaculture. While vaccinations offer an effective alternative to antibiotics and can aid in preventing some bacterial and viral diseases, the development time, specificity, and lack of efficacy that vaccines have in certain fish species or against certain types of pathogens leaves a demand for more alternatives in managing fish health (Magnadottir 2010; Ringø et al. 2014).

Furthering this problem, the intensification of global aquaculture has increased the production of feed-reliant species four-fold over the last two decades (Hasan 2017), increasing aquacultures dependency on commercial feed ingredients. Traditionally the intensive culture of finfish species in aquaculture, especially that of the higher value species like salmonids, relied on diets containing high amounts of fishmeal and fish oil as a means of providing balanced nutrition and palatability (Jobling 2016). However, rising costs and concerns of overexploitation of wild fish stocks used to supply those ingredients have shifted aquaculture diet formulations towards much higher inclusion of terrestrial feed ingredients, such as grains and legumes (Naylor et al. 2009). While these ingredients are more environmentally sustainable, they often contain antinutritional factors such as lectins, saponins, enzyme antagonists, antigenic irritants, or other phytochemicals (Krogdahl and Bakke 2015), for which fish have not evolved tolerance mechanisms, being evolutionarily naïve to most terrestrial ingredients. In many instances the replacement of high levels of fishmeal or fish oil with alternative ingredients produces some decrease in growth performance, nutrient utilization, gut health, microbiota composition, and overall health (Zhou et al. 2018; Gajardo et al. 2017; Kononova et al. 2019; Ringø et al. 2016). Despite these drawbacks, for aquaculture to continue growing and maintaining production in a sustainable manner, these alternative plant-based diet formulations are a certain part of aquaculture's future (Gatlin III et al. 2007; Naylor et al. 2009). Therefore, strategies to mitigate the deleterious impacts of antinutritional factors on digestive performance and gut health are required.

Direct fed microbials (DFM), namely probiotics, began receiving research and industry attention nearly three decades ago as a means of managing fish health in aquaculture (Gatesoupe 1994; Austin et al. 1995). Now as global aquaculture faces the present challenges, probiotics are the focus of much research. While probiotics appear to be a promising avenue for improving fish performance (Chauhan and Singh 2019; Hoseinifar et al. 2018), there are factors that must be considered for probiotic strategies to be implemented in a safe and effective manner in aquaculture. This chapter will focus on reviewing the current state of research related to the use of DFMs in aquaculture finfish species. While finfish represent 61.7% of the species (Zhou 2017) and 67% of the market share of global aquaculture (FAO 2018), our focus on finfish species is certainly not meant to discount the importance of probiotics in other areas of aquaculture, including the culture of crustaceans such as shrimp (Kumar et al. 2016; Farzanfar 2006) and various mollusks (Grandiosa et al. 2018; Huddy and Coyne 2014; Jiang et al. 2013; Prado et al. 2010; Desriac et al. 2014).

Current Understanding of the Homeostatic Microbiota of Fishes

Successful implementation of DFM strategies in aquaculture requires an understanding of the structure and function of the healthy homeostatic microbiota, how the microbial consortia interact with one another and their particular host, and the key factors related to the host and rearing environment that regulate the microbiota. Unfortunately, the study of microbiota in finfish aquaculture has been somewhat complicated by the diversity of species and conditions utilized in the industry, with the Food and Agricultural Organization of the United Nations (FAO) reporting 396 different finfish species, across numerous genera, being farmed globally in 2018, though the top twenty species account for 84% of total production (FAO 2018). Regardless, the diversity in aquaculture greatly surpasses the number of species raised in other forms of animal agriculture, which typically rely on a single species for most production. Fortunately, our basic understanding of fish microbiota has vastly increased over the last five to ten years, thanks in large part to advances in technologies like next-generation sequencing (NGS) and a surge in research interest (Kelly and Salinas 2017; de Bruijn et al. 2018; Wang et al. 2018b), much of which has been related to aquaculture species (Llewellyn et al. 2014; Romero et al. 2014).

Early studies on the microbiota of fish date back to at least 1915 when the stomach, intestine, their contents, and the gills of wild caught herring were analyzed for microbes via

plate culture, as described by Obst (1919). However, with early culture-dependent techniques, studies often failed to detect even a single culturable microbe from the fish intestines (Margolis 1953; Obst 1919), and therefore it was long thought that the gut microbiome, as it is now termed, was of little relevance to the physiology of fishes. Although by the 70's, seminal culture based studies on the normal "microflora", as it was called, of salmonids and other species were conducted that not only characterized the culturable microbiota, but also began to show the influence that host and environmental factors can have on the microbial composition (Yoshimizu and Kimura 1976). Now, with the common use of culture-independent molecular techniques such as NGS to bolster or replace traditional culture-based methods, our abilities to identify and characterize the microbiota of fish has greatly advanced. To date, the bacterial composition of the gut microbiota of nearly all the top aquaculture finfish species has been characterized and important sources of variation in the microbiota of farmed fish have been identified. Though by far the most valuable information that has arisen in recent years and will continue to be unveiled in the coming years, are lessons learned on the roles that microbiota play in their hosts' physiological and immunological functions, as well as the mechanisms of interaction between host and microbiota.

The microbiota of fish consist of a diverse consortium of microbes, and while many factors affect the phylogenetic composition of the microbiota, generally the microbiota are from within the bacterial phyla Actinobacter, Bacteroidetes, Chloroflexi, Fusobacteria, Firmicutes, Plantomycetes, Proteobacteria, Tenericutes, and Verrucomicrobia and include aerobes, facultative anaerobes, and obligate anaerobes, though each will vary in abundances depending on host and environment (Llewellyn et al. 2014; Sullam et al. 2012; Wang et al. 2018b; Nayak 2010b). Cyanobacteria are also commonly identified among the fish microbiota in NGS studies. However, this is often the result of detecting DNA reads of the prokaryotic phylogenetic marker gene 16S rRNA mapped to either the class Chloroplast, which represent genomic material derived from plant items in the diet, or environmental Cyanobacteria that transiently pass through the digestive tract (Tarnecki et al. 2017). Therefore, despite being reported as such in many studies, Cyanobacteria is typically not considered a functional member of the fish microbiota (Wong et al. 2013). In addition, yeast from two fungal phyla, Ascomycota and Basidiomycota, can be detected in commensal

association with fish, though their prevalence and function in fish gut microbiomes remains understudied (Navarrete and Tovar-Ramírez 2014; Huyben et al. 2017). The same is true for archaea, protozoa, and bacteriophage, for which even less is known (Merrifield and Rodiles 2015).

In general, the total microbial load of the gastrointestinal tract of fishes is considerably less than that of warm-blooded animals, and while estimates range drastically depending on host and environmental variables, aerobic and anaerobic microbial concentrations ranging from 10^4 to 10^{11} CFU g^{-1} are typically reported for fish intestinal contents, with bacteria by far making up the majority (Nayak 2010b; Egerton et al. 2018). The richness and diversity of microbiota detected in finfish species can range drastically, though typically is either slightly below or equal to that seen in terrestrial livestock such as poultry. Using NGS 16S rRNA gene surveys, it has been shown that the intestine of chickens and turkeys harbor some 900 and 500 species-equivalent operational taxonomic units (OTUs; 97% sequence identity), corresponding to 117 and 69 genera, respectively (Wei et al. 2013). Similar studies in Atlantic salmon, for example, have detected approximately 600 OTUs in the digesta and 180 OTUs in the intestinal mucosa (Gajardo et al. 2016) showing the similarity in microbial richness. However, it should be noted that differences in study design and analysis techniques can also greatly influence such comparisons between studies.

Physiological Function of Fish Microbiota

The microbiota of fish merits the significant amount of research attention that it has received because of its perceived, and in some cases proven, applications in improving fish performance. Much of the insight gained on host-microbiota interactions and the role of microbiota on fish physiology has been learned from germ-free or gnotobiotic models. At first this was primarily conducted using the zebrafish model, with work by Rawls and others showing that the presences of intestinal microbiota in the host regulates the expression of an array of genes involved in epithelial proliferation, promotion of nutrient metabolism, and activation of innate immunity (Rawls et al. 2004; Rawls et al. 2006). As a testament to the relevance of microbiota to host physiology, germ-free zebrafish are typically only able to survive to about one-month using sterile diets and current techniques, suggesting there are critical metabolic, nutritional, or immunological roles played by the microbiota (Melancon et al. 2017). More recently, gnotobiotic research on aquaculture species has shown that

colonizing a germ-free sea bass *Diecentrarchus labrax* with commensal microbes affects whole-body gene expression involving pathways in cell proliferation and turnover, cell adhesion and membrane permeability, reactive oxygen species metabolism, iron trafficking, and many aspects related to innate immunity (Schaeck et al. 2017).

The primary means by which the innate immune system of fishes is known to interact with microbiota is through interactions between microbe associated molecular patterns (MAMPs) such as liposaccharide (LPS) or peptidoglycan that are present on the surface of many commensal and pathogenic microbes alike. These MAMPs are then detected by host pattern recognition receptors (PRRs) such as the toll-like-receptors (TLRs) present on host epithelial and myeloid cells, which activates the release of various cytokines and induces intracellular MyD88 signaling pathway that is crucial to the maintenance of epithelial homeostasis (Kelly and Salinas 2017; Rauta et al. 2014). The release of these cytokines and activation of signal transduction pathways serves as a basal priming signal to the immune system of fish and helps maintain homeostasis, both in terms of morphology and immune defense. In addition, recent studies have even shown that mutualistic gut microbiota produce signaling proteins that have immunomodulatory effects on the host and which are required to maintain gut homeostasis and prevent excessive inflammation in the host (Rolig et al. 2018). In terms of disease protection, there are multiple mechanisms by which microbiota protect their host. The simplest means by which microbiota do this is competitive niche exclusion, in that the presence of a commensal microbe ensures that a pathogen cannot inhabit or adhere to potential infection sites in the host, thereby excluding pathogens from a host mucosal site that is already colonized by commensal microbes. Commensal microbiota can directly compete with other potentially harmful microbes by other means as well, either through the production of antimicrobial molecules or through nutrient competition. Bacteriocins, or antimicrobial peptides produced by bacteria that inhibit or are toxic to other bacteria, are one means by which commensal allochthonous microbiota can protect the host from invading pathogens (Desriac et al. 2010). Other antimicrobial mechanisms of microbiota include the production of organic acids, antimicrobial lipids, antibiotics, and hydrogen peroxide (Hoseinifar et al. 2018). Competition for nutrients, including iron, a common rate limiting nutrient in the microbial world, is yet another means by which commensal microbes can outcompete new, potentially harmful microbes. Siderophores, ferric iron chelating molecules

secreted by bacteria, produced by commensal microbes are thought to moderate the bioavailability of iron, thereby limiting the potential for growth of invading, unestablished microbes (Tan et al. 2016). Though it should also be noted that siderophores can be a virulence factor in pathogens as well if the high affinity iron binding molecules disrupt the host's homeostatic iron metabolism (Wilson et al. 2016).

The role of the gut microbiota in the nutrition and metabolism of fishes is not to be discounted either. The microbiota of fish are known to produce a range of enzymes which help their host harvest nutrients from their food items, including amylases (Sugita et al. 1996), cellulases (Ni et al. 2014), proteases, lipases, phytases, tannases (i.e. tannin acyl hydrolase), xylanases, and chitinases (Ray et al. 2012), though the presence of each may vary with host, diet, and environment. Additional evidence from the zebrafish model has shown that the gut microbiota of fish play a key role in fatty acid (FA) metabolism by showing that the presence of microbiota not only increases the capacity of enterocytes to uptake and accumulate lipids but also increases extra-intestinal lipid metabolism (Semova et al. 2012) and host gene expression related to lipid metabolism (Sheng et al. 2018). Furthermore, the lipid profile of *Shewanella putrefaciens* has been shown to be composed of approximately 40% eicosapentaenoic acid (EPA), an important polyunsaturated fatty acid in aquaculture finfish, therefore this common fish inhabitant and other fish microbiota may even be a source of EPA biosynthetic pathways that can be utilized by the host (Yazawa 1996; Austin 2006). Another major metabolic role of the gut microbiota, is the fermentation of non-digestible carbohydrates, into short chain fatty acids (SCFA) such as butyrate and acetate, which are known to improve cell turnover and proliferation in the gut and serve as an energy source for host enterocytes (Piazzon et al. 2017). Gut microbiota may even have a role in the proper development of pancreatic tissues that regulate glucose metabolism in fish through the production of specific bacterial proteins that stimulate proliferation of insulinogenic beta cells in the host (Hill et al. 2016). Vitamin production is yet another of the nutritional and metabolic attributes offered to the host by fish microbiota, as many common commensal fish microbes are capable of synthesizing cobalamin (vitamin B-12), as well as vitamin precursors (LeBlanc et al. 2013; Sugita et al. 1991), and the presences or absences of such microbes in a host can dictate whether or not that species of fish has nutritional requirements for certain vitamins (Sugita et al. 1991). In addition, research on man, as well as murine and

zebrafish models, suggest that microbiota have the potential to modulate the central nervous system control of appetite and energetics, both peripherally by influencing intestinal serotonin levels or centrally through effects on circulating metabolites and direct vagal nerve stimulation (Volkoff and Butt 2019).

Factors Influencing the Fish Microbiota

All metazoan microbiomes consist of dynamic microbial communities, which vary in their phylogenetic composition, abundance, and metabolic functions according to many intrinsic and extrinsic factors with varied time scales. The intrinsic, or host-associated factors are those features which are biologically inherent to a fish species such as aspects of its physiology or anatomy. The extrinsic factors represent environmental variables, like the physiochemical make-up of the water and diet, or the rearing density within an aquaculture system. The structural and functional composition of a fish microbiome is under constant influence from a concert of these co-occurring variables. A better understanding of the host-associated and environmental factors, which are responsible for shaping the microbiome of aquaculture finfish, will enable more efficient identification and implementation of effective DFM strategies. Understanding and controlling these variables in future research is crucial for facilitating interpretation of the complex interactions that occur in the fish microbiota, allowing better elucidation of the keystone microbial species or communities which confer physiological benefits to the host fish.

Host-Associated Factors

Host Phylogeny

Due to the number of host species in aquaculture it is important to understand the intricacies of each individual host species' microbiota, but also to be able to identify general mechanisms and beneficial host-microbe relationships that are conserved across groups of finfish species so that the knowledge can be applied in the diverse aquaculture industry. Host phylogeny somewhat indirectly influences the composition of a host microbiota because of its correlation with a multitude of differences in other host-associated and environmental factors (i.e. diet, environmental conditions, etc.) that cannot be separated from the genetic or phylogenetic background of the fish species. Li et al. (2012) showed that despite cohabitation, host phylogeny still yielded differences in the gut microbiota composition in larvae from four different aquaculture fish species from within the family Cyprinidae.

Furthermore, the impact of host phylogeny on fish microbiota structure and function, may be scaled relative to the genetic disparity of the hosts genetics, as it has been shown that differences in gut microbiota can be correlated with host genetic distances among populations of fish (Li et al. 2014; Smith et al. 2015), though similar correlations were not detected in a controlled aquaculture setting when diet and environment were controlled among hosts (Bledsoe et al. 2018). However, it is certainly known that host phylogeny and host genetics can impact the structure and function of fish microbiomes (Llewellyn et al. 2014; Tarnecki et al. 2017; de Bruijn et al. 2018; Wang et al. 2018b).

Host Ontogeny

The majority of finfish in aquaculture are oviparous, so the first interaction between fish and the environment occurs when eggs are deposited, or collected as is often the case in aquaculture, from a gravid female. Classically it has been thought that colonization of the surface of the egg begins immediately after being released in the environment, while the unhatched larvae within the eggs are precluded from interactions with environmental microbes by the protection of the egg chorion. However, it has also been shown that certain pathogens, including *Flavobacterium psychrophilum* and *Renibacterium salmoninarum*, can be vertically transmitted, or passed from mother to offspring through deposition within the chorion of an egg (Brown et al. 1997; Bruno and Munro 1986), suggesting fish may become inoculated with bacteria prior to hatching. Though this hypothesis merits more investigation, it is now relatively well accepted that microbes may not only be present on the exterior, but also to some extent the interior of fish eggs (de Bruijn et al. 2018). Once the eggs hatch, the environmental microbiota present on the egg and the surrounding water serve the important role of inoculating the fish mucosa with microbiota (Hansen and Olafsen 1989). While the colonization of the external and internal mucosal surfaces is first achieved by inoculation from the water, when the fish reach a first feeding stage the diet or live-feeds begin to have a substantial impact on shaping the microbiota, particularly in the gut (Ingerslev et al. 2014b). Though in aquaculture this colonization process is often confounded by egg disinfection practices that may be used to reduce the incidence of disease (Llewellyn et al. 2014). The long term effects of these early disinfection protocols on the microbiota of fishes in aquaculture has been poorly studied, though from an ecological perspective these practices represent a drastic perturbation to the homeostatic colonization dynamics and is likely to

increase the presence of fastidious opportunistic microbial species, which is the ecological niche of many fish pathogens (De Schryver and Vadstein 2014).

As a general rule, most fish species in aquaculture exhibit distinct life-stages (i.e. larvae, fry, juvenile, yearling, broodstock) which are known to be associated with unique changes in physiology and nutritional requirements. Not surprisingly, as the host's physiology and morphology undergo developmental changes, so too does their microbiota. The intestinal microbiota of the most farm-produced fish species in the U.S., channel catfish *Ictalurus punctatus*, has been shown by multiple groups to vary across host ontogeny, with results suggesting that gut microbiota are highly compositionally dynamic early in life, yet as the fish reach the juvenile-fingerling stage, microbial communities appear to begin stabilizing (Bledsoe et al. 2016; Burgos et al. 2018; Razak et al. 2019). Related studies conducted on other aquaculture species have also identified a similar pattern of temporal dynamics in fish microbiota composition (Giatsis et al. 2014). However, those studies, as well as most other studies on fish microbiota, only track the microbial community throughout the juvenile stage, potentially missing other important changes that may occur at later stages of the aquaculture production cycle including as the fish reach a marketable size or begin to achieve reproductive maturity. Because the early life stages of fish are so dynamic and represent a time when the fish are the most susceptible to stressors and disease, a majority of studies on fish microbiota have focused on these life stages (Llewellyn et al. 2014). Although, this has resulted in a shortage of studies that continue to track the microbiota of fish through later life stages, without confounding changes in environmental variables that are often inherent to aquaculture production.

Host Body Site

Commensal microbiota are known to inhabit every mucosal surface of fish, including the nares, gills, skin, and intestine (Lowrey et al. 2015). Recently, it has even been shown that commensal microbiota may also inhabit a visceral organ, the swim-bladder, in more ancestral physostomes fishes whose swim bladder is originally inflated via a direct connection to the gastrointestinal tract early in life (Villasante et al. 2018). By far, most research on fish microbiota has been focused on the gut microbiota, because of its expected role in not only disease but also host nutrition, metabolism, and growth performance. However, in recent years the skin, gill, and nare microbiome of fishes have begun receiving

more attention (Brown et al. 2019; Boutin et al. 2012; Guivier et al. 2018; Rosado et al. 2019) and while it is true that these host-associated microbiota sites serve less of a metabolic role, their utility in host immunity has certainly been proven (Lowrey et al. 2015). When comparing the microbiota composition of the various sites from a single host, there is some compositional overlap, but in general the various mucosal sites of a single fish host will show differences in microbiota structure and function (Brown et al. 2019; Boutin et al. 2012; Guivier et al. 2018; Rosado et al. 2019). Despite the discrepancies in composition between body site, the microbiota are influenced by the systemic immune system, and recently it's been shown that skin and gill microbiota composition may even be a biomarker for intestinal health (i.e. lymphocytic enteritis) in farmed yellowtail kingfish *Seriola lalandi* (Legrand et al. 2018). Not only are there compositional differences across body sites, but within a particular mucosal site there can even be spatial differences in microbiota composition. This has been best shown for the intestinal microbiome of fish, where the microbiota composition is known to change in accordance with spatial differences in pH, nutrient content, and oxygen concentration associated with different sections of the intestinal tract (Lowrey et al. 2015; Yang et al. 2019). Although in some cases, the difference between the allochthonous (transient) microbiota found in the digesta and autochthonous (mucus colonized) microbiota associated with the host intestinal mucosa can be even more pronounced than spatial differences (i.e. proximal vs. distal) (Gajardo et al. 2016).

Host Immunity

Understanding of the teleost immune system has advanced greatly in the last decade with many interesting new discoveries pertaining to host immune regulation of microbiota. Just as in other animal species, the host-associated microbiota of fishes is under immune regulation from the host, but there are some key differences in the immune system of teleost finfish worth considering. Finfish exhibit both innate (non-specific) and adaptive (acquired) immunity similar to higher vertebrates, and many of the key cellular and humoral factors known in mammals are also present in fish. Typically fish are thought to have a slightly higher reliance on the non-specific or innate immune response, especially early in life, and have been found to possess a larger diversity of genome encoded complement pathway components (Magnadóttir 2006) and antimicrobial peptides (AMP) (Shabir et al. 2018) in comparison to mammals. The mucus layer which is continuously produced by goblet cells

along the mucosal surfaces of fish also serves as an important buffer between the microbiota or potential pathogens and the host, and this mucus often contains a range of immunologically active molecules, including complement components and AMP factors, which further protect the host (Kelly and Salinas 2017). The potential for higher levels of non-specific regulation from the fish immune system likely impacts the microbiota structural dynamics. In fact, it has been suggested that host-microbiota interactions served an important evolutionary pressure in selecting for greater reliance on adaptive immunity in higher vertebrates, because it enables the host's immune system to differentiate between the beneficial microbiota and targeted pathogens (Lee and Mazmanian 2010). This implies that the microbiota in fish is regulated with a greater level of interaction yet less specificity than that of higher order organisms, which may increase compositional variance and phylogenetic turnover of the microbiota in fish.

Despite the exaggerated reliance on innate immunity in fish, the adaptive immune response in most finfish species in aquaculture is certainly of importance. Fish lack bone marrow and lymph nodes, which serve significant roles in mammalian acquired immunity, and instead, fish rely on the thymus, head kidney, and spleen as central immune tissues (de Bruijn et al. 2018). Peripherally, each mucosal surface has an associated lymphoid tissue (MALT), including the skin (SALT), gill (GiALT), nasal pharyngeal (NALT), and gut (GALT), which are dedicated to the local immune defense of that particular mucosal site (Salinas 2015). The MALT of fish is typically more diffuse and less structured than comparable lymphoid tissues in higher order vertebrates. As an example, fish lack Peyer's Patches, the primary site of antigen sampling by dendritic cells in the mammalian gut (Pérez et al. 2010), though recently an M-like cell that enables antigen sampling by dendritic-like cells similar to that found in Peyer's Patches was discovered in trout and salmon (Fuglem et al. 2010). Similarly, it was discovered that salmon possess an organized interbranchial lymphoid tissue in their gills where T-cells appear to aggregate (Haugarvoll et al. 2008), although it still lacks the organized topology of mammalian primary and secondary immune tissues (Kelly and Salinas 2017). However, these examples of semi-organized lymphoid tissue in fish emphasizes the evolutionary importance of the teleost immune system from a comparative perspective and shows the relative importance of adaptive immunity within fish.

Another difference in adaptive immunity between fish and higher order vertebrate is related to the immunoglobins (Ig). Only three isotypes of Ig are found in finfish, including IgM, IgD, and IgT/Z, but because IgD is not found on the mucosal surfaces, IgM and IgT are the most likely immunoglobulins to interact with microbes in fish (Parra et al. 2015). In addition, teleost B-cells do not undergo class switch recombination despite the fact that most teleost fish express multiple Ig isotypes, as well as the enzyme essential for Ig class switching, activation-induced cytidine deaminase (AID) (Parra et al. 2015). Just like in mammals, immunoglobulins can be membrane bound on the surface of B-cells or secreted as soluble Ig by activated plasma cells. While IgA is the mucosal immunoglobulin in mammals, IgT serves that function in fish, and along with IgM can be secreted into the lumen of the intestine or along the mucosa of other tissues where they can antagonize microbes by coating their surface, preventing adherence to host cells (Kelly et al. 2017; Xu et al. 2013). As in mammals, most teleost fish B and T-cells can differentiate into memory subpopulations that are long-lived and can provide highly specific protection against previously recognized pathogens or antigens to protect the host from secondary infections.

While some of the conserved traits of finfish immunity are unique compared to higher order vertebrates, the diversity of fish species used in aquaculture is accompanied by a range of unique immune adaptations within fish as well. One such example is seen in Atlantic cod *Gaddus morhua*, who are known to lack MHC II molecules and the associated ability to activate CD4 helper T-cells (Star et al. 2011). As a trade-off, these cod possess a much greater repertoire of TLRs and genes encoding MHC I proteins (Solbakken et al. 2016; Star and Jentoft 2012). Together, these unique traits of the cod immune system are sure to impact how the fish regulates not only pathogens, but also their commensal microbiota. This is just one example of the immunological diversity displayed by the wide-range of finfish species reared in aquaculture and highlights the importance of understanding the immune system of the host species while considering the fish microbiota structure or function. Furthermore, the immune regulation of microbiota during disease has been poorly studied in finfish (de Bruijn et al. 2018), but the immunological burden of pathogen infection is sure to modulate regulation of commensal microbes as well. Currently, few studies have tracked the effects of disease on host microbiota or compared that of healthy and diseased hosts (Brown et al. 2019; Legrand et al. 2018; Parshukov et al. 2019). More such research on the effects of

disease and compromised immunity on regulation of fish microbiota would certainly provide greater insights into fish microbiota structural dynamics.

Host Trophic Level

Trophic level (i.e. herbivore, omnivore, carnivore) has been shown to be the strongest biotic (host-associated) influence in terms of explaining differences between the microbiota of different fish species (Sullam et al. 2012). A fish's so-called trophic level is a product of evolutionary forces surrounding its natural diet in the wild and associated position in the food web, as well as the digestive morphology that evolved in that species to allow it to efficiently digest and assimilate its natural diet. For example, a highly carnivorous fish species such as Atlantic salmon, who's natural diet consists of high protein and lipid content items, possess a relatively large, muscular, low-pH cardiac stomach, high surface area pyloric ceca for fat digestion and absorption, and a relatively short, straight intestinal tract. On the other hand, an herbivores species, such as the grass carp, lacks a stomach and possess a rather long coiled intestinal tract that permits more time for microbial fermentation of the hosts' otherwise less digestible diet. These stark differences in digestive morphology and physiology explain a significant portion of the differences in microbial composition between these two species' microbiota (Ni et al. 2014; Gajardo et al. 2016). Generally speaking, the diversity and richness of microbes present in the gut of fishes is positively correlated with their degree of herbivory (Wang et al. 2018b), although other factors can certainly influence such comparisons. In an intriguing study design, reciprocal hybrid fish lines were generated by making separate crosses between a carnivorous (topmouth culter *Culter* alburnus) and herbivorous species (blunt snout bream *Megalobrama amblycephala*) to investigate the role of genetics and digestive morphology on gut microbiota composition (Liu et al. 2018). The two original parent species showed significant disparity in intestinal morphology (relative density, relative mass, and relative length) and microbiota composition (alpha diversity and beta diversity), but interestingly the hybrid strains were almost exactly intermediate to that of the two parent species in nearly all indices measured, morphological and microbiological (Liu et al. 2018), highlighting the role of host trophic level on structuring microbiota composition. The influences of host trophic level on the microbiota of marine fishes are well discussed by Egerton and colleagues (2018) and their review shows that while many

phylogenetic groups of bacteria are detected across hosts from disparate feeding guilds, the prevalence of certain microbial clades can often be correlated to the trophic level of the host.

Environment-Associated Factors

Diet and Feeding Pattern

While consideration of trophic level is important when comparing the microbiota structure and function between different finfish hosts, differences in dietary ingredients or feeding rates can alter the microbiota of finfish as well. With alternative dietary formulation an active area of research in aquaculture, there are a wide range of dietary ingredients tested and utilized in both aquaculture research and industry, all of which are likely to have some unique influences on the structure and function of the homeostatic commensal microbes of the host. Dietary components which are known to influence the microbiota of finfish include diet-type (live vs. pelleted), and dietary sources of lipid (levels, sources, and biochemical profile), protein (level, source), minerals (i.e. iron), and carbohydrates (levels, structure, host-digestibility) (Ringø et al. 2016). In particular, the transition in aquaculture towards plant-based diets has been shown numerous times to alter the gut microbiota of fish (Gajardo et al. 2017; Zhou et al. 2018). A recent comprehensive review by Kononova et al. (2019) of the effects of soy proteins on salmonids, a high-value group of aquaculture fishes that includes Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss*, highlights the often negative implications that plant proteins have on the gut microbiota of fish, particularly in salmonids who are among the worst affected by such ingredients. Though in certain situations alternative dietary ingredients have also been found to have minimal impacts on the gut microbiota in other fish species such as channel catfish (Schroeter et al. 2018). The impacts of diet on the microbiota appear to be particularly important in early life-stages of fishes, as the diet-type at the first feeding stage is thought to have lasting impacts on the future trajectory of the host microbiome (Ingerslev et al. 2014a; Ingerslev et al. 2014b). In addition to dietary formulation, the feeding rate can also impact host microbiota. In grass carp, it has been shown that feed-deprivation reduces the abundance of microbiota in the phyla Dictyoglomi, Firmicutes, Fusobacteria, and Synergistests while increasing Euryarchaeota, Actinobacteri, and Fibrobacteres (Ni et al. 2014). Similarly, in the Asian seabass *Lates calacerifer,* starvation shifted the microbiota composition towards a higher abundance of Bacteroidetes and a reduction in Betaproteobacteria, which also significantly

altered the abundance of nine functional orthologous gene clusters in the microbiome (Xia et al. 2014).

Environmental Microbiota

The most obvious and differentiating feature between terrestrial livestock and finfish is the aquatic environment. While terrestrial livestock interact with environmental microbes that influence their microbiota compositions, such interactions are exacerbated in the aquatic environment owing to waters capacity to sustain and transfer viable microbes. This property of the aquatic environment results in greater potential for translocation of microbes between the environment and fish host (De Schryver and Vadstein 2014). In a study comparing the impact of rearing habitat on the microbiota of Nile tilapia *Oreochromis niloticus* larvae, it was observed that the influence of the dietary microbiota (microbes present in the diet) in shaping the fish gut microbiota was minimal in comparison to that of the water, as microbiota found in the water had much greater overlap in composition with the fish gut than did that of the diet (Giatsis et al. 2015). Furthermore, results from that study show that physiochemical parameters can influence the presence or abundance of microbes in the water, and therefore physiochemical parameters can indirectly impact fish associated microbiota by altering environmental inoculation pressures (Giatsis et al. 2015). Other influences on the microbial load and composition of the culture water in aquaculture include biological variables such as fish density and nutrient load (i.e. feeding rate), which may be positively correlated with an increase in the level of heterotrophic opportunistic pathogens that are a constant presence among the water microbiota of most aquaculture operations (Blancheton et al. 2013; Derome et al. 2016; Rosado et al. 2019). Of course, the impacts of environmental microbiota in aquaculture will vary with the type of production system used, as aquaculture encompasses an array of production techniques that include methods as diverse as land-based recirculating aquaculture systems (RAS), outdoor constant flow-through systems, static pond culture, or net-pen cages sited in natural waters. Although the aquatic environment places fish and their microbiota under greater influence by environmental microbes, especially early in life, the microbial communities associated with fish hosts are maintained structurally separate from the environment (i.e. diet and water) by factors within the host (Bakke et al. 2015; Giatsis et al. 2016; Bledsoe et al. 2016).

Environmental presences of antibacterial agents or other xenobiotics, as may occur in aquaculture either intentionally as an administered disease treatment or unintentionally from environmental contamination of water sources, can have a strong influence on shaping fish microbiota communities as well. Work by Mohammed and colleagues (2015) has shown that the intentional use of chemical therapeutants in aquaculture to control disease outbreaks, such as the potent disinfectant potassium permanganate, can disrupt the homeostatic regulation of fish microbiota, particularly the skin microbiota, resulting in a microbial imbalance that inadvertently leaves the host even more vulnerable to disease. It has also been shown that feeding of antibiotic-treated diets in high-intensity pond aquaculture can have indirect effects on the environmental microbes, drastically shifting the phylogenetic composition of the microbial communities present in the environment (water and sediment), but also the genetic structure of those microbes including a dynamic gain and loss of antibiotic resistant genes (Zeng et al. 2017). While this alone represents an environmental concern, it is also problematic for the health of the fish population, as it suggests host-associated microbes are also gaining resistance to the therapeutants.

Physiochemical Properties

Salinity has been shown to be the major environmental determinant of microbial community composition across the globe (Lozupone and Knight 2007) and the influence of salinity is certainly pertinent to the composition of fish microbiomes as well. Aquaculture species exhibit a wide range of osmoregulatory preferences and adaptations. These include freshwater or seawater stenohaline species who can only tolerate minor changes in environmental salinity, euryhaline species than can adapt to relatively wide ranges of water salinities with some acclimation, as well as anadromous and catadromous species which vary their osmoregulatory capabilities by life stage. A meta-analysis of fish microbiome data by Sullam et al. (2012) suggested that salinity (freshwater vs. marine host) was the abiotic factor responsible for explaining the greatest amount of variation among fish gut bacterial community composition. An experiment using a euryhaline aquaculture species, tilapia, showed that after acclimatizing to hypersaline conditions (24 psu) the gut microbiota community as a whole shifted in comparison to the control, with the abundance of microbes within the phyla Actinobacteria decreasing significantly with salinity, while Fusobacteria were increased (Zhang et al. 2016). The anadromous Atlantic salmon also offers insights into

the effects of salinity. Two different groups have tracked the gut microbiota of Atlantic salmon transitioning from freshwater to saltwater within an aquaculture setting, and while there is disagreement in the microbial compositions and patterns of diversity detected between the two studies, a highly significant shift in overall microbial structure and function was detected in both (Dehler et al. 2017; Rudi et al. 2018), with similar shifts known to occur for the skin microbiota of Atlantic salmon as well (Lokesh and Kiron 2016).

Temperature is also an important physiochemical parameter to consider. Most finfishes in aquaculture are poikilothermic, meaning their body temperatures vary with the surrounding temperature of the environment, therefore temperature can have a rather strong influence on fish metabolism, immunity and behavior (Bowden et al. 2007). In a poikilothermic host, the effects of temperature on the microbiota are both direct and indirect because environmental temperature not only effects the metabolism of the microbes directly, but also indirectly through alterations made to their host's physiology. In comparison to the stable, regulated body temperature of mammalian terrestrial livestock, thermal variation likely plays a larger role in shaping the composition and function of fish microbiota. Huyben et al. (2018) showed that even moderate differences in temperature (11°C vs 18°C) effected the microbiota composition of rainbow trout more so than a dietary treatment that replaced 40% of the fishmeal with a dried *Saccharomyces cerevisiae* product. Further evidence of temperature having a more profound effect on the gut microbiota than a dietary change was shown in Atlantic salmon reared in marine net pens, as Neuman et al. (2016) used culture techniques to show that lactic acid bacteria were replaced by potentially pathogenic *Vibrio* species during warmer months, with a correlated plummet in the metabolic capacity of the microbiota, an index based on the microbiome's ability to metabolize various carbon sources. In addition to temperature and salinity, other physiochemical parameters of a fish's environment are likely to influence bacterial communities as well, as Giatsis et al. (2015) found significant correlations between water conductivity, pH, PO_4-P, and NO_3-N and differences in bacterial communities in the water of a tilapia aquaculture systems. Unfortunately, few studies on the microbiota of fish report details of physiochemical properties of the rearing water and therefore little is currently known about their influence on finfish microbiota.

Inter-individual Variations

Despite the many host-associated and environmental factors that are known to alter the presence and abundance of some microbial constituents of fish, there is also evidence of an often rather large group of core microbiota within many fish species. The concept of core microbiota, a set of microbes that are found in association with a particular host, irrelevant of intrinsic or extrinsic factors, was originally coined in humans (Turnbaugh et al. 2009; Caporaso et al. 2011) and was later popularized in fish when a shared core of microbe were found in zebrafish of disparate origins and life histories (Roeselers et al. 2011). It is now rather common for studies on the microbiota of fish to identify at least some portion of microbiota which are resilient to varying conditions. For instance, separate studies on rainbow trout have shown a relatively large proportion of the microbes that inhabit the trout gut to be present irrelevant of diet and rearing density (Wong et al. 2013) or habitat (laboratory aquarium vs. freshwater net pens) (Lyons et al. 2017). While salinity is known to have a strong influence on microbial communities and freshwater to seawater transfer is known to invoke a shift in the gut and skin microbiota of Atlantic salmon, as discussed above, all studies on the topic have also identified a subset of microbes that remain associated with the host despite drastic changes in the environment (Lokesh and Kiron 2016; Dehler et al. 2017; Rudi et al. 2018). Furthermore, stability in the functional metabolic capacity of a host microbiome is likely more relevant than compositional stability, and in some cases changes in the taxonomic composition of a microbial community can yield little to no change in functional capacity (Huttenhower et al. 2012).

Contrary to the sharing of core microbes among many hosts, it is also common to observe rather high inter-individual variation in microbiome compositions of some fish populations (Boutin et al. 2014; Fjellheim et al. 2012; Bakke et al. 2013). Because many sectors of finfish aquaculture rely on relatively unselected stocks that have experienced minimal domestication and selective-breeding pressure as compared to terrestrial livestock, within-population genetic variance and phenotypic plasticity can be relatively high in aquaculture (Teletchea and Fontaine 2014). Therefore, the genotypic variance in many aquaculture populations further helps to explain inter-individual variations in microbiota compositions (Boutin et al. 2014). The number of ecological perturbations to the colonization dynamics of the finfish microbiota within aquaculture (i.e. disinfection practices, host stress,

antibiotics, etc.) is yet another source of within-population variance, as such perturbations have been shown to results in stochastic alterations and temporal instability in the composition of animal microbiomes (Zaneveld et al. 2017).

Manipulating Fish Microbiota: Current Status of DFMs in Aquaculture

According to the FAO and an expert panel of the International Scientific Association of Probiotic and Prebiotics (ISAPP), probiotics are considered to be "*live microorganisms that, when consumed in adequate amounts confer a health benefit on the host*", though it is also suggested that more precise definitions may improve differentiation of a diverse set of microbial products now available (Hill et al. 2014). As a more aquaculture specific definition, Merrifield et al. (2010) posited *"a probiotic organism can be regarded as a live, dead or component of a microbial cells, which is administered via the feed or the rearing water, benefiting the host by improving disease resistance, health status, growth performance, feed utilization, stress response, or general vigor, which is achieved at least in part via improving the hosts microbial balance or the microbial balance of the ambient environment"*. In line with that view, Hai (2015a) provided an even more all-encompassing definition for aquaculture probiotics as *"live and/or dead microbial feed supplements or water additives in the form of mono-, multiple-strains, or in combination with prebiotics or other immunostimulants, which are administered to improve the rearing water quality, to enhance the physiological and immune response of aquatic animals, and to reduce the use of chemicals and antibiotics in aquaculture".* However, there is still debate as to whether in-activated, dead, or lysed microbial products should be considered as true probiotics as stated by the ISAPP, *"development of metabolic by-products, dead microorganisms, or other microbial-based, nonviable products has potential; however, these do not fall under the probiotic construct*". Here, our focus is primarily on live, viable probiotics aimed at improving fish performance, at least partially through alterations to the native host microbiota, though this should not discount the utility of inactivated or otherwise non-viable microbial products and their immunomodulatory effects on finfish in aquaculture (Newaj-Fyzul and Austin 2015).

Bibliometric Analysis of Aquaculture Finfish Probiotic Literature

To assess the current status of probiotics research in aquaculture, a bibliometric analysis was conducted on a query of the current literature in the ISI Web of Knowledge

(www.webofknowledge.com) and Scopus (www.scopus.com) scientific databases. The search terms “fish” AND “probiotic” AND “aquaculture” were used, with results filtered to include only primary research articles. These search terms were chosen to minimize unrelated articles, while still capturing a good representation of the current literature. The results from the two literature databases were merged and duplicate entries were removed, resulting in a total set of 655 unique articles published between 1995 and 2019. The articles were published across 184 different journals in total and with a 12.3% average annual increase in publications (Figure 1.1 A), the interest in probiotics for aquaculture fish species is obviously rather strong and continuing to grow. Network analyses were further conducted on the literature, by extracting words from the titles and abstracts of each article and constructing a co-occurrence network based on those keywords detected over twenty times. According to the network analysis and the keywords associated with topic clusters, research in aquaculture has primarily had two to three main focuses: 1) *in-vitro* isolation and characterization of new candidate probiotics (red cluster; Figure 1.1 B), 2) *in-vivo* testing of probiotics and their effects on finfish performance (green cluster; Figure 1.1 B), as well as 3) a smaller subset of studies focused on the effects of probiotics to control environmental microbes and establish the microbiota in early larval life stages (blue cluster; Figure 1.1b). By mapping the chronology of important keywords (Figure 1.1 C), it is apparent that the earliest studies were primarily aimed at *in-vitro* work related to the isolation and testing of potential probiotics and as the field has progressed, more focus has been placed on characterizing the *in-vivo* effects of probiotics on fish performance with keywords of recent popularity in the literature including “disease resistance”, “expression”, “microbiota”, and “diversity”. To summarize the bibliometric review of the current published literature, interest in probiotics is rapidly gaining interest in the aquaculture industry and expected to continue increasing in popularity. As the research advances, studies have moved towards gaining a more mechanistic understanding of the effects of direct-fed-microbials on their hosts in aquaculture.

While the set of articles analyzed are representative of the research currently being conducting on aquaculture fish probiotics, there are many other articles published on the topic as well, which fell outside of our query parameters, including a surplus of review articles. Many of the reviews published on the topic over the last two decades have laid the

groundwork for recent research, as is made apparent by the ten most cited articles from within our bibliometric dataset (Verschuere et al. 2000; Gatesoupe 1999; Balcazar et al. 2006; Nayak 2010a; Kesarcodi-Watson et al. 2008; Irianto and Austin 2002a; Merrifield et al. 2010; Fuller 1989; Gomez-Gil et al. 2000; Irianto and Austin 2002b). Within the last five years, a plethora of new reviews have been published (Chauhan and Singh 2019; Hindu et al. 2019; Vanderzwalmen et al. 2019; Dawood et al. 2018; Haygood and Jha 2018; Hoseinifar et al. 2018; Ringo et al. 2018; Wang et al. 2018a; Banerjee and Ray 2017; Carnevali et al. 2017; Hossain et al. 2017; Hoseinifar et al. 2017a; Rather et al. 2017; Wang et al. 2017; Carbone and Faggio 2016; Dawood and Koshio 2016; Tan et al. 2016; Zorriehzahra et al. 2016; Akhter et al. 2015; Hai 2015a; Hai 2015b; Lazado et al. 2015; Newaj-Fyzul and Austin 2015), which are sure to guide future research efforts. Much knowledge has been gained about DFM in aquaculture in recent years, yet old questions remain, and new ones often arise from novel findings, making this an exciting and active area of research going into the future.

Probiotics Used in Aquaculture

The diversity in finfish species and rearing situations in aquaculture is nearly matched by the number of probiotic microbes which have been tested or implemented, with most finfish probiotic candidates being from the bacterial phyla *Firmicutes*, *Proteobacteria*, and *Actinobacteria*, though fungal probiotics are commonly used as well (Table 1.1). Other non-bacterial probiotic candidates including bacteriophage and microalgae have been tested in aquaculture as well (Newaj-Fyzul et al. 2014). The probiotics tested in aquaculture thus far have originated from many sources, including commercially available probiotics originally developed for human or terrestrial livestock (He et al. 2013; Hernandez et al. 2010), as well as those isolated from the aquaculture environment (i.e. water, pond sediment, or tank biofilms) (Chen et al. 2016; Chen et al. 2019) or directly from host-associated microbiomes of wild (Selim et al. 2019) or farmed finfish (Burbank et al. 2011). Typically, it is preferred to utilize host-derived probiotics, or those isolated from the target host species or its environment because these probiotics are thought to have co-evolved with the host and consequently should possess the traits necessary to thrive in a particular host and environment (Lazado et al. 2015). Though this has rarely been tested with side-by-side comparisons of probiotics of disparate origins. For more in-depth discussion on individual

genera and species of probiotics tested in aquaculture see the following reviews (Hoseinifar et al. 2018; Newaj-Fyzul et al. 2014; Merrifield and Carnevali 2014; Ringo et al. 2018)

Gram Positive Bacteria

Gram positive bacteria are those which possess a thick outer peptidoglycan layer surrounding their cytoplasmic membrane yet lack any outer cell membrane. By far the most common subset of gram-positive bacteria used as probiotics, not only in aquaculture but in general, are the lactic acid bacteria (LAB) (Figure 1.1c). LAB are non-spore forming bacteria from within the order Lactobacillales, which are known for their abilities to ferment sugars into lactic acid and produce antimicrobial compounds that reduce or control harmful bacteria involved in pathogenesis or spoilage (Ringo et al. 2018). Among the LAB, those within the genera *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pedicoccus, Streptococcus, Vagococcus and Weisella* have been evaluated as probiotics in aquaculture (Ringo et al. 2018; Newaj-Fyzul et al. 2014). The popularity of LAB has led to a wealth of literature on their use in aquaculture (Gatesoupe 2008; Hoseinifar et al. 2018; Ringø and Gatesoupe 1998; Ringo et al. 2018; Merrifield et al. 2014). Gram positive probiotics from the genus *Bacillus*, especially *B. subtilis*, are also quite common in an aquaculture setting, and as spore forming microbes these probiotic strains are thought to be more resilient and heat tolerant during processing, transport, storage, and implementation (Bajagai et al. 2016). Other gram positive bacteria also commonly evaluated as probiotics in aquaculture, include bacteria from the genera *Arthrobacter, Brevibacillus, Brochothrix, Clostridium, Kocuria, Microbacterium, Micrococcus, Rhodococcus, Streptomyces* (Balcázar et al. 2006; Newaj-Fyzul et al. 2014; Tan et al. 2016).

Gram Negative Bacteria

Bacteria which possess both an inner and outer lipid membrane with only a thin layer of peptidoglycan in the periplasmic space are classified as gram negative. The presence of an outer cell membrane influences how these microbes interact with host pattern recognition receptors (Rauta et al. 2014), and a recent review suggests that gram-negative probiotics may be more effective in modulating gut immunity towards greater protection from enteric disease in mammals (Kandasamy et al. 2017). Gram negative microbes evaluated as probiotics in aquaculture include *Aeromonas, Agarivorans, Alteromonas, Bdellovibrio, Burkholderia, Citrobacter, Enterobacter, Neptunomonas, Phaeobacter, Pseudoalteromonas,*

Pseudomonas, *Rhodopseudomonas*, *Roseobacter*, *Shewanella*, *Synechococcus*, *Thalassobacter*, *Vibrio*, and *Zooshikella* (Balcázar et al. 2006; Newaj-Fyzul et al. 2014; Akhter et al. 2015).

Synbiotics

Synbiotics involve the co-administration of viable probiotics and prebiotic ingredients intended to serve as nutritional substrate for the probiotic strain. Rodriguez-Estrada et al. (2009) were the first to study the synergistic effects of combining probiotic and prebiotic supplements in aquaculture, and interest in their application has only grown since. Prebiotics are non-digestible feed ingredients that beneficially effect one or more of the host-associated microbes. Examples of prebiotics used in aquaculture include various oligosaccharides, such as fructo- (FOS), galacto- (GOS), arabino-xylo- (AXOS), xylo- (XOS), isomalto- (IMO), and mannan-oligosaccharides (MOS), as well as β-glucan, inulin and chitosan (Huynh et al. 2017; Nawaz et al. 2018). In addition, various plant extracts and immunostimulants, including date palm and fenugreek seed, taken from traditional medicine have also been evaluated from their synbiotic effects in combination with various probiotics (Guardiola et al. 2016; Hoseinifar et al. 2015a; Bahi et al. 2017). The prebiotic portion of a synbiotic is thought to serve as a nutritional substrate for the co-administered probiotic, however, the prebiotic can also exert direct effects on the non-specific immune system of host fish and modulate the commensal microbiota composition (Nawaz et al. 2018). Typically, when studies include both individual prebiotic and probiotic treatments, as well as a combined synbiotic treatment, results show that the synbiotic produces the greatest improvement to host performance, suggesting the pre- and probiotic act in a synergistic manner (Table 1.1) (Lee et al. 2019; Guardiola et al. 2016; Hoseinifar et al. 2015b). Similarly, Modanloo (2017) found that GOS and *Pediococcus acidilactici* stimulated serum and skin Ig levels significant more so than the pre- or probiotic alone in carp *Cyprinus carpio*, although the probiotic in that study was found to stimulate non-specific humoral response (i.e. serum complement and skin and serum lysozyme) significantly more than the prebiotic or synbiotic combinations. Furthermore, prebiotic blends can be optimized, both in terms of source and dose, using *in-vitro* experiments with the probiotic candidate strain to ensure the prebiotic portion of the synbiotic serves as an adequate substrate for the probiotic to better colonize and persist in a host (Hoseinifar et al. 2017b; Lee et al. 2019).

Yeast

Bacteria are not the only origin of DFMs in aquaculture, as a variety of single-celled fungi, or yeast, have been administered to finfish in aquaculture as probiotics as well. The genera of yeast tested as probiotics in aquaculture include *Debaryomyces, Wickerhamomyces, Phaffia,* with by far the most common being *Saccharomyces* (Navarrete and Tovar-Ramírez 2014; Huyben et al. 2017). Yeast, specifically *S. cerevisiae,* are also commonly used as a single-cell protein source irrespective of their probiotic abilities, typically included in diet formulations as a hydrolyzed extract or lyophilized whole cells (Nayak 2010a). To date, few studies have used culture-independent NGS techniques to characterize the mycobiome of finfish species (Marden et al. 2017), with nearly no such data on aquaculture species. Therefore, there remains a paucity of information related to the diversity and function of homeostatic fungal microbiota in finfish (de Bruijn et al. 2018). Recent studies by Huyben and colleagues used viable dried yeast as a DFM that also served as a fishmeal replacement in the diet, and found that rainbow trout receiving the yeast based diets had only slightly higher culturable levels of yeast in the gut, which included an increase in the proportion of typically harmful *Candida* species, though it did alter the composition of the indigenous bacterial and fungal microbiota (Huyben et al. 2017; Huyben et al. 2018).

Known Effects of Probiotics on Fish Performance

By definition, a probiotic must confer some positive attribute to the host to which it is administered. In aquaculture, growth performance and feed utilization, pathogen resistance, digestive morphology, immune performance, tolerance of stress, and microbiota composition are among the most common traits that aquaculture practitioner aim to improve with probiotics (Table 1.1). Enhancement of growth performance and feed utilization is typically assessed by 8-12 week feeding trails with focus on production metrics such as weight gain, feed conversion ratio (FCR), and specific growth rate (SGR), or the like. Many probiotics have been shown to improve growth performance metrics in finfish, though certainly some fail to do so as well (Table 1.1) (Fuchs et al. 2015). Effects of probiotics on growth have been further assessed using measures such as quantitative gene expression of growth hormone (GH) and insulin like growth factor-1 (IGF-1), the genes responsible for hormonal control of the endocrine growth axis, as well as histological analyses of muscle fiber morphology. Recently, both techniques were used to show that host-derived probiotics

(*Alcaligenes sp.* and *Bacillus sp.*) could increase the growth performance of Malaysian mahseer through upregulation of the GH-IGF axis and hypertrophy of myofibers (Asaduzzaman et al. 2018). Mechanistic insights such as these are valuable in providing useful information regarding future use and development of growth promoting DFM strategies.

In many situations the driving motivation behind the development of a probiotic is to improve the resistance of a population of farmed finfish to a specific disease or an array of commonly encountered pathogens. In such a situation, a probiotics antimicrobial activity against a target pathogen can be assessed using *in-vitro*, *in-vivo*, or molecular techniques (Figure 1.2). Typically, *in-vitro* screening of candidate isolates or their cell free supernatant (CFS) is the most common first step (Araujo et al. 2016; Araujo et al. 2015a; Amin et al. 2017; Burbank et al. 2011; Gonzalez-Palacios et al. 2019; Selim et al. 2019). In order to isolate a probiotic aimed at improving resistance to *F. psychrophilum*, the causative agent of bacterial coldwater disease in rainbow trout, Burbank et al. (2011) used *in-vitro* inhibition assays to test hundreds of isolates for inhibitory capabilities. Later, Schubiger et al. (2015) used reverse genetics to prove the *in-vivo* utility of a bacteriocin within one of the effective *Enterobacter* probiotics identified by Burbank's (2011) *in-vitro* trials. After identifying the putative bacterial protein and its associated gene (*ecnAB*), mutant probiotic populations were generated which either lacked the *ecnAB gene* locus or were complemented with an additional copy. Results of a *F. psychrophilum* challenge following ten days of direct-fed administration of either the wild-type or one of the two mutant probiotic strains to rainbow trout highlight the potential utility of probiotic bacteriocins, as survival was greatest among fish receiving the *encAB* complemented probiotic, followed by the wild-type, while the *encAB* knock-out probiotic strain performed no better than the control group (Schubiger et al. 2015). Araujo et al. (2015b) also used knockout techniques to show that the bacteriocin nisin-Z was an important mechanism behind the protection conferred to rainbow trout by a *L. lactis* probiotic strain against a congener pathogen, *L. garvieae*. Bacteriocins are not the only molecules probiotics can produce to generate antimicrobial activity. Other antimicrobial molecules produced by probiotics include siderophores, SCFAs, peroxides, lytic enzymes, or organic acids which can all help to control populations of unwanted microbes (Pérez-Sánchez et al. 2018). To date, probiotics derived from and used in aquaculture have been shown to

have antimicrobial activities against a wide range of bacterial, fungal, viral and protozoan fish pathogens (Pieters et al. 2008; Chauhan and Singh 2019).

Not only are probiotics useful in promoting disease resistance through their direct effects on potential pathogens, but also by promoting improvements to the immune performance of their host. Many studies have shown the ability of probiotics to upregulate activity of the innate immune system of fishes. This non-specific activation can occur locally through interactions with the MALT at the site of probiotic colonization, as well as systemically through modulations to peripheral immune responses. Non-specific activation commonly includes increasing the activity of host enzymes (i.e. lysozyme, peroxidase, alkaline phosphatase and anti-protease), increasing respiratory burst activity of neutrophils and macrophages, altering the cytokine milieu, especially the pro- and anti-inflammatory cytokines, and upregulating host complement activity (Table 1.1) (Akhter et al. 2015; Nayak 2010a). Unfortunately, little research has been conducted in fish on the interactions between microbiota and a host's adaptive immune response in fish, though in other organisms it has been shown that microbiota composition can influence the efficacy of host vaccination (Valdez et al. 2014). In mice, the gut microbiota facilitate efficient host vaccination by increasing basal activation of the TLR and NOD-2 immune pathways, which generate more robust immunization responses to the initial vaccine and adjuvant within a host (Oh et al. 2014; Kim et al. 2016). This suggests that the commensal microbiota have an important indirect role in determining the efficacy of vaccines and as vaccination practices continue to increase in aquaculture, a better understanding of microbiota interactions is likely to unveil insights which improve vaccination practices and overall management of fish health in aquaculture (Ringo et al. 2014). One of the few studies conducted on the interaction between fish microbiota and vaccination showed that an *Aeromonas* vaccine given to grass carp had minimal influence on the commensal microbiota, although regulation of *Aeromonas* within the intestine was altered (Liu et al. 2015). Unfortunately, sample size was very limited in that study, hence much more research on this topic is needed in aquaculture.

The increased use of alternative dietary ingredients in aquaculture often leads to dietary formulations which irritate either the host intestinal epithelia or the intestinal microbiota, resulting in chronic inflammation or enteritis. This results in increased gut permeability which impairs nutrient digestion and greatly increases disease susceptibility in

fish, and probiotics are often called upon as a potential remedy for such maladies in aquaculture. In order to illustrate the effects of *Lactobacillus plantarum* and *Lactococcus lactis* probiotics on gut permeability of olive flounder *Paralichthys olivaceus*, Beck et al. (Beck et al. 2016) used fluorescein isothiocyanate labeled dextran (FITC-dex). After a 30 d period of probiotic feeding, fish were fasted and gavaged with FITC-dex, then by measuring the levels of FITC in the plasma at later timepoints the authors showed that both probiotics significantly improved gut permeability compared to a control group, particularly after disease challenge (Beck et al. 2016). Gene expression and protein expression analysis of claudin, occludin or other tight junction genes are also commonly used to evaluate the *in-vivo* effects of probiotics on gut permeability (Dong et al. 2018).

In conjunction with the deleterious effects on gut morphology caused by some alternative dietary formulations, a stark shift in the homeostatic composition of gut microbiota often referred to as dysbiosis is also often observed (Kononova et al. 2019; Ringø et al. 2016). Dysbiois is typically caused by both direct impacts of the dietary ingredients on the microbiota, as well as the indirect effects due to changes in the host morphology and immunity caused by the dietary alterations (Ringø et al. 2016). As a result, probiotics or other DFM strategies are also often investigated for their abilities to alter the microbiota composition of a host, although characterization of the effects of probiotics on the finfish host microbiota are surprisingly sparse relative to the total number of studies on fish probiotics (Merrifield and Carnevali 2014). Interestingly, in many studies probiotics are found to confer positive benefits to their host without causing much impact on the general microbiota composition. A probiotic strain of *Bacillus velezensis* was administered to channel catfish in multiple experiments, and showed 30-40% improvements in fish growth performance and water quality indices were significantly improved, yet little to no differences in overall composition of fish or water microbiota were detected (Thurlow et al. 2019). Similarly, Huyben et al. (2018) fed live yeast probiotics to trout for six weeks and saw minimal impacts on the native bacterial microbiota. Schmidt et al. (2017) co-administered the antibiotic streptomycin and two streptomycin resistant probiotic strains, and while the probiotics did briefly colonize the fish gut microbiota and provided significant protection to the host during disease challenges, there was no overall effect of the probiotics on gut microbiota diversity or structure at the conclusion of the trial. These results suggest

probiotics alone may not be effective in drastically shifting the microbiota composition of fish, though the paucity of studies on the topic merits further investigations.

As aquaculture intensifies, so too does the number of stressors that impact a fish's physiology. Severe acute stressors, as well as mild but chronic stressors represent a possible and common etiology of mortality in aquaculture operations, and chronic stress at any level will interfere with the energetic regulation of fish, reducing their capacity for growth (Barton 2002). As such, mitigating the stress response of a host finfish species, is another common aim of DFM interventions in aquaculture (Mohapatra et al. 2013). For instance, it has been recommended that the administration of heat-tolerant LAB probiotics may be an effective means of mitigating the effects of heat-stress in Atlantic salmon reared in marine net pens, as elevated temperatures tend to deplete LAB levels in the salmon gut microbiome, while increasing potentially harmful *Vibrio* bacteria and decreasing the functional potential of the commensal microbiota (Neuman et al. 2016). In addition, a study on a popular species from the aquarium trade sector of aquaculture, angelfish *Pterophyllum scalare*, showed that pre-, pro-, and synbiotics could completely mitigate high salinity stress, while only the probiotic and synbiotic provided slight protection to temperature stress (cold) (Azimirad et al. 2016). A recent study even showed the utility of mixed *Bacillus* probiotics administered through either the water or both water and live feeds in reducing transport stress and associated mortality in an emerging marine aquaculture species, common snook *Centropomus undecimalis* (Tarnecki et al. 2019).

In addition to growth, disease, digestive morphology, and stress response as just discussed, reproduction rounds out those variables in aquaculture which represent the greatest bottlenecks to production. Many of the difficulties in aquaculture with reproduction are related to nutritional and metabolic processes that ensure successful vitellogenesis and oogenesis. The physiologic stress put on spawning females due to both the reproductive processes and the handling stress associated with the logistics of spawning in intensive aquaculture operations leads to increased disease susceptibility (Gioacchini et al. 2014). Despite these maladies having been shown to be potentially ameliorated by probiotics individually, little research has evaluated the potential of DFMs to mitigate the concert of stressors placed on finfish during reproduction (Mehrim et al. 2015; Carnevali et al. 2017; Gioacchini et al. 2014). Results of one of the only few such studies in an aquaculture finfish

species was conducted in tilapia *Oreochromis niloticus* and suggests DFM strategies could improve aquaculture reproduction. In that study, beneficial effects of probiotics were observed on male gonadosomatic index (GSI) and sperm quality, as well as female GSI, ovarian-specific gravity, egg diameter, and fecundity (absolute and relative) (Mehrim et al. 2015).

Important Considerations for Future Selection of Probiotics for Aquaculture

While a diverse range of microbes have been evaluated for their probiotic potential in aquaculture, the continually growing diversity in fish species and rearing condition in aquaculture calls for continued efforts in the isolation and characterization of new probiotic candidates. A recent deep-sequencing shotgun-metagenomic survey of the homeostatic microbiota of an important freshwater carp species *Labeo rohita* showed that common probiotic strains (i.e. *Bacillus*, *Lactobacillus*, *Streptococcus*, and *Lactococcus*) were either at very low abundance or completely absent, suggesting development of more host-specific probiotic candidates are needed in aquaculture (Tyagi et al. 2019). Over the last few decades, lessons have been learned regarding the development of aquaculture probiotics, and as will be discussed below, considerations of certain aspects of probiotic implementation are now known to improve the likelihood of beneficial outcomes (Figure 1.2) (Merrifield et al. 2010; Cui et al. 2017; Chauhan and Singh 2019).

Safety to the Host and Environment

Safety, both that of the intended host and the environment, should be among the first consideration when identifying new candidate probiotic for aquaculture. As a first safety assessment, it must be ensured that the probiotic is not virulent to the host. This can be assessed by the detection of virulence genes using molecular techniques, or by *in-vivo* intraperitoneal (IP) or intramuscular (IM) administration of the candidate probiotics (Figure 1.2). *Ex-vivo* intestinal sac assays can also be used to evaluate the effects of probiotic candidates on host intestinal cells, including any cellular disruption that a probiotic may cause to the host gut (Løvmo Martinsen et al. 2011). This is an important consideration, because often there is only minor phylogenetic distance between probiotic candidates and potential pathogens, especially when considering probiotic candidates from genera known for their pathogenicity in fish, such as *Aeromonas*, *Carnobacterium*, *Streptococcus*, *Vibrio* and the like. If a probiotic causes infection, shows signs of host cellular disruption, or elicits an

exaggerated immune response in the host then that microbe should no longer be considered for probiotic development, at least not for that host species. While probiotics should certainly not be injurious to a host fish species, probiotics that exhibit minor immunogenicity may act as a hormetic stressor, thereby explaining the up-regulation of non-specific immunity often seen in finfish following DFM interventions (Table 1.1) (Nayak 2010a).

After decades of antibiotic use, resistant microbes are now a rather common presences in the environment and represents a global threat to public health and food production. Due to the potential ease at which bacteria can share plasmid-encoded antibiotic resistance, via horizontal gene exchange, new candidate probiotics should always be assessed for antibiotic resistance. Administering probiotic bacteria that possess antibiotic resistance runs the risk of transferring the antibiotic resistance from the probiotic to a pathogen residing in the host, leaving the host susceptible to antibiotic resistant infections in the future. While absences of antibiotic resistance is recommended as a crucial trait for a microbe to be considered a probiotic candidate in aquaculture (Hai 2015a; Chauhan and Singh 2019), in certain situations antibiotic resistant probiotics could be useful to administer during antibiotic chemotherapies in order to maintain colonization of the gut with probiotic microbiota (Schmidt et al. 2017). Introduction of probiotic strains to the environment may also be a potential safety concern if the probiotics have been sufficiently modified, particularly using transgenic techniques (Lin et al. 2019; Saputra et al. 2016). The potential for downstream environmental impacts and legality associated with the use of such additives should be fully considered before being administered on aquaculture farms.

Route of Delivery

Probiotics are typically found in one of two final forms: a lyophilized dry powder containing viable but dormant microbes or a liquid live culture of active probiotics. These products are administered to the fish either by means of static bath immersion, live-feeds enrichment, or mixing into or onto formulated diets. Each of these routes has its own advantages and disadvantages as a method of administration. For example, larval marine finfish typically rely on live feeds of *Artemia* or copepods for nutrition and therefore enriching the live-feed organisms with probiotic microbes can be an effective means of delivering the probiotics to larvae, especially because the *Artemia* can serve as bio-encapsulation for the probiotics, safeguarding the delivery of the probiotic to the host gut in a

viable form (Tarnecki et al. 2019; Skjermo et al. 2015). The benefits of encapsulation to preserve probiotic viability and ensure delivery of competent probiotics to the host target site have been further applied to probiotics included in formulated diets as well. These encapsulations typically involve mixing the probiotic, finely ground formulated diet, and a biopolymer such as alginate, before co-extruding droplets into calcium chloride, which yield microencapsulated probiotic diet pellets (Rosas-Ledesma et al. 2012; Cordero et al. 2015). This encapsulation technique not only improves the delivery of the probiotics to the host gut by protecting the probiotic from the harsh conditions encountered in route to the gastrointestinal tract (low pH, bile acids, digestive enzymes, etc.), but also have been shown to improve shelf-life and storage-viability, an important consideration for ensuring commercially viability (Tripathi and Giri 2014).

While direct feeding is certainly the most common method of administering probiotics in aquaculture, the capacity of water to efficiently support and transfer microbes also makes water-based administration methods rather effective in aquaculture (Jahangiri and Esteban 2018). Strains of *Pseudomonas fluorescens* administered to rainbow trout resulted in a 58-75% reduction in the infection rate with the fungal pathogen *Saprolegnia parasitica* following disease challenge, not when direct-fed but only when administered through repeated 6 h static water baths. In the case of *Saprolegnia*, secondary zoospores are known to continue the infection cycle by adhering to fish skin and gills, and therefore for this pathogen a water based administration strategy was likely most effective because the probiotics could better colonize the fish skin to provide protection from secondary *S. parasitica* zoospores (Gonzalez-Palacios et al. 2019). This highlights that the correct route of delivery for a probiotic can be dependent upon the goals in mind for a particular probiotic and shows the utility of optimizing delivery routes prior to full implementation.

Injection may be another viable route of administering probiotics in aquaculture (LaPatra et al. 2014; Jahangiri and Esteban 2018). A probiotic strain of *Enterobacter* isolated from the gut of healthy rainbow trout that was shown to improve resistance to *F. psychrophilum* when used as a direct-fed probiotic (Burbank et al. 2011), was later tested for probiotic activities following IP and IM injection (LaPatra et al. 2014). Results showed that rainbow trout fry injected either IM or IP with live probiotic seven days prior to a *F. psychrophilum* challenge had much great survival and adaptive immune responses (LaPatra et al. 2014); however, this

route of administration is likely working through more of an immunostimulant mechanism, as opposed to a probiotic effect on the host's microbiota. Although, strong immunostimulant effects can certainly produce systemic changes in immunity, which yield altered regulation and composition of host microbiota (i.e. indirect probiotic effects).

Dosing should also be considered with the route of delivery in mind. Often when probiotics are tested at graded levels, there is a positive correlation among performance and inclusion level (Safari et al. 2016; Abass et al. 2018; Adel et al. 2017). Salinas et al. (2006) suggested that *in-vitro* methods should be used to make early evaluations into effective dosing prior to *in-vivo* administration. While higher doses often improve results, it is possible to overdose probiotics resulting in overstimulation and suppression of the innate immune response (Hai 2015a). In addition to the dose, duration has been shown to have implications on host immune stimulation, with results suggesting cyclical, short-term administration is preferable over long-duration administration, in order to avoid host attenuation and diminished prophylactic effects to the probiotic over time (Hai 2015a).

Host Colonization and Persistence

The ability of a probiotic strain to colonize and persist in the host is another trait which receives much focus in the delineation of probiotic candidates. In order to colonize among the native gut microbiota, a probiotic must enter the alimentary canal, survive the low pH environment of the stomach, if present (20% of fish lack a true stomach (Egerton et al. 2018)), tolerate bile salts, be capable of adhering to host cells to establish itself, and finally proliferate, persist, and replicate. Many times, the ability of a probiotic to tolerate these conditions is tested *in-vitro* by simulating gastrointestinal conditions and measuring adhesion to epithelial cells during the identification of probiotics candidates (Guo et al. 2016; Lee et al. 2019). However, less studies measure the *in-vivo* colonization efficacy of probiotics, which in many cases is required to yield true probiotic effects (Hill et al. 2014). To assess the colonization capabilities of their probiotic candidate, Dong et al. (Dong et al. 2018) used carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) to fluorescently label *Lactococcus lactis* probiotics prior to dietary administration to crucian carp *Carassius caracssius* for one week. Microbiota were then isolated from the mid-intestinal contents at the end of the 7-d probiotic treatment, and again 14-d later. Samples were then analyzed by flow cytometry to quantify the level of probiotic colonization according to the CFDA-SE

fluorescence. After administering the direct-fed probiotic at a dose of 1 x 10^9 CFU mL for one week, the colonization level was 6.6 x 10^3 CFU cm^{-1} at the end of feeding period, and persisted at 2.2 x 10^3 CFU cm^{-1} two weeks after probiotic feeding concluded (Dong et al. 2018). Results from this study show the promise of flow-cytometry as an effective tool in the study of host-microbiota-probiotic interactions, but also that effective probiotics are capable of colonizing and persisting in the host, even after administration has ceased.

Efficient colonization and persistence are not observed for all probiotic strains or situations. In rainbow trout, it was found that two separate probiotic candidates were able to colonize the trout gut and represented a high proportion of the total detected microbiota after 14-d of feeding; however, neither probiotic strain was detectable after administration of the probiotics ceased (Sharifuzzaman et al. 2014), suggesting the probiotic was never able to integrate with the autochthonous microbiota. Similarly, Skjermo et al. (2015) used a mixture of four host-derived probiotics administered to Atlantic cod larvae via artemia enrichment to steer the development of the gut microbiota. Of the four probiotic strains used, only one strain represented a dominant fraction of the hosts microbiota (Skjermo et al. 2015), highlighting the potential benefits of using multiple strains of probiotics within a single supplement to increase the odds of introducing a probiotic strain that is capable of host colonization (Table 1.1) (Standen et al. 2016; Wang et al. 2019; Skjermo et al. 2015).
In some cases, it may not be imperative that probiotics dominate the relative abundance of the total microbiota or that they even persist at all in the microbiota communities after administration has ceased to yield benefits to the host. Using axenic tilapia larvae, it has been shown that administering *B. subtilis* probiotics through the rearing water for one week could generate a significant shift in the microbiota population of those fish compared to a control group two weeks after the administration of the probiotic ceased, though surprisingly the administered probiotic strain was not detected at any point after administration ended (Giatsis et al. 2016). These “legacy effects” of probiotics, as coined by the authors of that study (Giatsis et al. 2016), indicate that the effects that probiotics have on shaping the microbial ecology and host developmental trajectory may long out last the presence of the probiotic microbes. However, these effects might only be seen when probiotic treatments are administered at early life stages when the host and its microbiota are more amenable to alterations that could have profound effects on the long-term trajectory of microbiota

structure and function. It has even been shown that in some cases heat-inactivated or killed probiotics can yield a greater benefit to a host finfish than that of live probiotics (Selim et al. 2019), which brings into question the theory that colonization is necessary for a successful probiotic effect. But as mentioned before, these heat-inactivated DFMs are likely to be acting more as a short-term immunostimulant and not a true probiotic because they do not exert direct ecological effects on the host microbiota.

Future Directions for DFM Strategies in Aquaculture

The immense variation in aquaculture including the number of host fish species, rearing situations, and probiotic candidates suggest it is unlikely there will ever be a "silver-bullet" probiotic which will be effective in all aquaculture situations (Lazado et al. 2015). As a result, it is imperative that probiotics continue to be discovered and further developed for the many different situations encountered across aquaculture. Awareness of the factors that negatively influence the homeostatic microbiota of fish and working to manage aquaculture operations in a way that maintains and promotes diverse commensal microbiota (i.e. minimal antibiotic use and informed use of DFMs) while limiting the presence of pathogens through rigorous bio-security will produce the best outcomes in the management of fish health (Oidtmann et al. 2011).

Because host-associated microbiota function as an ecologically linked community whose composition and metabolic activities are reliant upon one another (Layeghifard et al. 2017), it is important that future implementation of probiotics in aquaculture be made from an ecologically informed perspective (De Schryver and Vadstein 2014). With microbial ecology in mind, it may be more effective to administer multi-strain communities of probiotic microbes, which ideally have known symbiotic interactions based on both *in-vitro* and *in-vivo* data, as opposed to the mono-strain probiotic approach that is commonly in practice today. Fecal microbiota transplants (FMT), in which entire fecal microbiota communities are translocated between hosts, have been implemented in human medicine to remedy persistent intestinal infections of *Clostridium difficile* or other gastrointestinal abnormalities (Zhang et al. 2018). FMT has also shown promise in reducing disease, particularly following antibiotic administration, in a range of other livestock and domestic pets (Niederwerder 2018). Such results suggest more complex communities of microbes may increase the effectiveness of probiotic supplements in producing the desired compositional

and functional alterations to fish microbiomes. In addition, FMT studies have already been successfully done in zebrafish (Rawls et al. 2006; Ran et al. 2016), and germ-free or gnotobiotic models of a few aquaculture species already exist (Schaeck et al. 2016; Forberg et al. 2011). In practice, FMTs function as unselected multi-strain host-derived probiotics, that if derived from a healthy donor, have the proper proportional abundance of diverse microbes that is necessary to achieve functional homeostasis within the microbiota communities. However, the downfall of such a strategy is the potential for unintended transfer of harmful microbes between hosts, especially when transferring into an already immuno-compromised individual, which may limit the practical applicability of this technique. Even if FMTs do not represent a practical DFM strategy, their use will certainly be valuable in future studies on fish microbiota and probiotics.

As DNA sequencing and other technologies continue to advance and the hurdles associated with the analyses of such data are diminished, a wealth of information will be gained by the more routine implementation of advanced techniques to get at the mechanisms behind probiotic effects in finfish hosts. As has already begun, it is expected that future research on DFMs in aquaculture will focus more on characterizing, through the means of amplicon and shotgun metagenomic analyses, the effects of DFMs on the structure and function of the full microbial communities of not only the fish gut, but also that of the skin, gills, and nares. In this regard, more focus should be paid to the functional aspects of the microbiota in the future, because it is possible for a stable microbiota composition to yield different levels of metabolic function, just as it is possible for relatively large changes in taxonomic composition to have no effect on microbiota function due to conserved functionality across microbial phylogeny. In addition, as more probiotics are intensively developed for aquaculture, probiogenomic studies focusing on understanding the genomic architecture underlying successful probiotics and tracking the genetic stability of probiotic strains, as have been conducted for human probiotics (Ventura et al. 2009; Ventura et al. 2012), are likely to better inform probiotic selection and implementation in aquaculture. Further evaluating host-microbiota interactions in a more holistic, systems-based approach, including the coupling of host -omics data (i.e. transcriptomics, proteomics, metabolomics) with microbiota meta-omics data, should be the aim of future studies. Hologenome theory suggests that the genomes of host organisms and that of their associated microbiota are under

constant interaction and therefore cannot be viewed as evolutionarily independent entities (Limborg et al. 2018). Applying this paradigm while undertaking physiological studies in aquaculture is likely to produce new and valuable insights on host-microbiota interactions and is sure to be accompanied by gains in finfish performance.

Another promising yet underexplored aspect of DFM research in aquaculture is the use of bacteriophages as a means of manipulating the host microbiome for beneficial outcomes. Bacteriophages, viruses which infect bacteria, have been applied as a means of managing microbial communities in the food industry (Endersen et al. 2014) and other areas of agriculture with some success (Svircev et al. 2018), yet research on the use of bacteriophages in aquaculture has been minimal. Bacteriophages of various important aquaculture pathogens have been isolated, including phage that infect *Edwardsiella tarda, E. ictaluri, Lactococcus garvieae, Pseudomonas plecoglossicida, Streptococcus iniae, Flavobacterium columnare, F. psychrophilum, Aeromonas salmonicida, A. hydrophila, Vibrio anguillarum, V. harveyi, and V. parahaemolyticus*, although more research is needed on their implementation (Gon Choudhury et al. 2017). In addition, direct feeding of bacteriophages in aquaculture is likely to be further improved by techniques such as those recently promoted by Huang and Nitin (2019), whereby aquafeeds are coated with an edible protein coating that protects the bacteriophage, increases delivery of viable phages to the fish, and boosts functional shelf-life. Bacteriophage technology has also been implemented in finfish aquaculture indirectly as a means of controlling pathogen outbreaks (i.e. *Vibrio*) in live-feed cultures of artemia (Quiroz-Guzmán et al. 2018). In their experiment, both single and mixed populations of bacteriophages significantly improved hatching and survival of live-feeds, in addition to greatly reducing the growth kinetics of *Vibrio* pathogens, thereby reducing the likelihood of disease outbreak in the fish population (Quiroz-Guzmán et al. 2018). Furthermore, *in-vitro* studies have shown that introduction of a bacteriophage specific to a bacterial fish pathogen can exert selective evolutionary pressures on the pathogen, which can yield a complete loss of virulence (Laanto et al. 2012). Because of the positive results from early studies and the highly targeted nature of bacteriophages, more research in the future should be focused on the use of phages as a dietary additive for selective control of pathogens or targeted management of microbial communities in aquaculture (Gon Choudhury et al. 2017).

Disruption of quorum sensing is yet another DFM strategy that should be further explored in aquaculture. Quorum sensing is a bacterial mechanism for interindividual communication by which signaling molecules released by one microbe are received by an intraspecific bacterium in close proximity, thereby activating gene expression in the bacteria in response to detection of cohorts (i.e. density dependent gene expression). Quorum sensing is especially common in fish pathogens such as *Aeromonoas*, *Edwardsiella tarda*, and *Vibrio sp.*, which utilize acyl-homoserine lactone signaling molecules to regulate expression of virulence genes (Zhou et al. 2016). It has been shown that some probiotics produce various metabolites that can interfere with this process in adjacent pathogens. Some strains of probiotics are known to produce acyl-homoserine lactonase enzymes which can degrade the signaling molecules of pathogens, as has been shown for a few probiotics isolated from fish such as *Bacillus sp.* QS-1 (Zhou et al. 2016) and *Flaviramulus ichthyoenteri* Th78 (Zhang et al. 2015). Furthermore, some probiotics, including some *Bacillus sp*., produce fengycin lipopeptides, which block reception of quorum sensing signals (Piewngam et al. 2018), in turn diminishing or eliminating pathogen virulence. In fact, quorum sensing may be required for a pathogen to even establish or remain colonized in or on a host, and hence disruption of this process may serve to completely eliminate a pathogen from a host (Piewngam et al. 2018). Disruption of quorum sensing holds promise as another means of microbial intervention in aquaculture and merits more research effort in the future.

Lastly, despite the large number of microbial strains that have been tested and shown useful as DFMs in aquaculture, there still remain only a handful of commercially available probiotics, which limits the aquaculture industries ability to apply DFM strategies (de Bruijn et al. 2018). Logistical and regulatory hurdles receive most of the blame for the paucity of marketed DFM products in aquaculture. Hurdles which have detracted from the commercialization of probiotics in aquaculture include difficulties scaling up the production of probiotic microbes, navigating the regulatory framework of the many countries in which aquaculture is practiced, and ensuring sufficient market demand (Bajagai et al. 2016). However, we expect that the continued and increasing pressure to eliminate antibiotic use and implement alternative diet formulations will continue to make the commercialization of probiotics a more economically viable prospect for aquaculture feed- and drug manufacturing

companies, hopefully leading to more options for aquaculture producers interested in implementing DFM interventions in the future.

Conclusion

Our understanding of the physiological relevance of microbiota in finfish once seriously lagged behind that of other livestock industries, although an explosion of research efforts focused on the fish microbiome in recent years has substantially advanced our understanding of host-microbe interactions in finfish. Along with these newfound insights, the use of DFMs, namely probiotics and synbiotics, has continued to gain interest in aquaculture. While research on probiotics in aquaculture has been abundant, the utilization of such supplements in the commercial industry is still rather limited, though the rate of implementation is expected to increase. To achieve this, more work is required to tailor the microbial composition of probiotic supplements to achieve the maximum benefit for each of the various species and life stages of finfish utilized in aquaculture, and fill in the knowledge gaps related to probiotic function, phenotypic stability, dosing, delivery route, storage and shelf-life. Furthermore, the efficacy of these treatments will need to be demonstrated outside of the laboratory setting, under commercial production conditions to be viable at an industry level. Current research efforts on the finfish microbiome are rapidly improving our understanding of the microbial ecology and physiological function of fish microbiota and continued work on modulating fish microbiota through the use of DFMs and other similar additives has the potential to generate substantial improvements in aquaculture production within the near future.

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Table 1.1. Overview of probiotics tested in aquaculture over the last five years (2014-2019). Probiotics are categorized by phylum, genus, and species with all probiotics being bacteria, with the exception of those from the fungal phylum Ascomycota listed at the bottom.

Phylum	Genus	Species	Host Species	Effect of probiotic on host performance	Citation
Firmicutes	*Bacillus*	*velezensis*	*Ictalurus punctatus*	Improved growth performance, survival, and water quality; Altered the gut microbiota	Thurlow et al. 2019
		circulans	*Tor tambroides*	Improved growth performance, expression of growth axis genes, and muscle fiber hypertrophy	Asaduzzaman et al. 2018
		subtilis	*Ctenopharyngodon idella*	Inhibited six pathogens in-vitro; Promoted anti-inflammatory cytokine environment of intestine	Guo et al. 2016
				Increased protective intestinal apoptosis following oral *Aeromonas hydrophila* challenge and reduced pathogen induced intestinal damage	Zhang et al. 2019
			Scophthalmus maximus	No effect on any growth performance metric	Fuchs et al. 2015
		licheniformis	*Centropomus undecimalis*	Improved retention of yolk-sac and survival to transport stress (reproducible); No effect on NS immunity; Varied impacts on microbiota	Tarnecki et al. 2019
		amyloliquefaciens	*Oreochromis niloticus*	Altered NS immune gene expression; Improved hepatic enzyme activity and survival	Saputra et al. 2016
				Improved growth performance, intestinal histology, and hematology metrics	Reda and Selim 2015
			Labeo rohita	Improved NS immunity in sera, hematology, serum lysozyme, and serum IgM	Nandi et al. 2018

Paenibacillus	*ehimensis*	*O. niloticus*	Improved growth performance, NS immunity and survival to *Aeromonas hydrophila* and *Streptococcus agalactiae*	Chen et al. 2019
Lactococcus	*lactis*	*Paralichthys olivaceus*	Activated a regulatory immune environment according to intestinal gene expression; Reduced gut permeability and mortality following *Edwardsiella tarda* challenge; More effective than *Lactobacillus plantarum* probiotic	Beck et al. 2016
		Carassius carassius	Improved serum NS immunity, intestinal immune gene expression, colonized and persisted in the host, and reduced pathogen colonization during *Aeronomos hydrophila* disease challenge	Dong et al. 2018
Lactococcus	*lactis*	*Pagrus major*	Improved growth performance, digestive protease activity, hematocrit, NS immunity in sera, plasma total protein, mucus secretions, and bactericidal activity of mucus and serum	Dawood et al. 2016
Lactobacillus	*rhamnosus*		Improved growth performance, digestive protease activity, hematocrit, plasma total protein, alternative complement, mucus secretions, bactericidal activity of mucus and serum	Dawood et al. 2016
	plantarum	*P. olivaceus*	Activated a proinflammatory immune environment according to intestinal gene expression; Reduced gut permeability and mortality following *Edwardsiella tarda* challenge; Less effective than *L. lactis* probiotic	Beck et al. 2016
Enterococcus	*faecium*	*Pterophyllum scalare*	Showed best *in-vitro* performance among tested isolates; Significantly improved growth performance	Dias et al. 2019
	casseliflavus	*O. mykiss*	Improved growth performance, digestive enzyme activity, TVAC and LAB in the intestine, hematology, and serum NS immunity; Reduced mortality in *Streptococcus iniae* infection	Safari et al. 2016

Proteobacteria	*Leuconostoc*	*mesenteroides*	*Clarias gariepinus*	Best performing host-derived candidates based on *in-vitro* screening; Heat-inactivated form showed best performance for both probiotics, yet the live forms did still outperform the control	Selim et al. 2019
	Edwardsiella	*sp.*			
	Shewanella	*putrefacians*	*Solea senegalensis*	Reduced negative effects of oxytetracycline administration; Increased expression of head-kidney immune genes and microbiota diversity following antibiotics; Reduced intestinal apoptosis induced by antibiotics	Tapia-Paniagua et al. 2015
			Sparus aurata	Increased serum IgM and peroxidase level; DDGE profiles showed no increase in richness or diversity, but range-weight richness increase with probiotic; Enriched the microbiota with LAB	Cordero et al. 2015
	Enterobacter	*sp.*	*O. mykiss*	Improved survival to *Flavobacterium psychrophilum* disease challenge; Enterocidin gene is shown responsible for probiotic effects.	Schubiger et al. 2015
	Alcaligenes	*faecalis*	*T. tambroides*	Improved growth performance; Upregulated expression of GH-IGF1 growth axis; Induced hypertrophy of muscle fiber	Asaduzzaman et al. 2018
	Psuedomonas	*fluorescens*	*O. mykiss*	Reduced number of infected fish (58-75%) following *Saprolegnia parasitica* challenge	Gonzalez-Palacios et al. 2019
Actinobacteria	*Rhodococcus*	*sp.*	*O. mykiss*	Improved gut morphology; Inhibited three known pathogens *in-vitro*	Sharifuzzaman et al. 2014 and 2018
	Kocuria				
Multistrain	*B. subtilis, E. faecium and P. acidilactici*		*O. niloticus*	Increased the levels of culturable LAB and probiotic strains in the gut; Altered microbiota communities, increase diversity	Standen et al. 2015

	B. velezensis and Rhodotorula mucilaginosa		*S. salar*	Improved growth performance, NS immunity, antioxidant capacity, and basal cortisol; Reduced mortality after *Aeromonas salmonicida* challenge	Wang et al. 2019
	Microbacterium, Ruegeria, Pseudoalteromonas, and *Vibrio*		G. morhua	*Microbacterium* probiotic showed the greatest ability to colonize and persist in the host out of the four host-derived candidates, though no probiotic strain dominated	Skjermo et al. 2015
Synbiotics	*Bacillus sp.* and barley β-glucan		*P. olivaceus*	Prebiotic source optimized *in-vitro*; Improved growth performance and survival following *Edwardsiella tarda* challenge: Synbiotic > Probiotic > Prebiotic	Lee et al. 2019
	Shewanella putrefacians and date palm extract		*Dicentrarchus labrax*	Pre-, pro-, and synbiotic diets performance varied by metric; Synbiotic treatment showed highest expression of immune genes in head kidney and greatest serum phagocytic ability	Guardiola et al. 2016
	Pediococcus acidilactici and GOS		*Cyprinus carpio*	Probiotic alone was most effective in stimulating serum humoral immune factors, and showed decreased IL-1B expression; Synbiotic stimulated highest levels of serum and skin mucus	Modanloo et al. 2017
			O. mykiss	GOS selected based on in-vitro performance; Improved growth performance; No effect on hematology; Increased relative proportion of LAB while not effecting total bacteria	Hoseinifar et al. 2017
Ascomycota	*Saccharomyces*	*elipsoedas*	*O. mykiss*	Growth increased with dietary inclusion level; Increased intestinal bacteria and LAB; Increased digestive enzymes and NS immunity; Increased skin total protein, lysozyme, and bacteriocidical activity	Adel et al. 2017
		cerevisiae	*O. niloticus*	Improved growth performance; Reduced heat stress/hypoxia mortality; Reduced mortality to *Aeromonas hydrophila*	Abass et al. 2018

		O. mykiss	Increased culturable yeast in intestine; Reduced abundance of *Leuconostocaceae* and *Photobacterium*; Probiotics had less impact on microbiota composition than temperature	Huyben et. 2018
Wickerhamomyces	*anomalus*		Diet extrusion reduced in-diet viability; LAB and *Debaryomyces hansenii* abundance increased in the gut, but not the probiotic strains; Altered gut microbiota communities	Huyben et. 2017

LAB – Lactic acid bacteria, NS – non-specific, GOS – galactooligosaccharides, DGGE – denaturing gradient gel electrophoresis, TVAC – Total Viable Aerobic Count

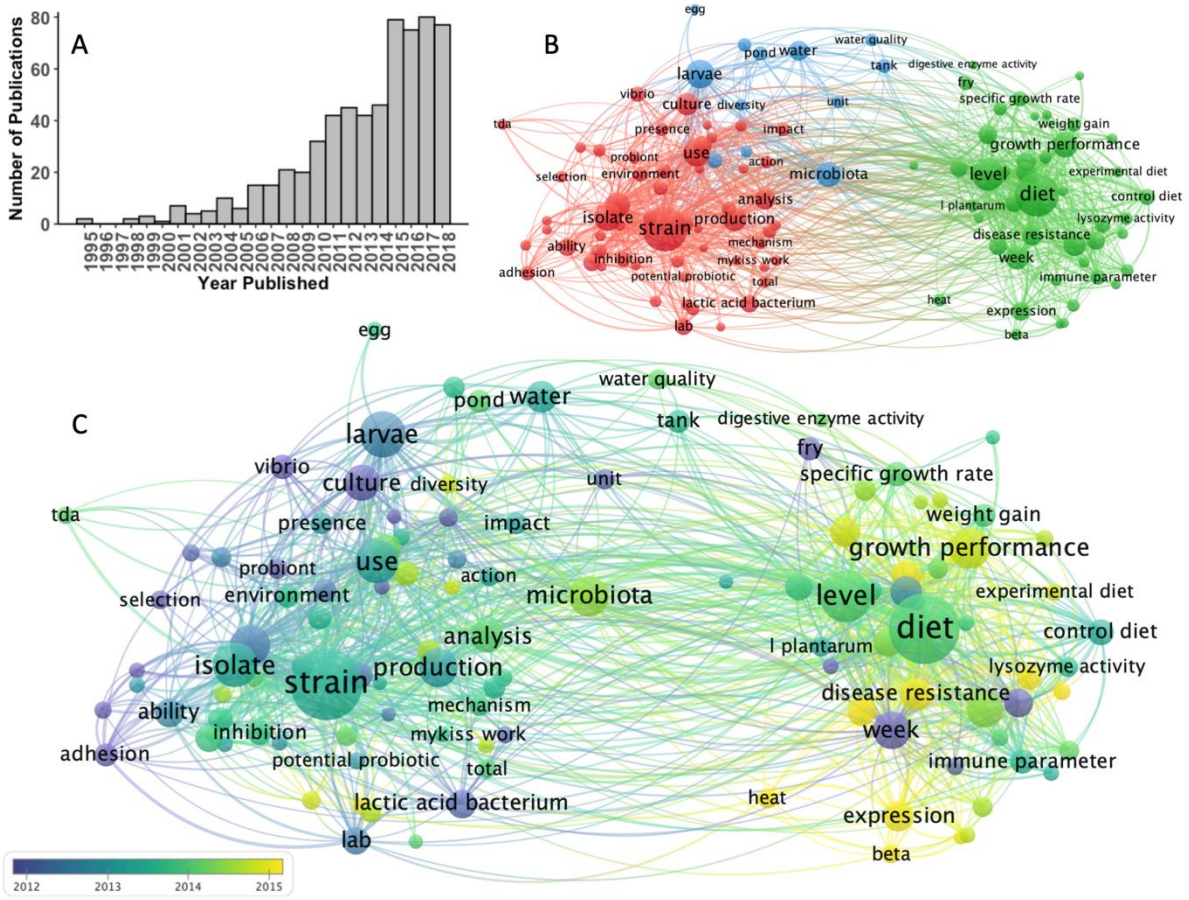

Figure 1.1. Bibliometric analysis of probiotic research in finfish aquaculture. The search terms "fish" AND "probiotic" AND "aquaculture" were used to query the Scopus and Web of Science databases. The resulting deduplicated entries were filtered to include only primary research articles (n = 655). (A) A histogram plot of the number of articles published on the topic each year between 1995-2018. (B and C) Co-occurence network analysis plots based on keywords extracted from article titles and abstacts. Node size (circles and text) is based on total occurrences (i.e. larger nodes observed more often in the literature). Nodes and edges are colored by either keyword clustering based on (B) co-occurrence association strength or (C) average year of source publications (i.e. keyword chronology).

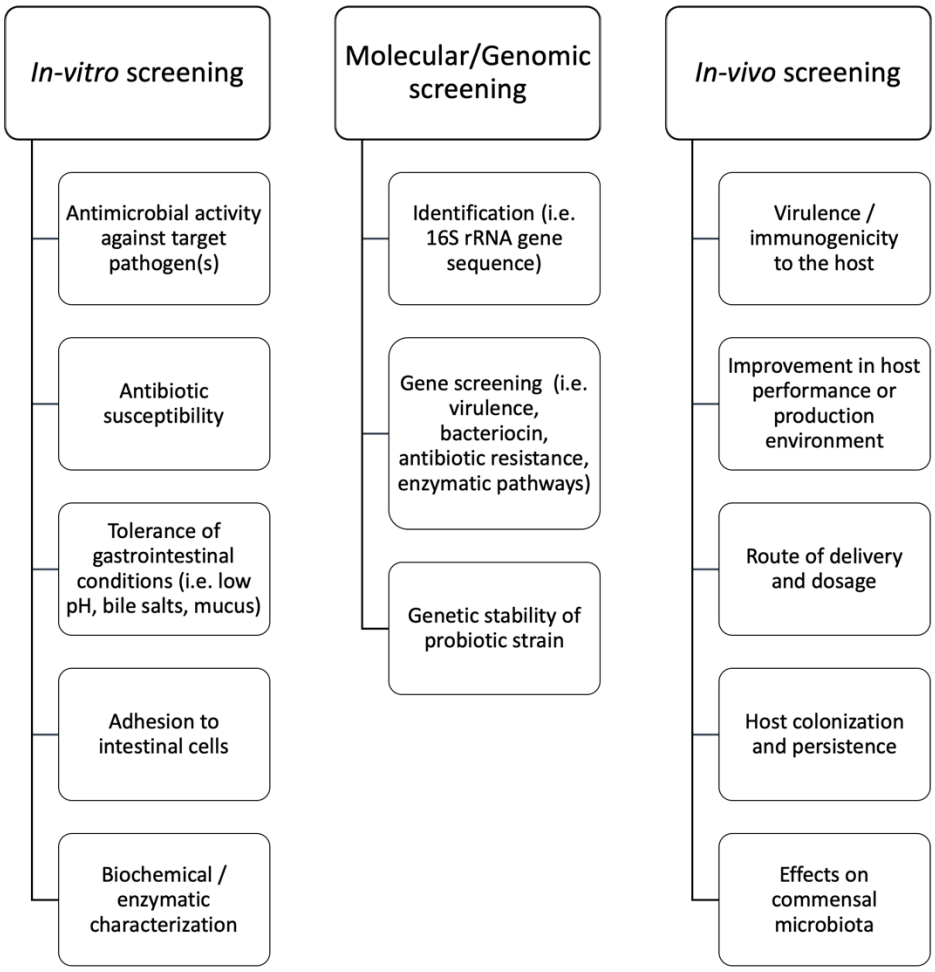

Figure 1.2. Selection criteria recommended for screening and identifying new host-derived probiotic candidates in aquaculture.

Chapter 2: Integrated functional analysis of host immunity and microbiota across mucosal tissues of Atlantic salmon identifies impacts of functional feeds and tissue-specific coadaptation

Abstract

Understanding of mucosal immunity in teleost fishes has greatly advanced over the last two decades, from the discovery of a mucosal antibody (IgT) and delineation of mucosa-associated immune systems, to the characterization of mucosal microbiota and their impacts on the host. With mucosal surfaces providing the cardinal defense against many of the costliest maladies facing aquaculture today, including intestinal enteritis, pathogenic or environmental irritation of the gills, and parasitic or bacterial skin infections, a more integrated understanding of host immunity and microbiota in these mucosal tissues would be advantageous. Here the interactions between the host mucosal immune system and mucosal microbiota are characterized and compared across the gut, gill, and skin of Atlantic salmon receiving diets containing functional feed ingredients. Dietary treatments included a basal control diet (Control), a 1% mannan-oligosaccharide supplementation diet (MOS), a diet with 96% lipid replacement with coconut oil (CoconutOil), and a diet combining both ingredients (CocoMOS). On average, fish grew 288.2 g ± 38.3 to a size of 646.5 g ± 35.8 (mean ± SD) during the 12-week trial with no dietary differences in growth. Bacterial richness and diversity were reduced in gut mucosa ($p \leq 0.001$) compared to skin and gill, and dietary effects were detected in richness ($p = 0.032$), but not diversity. The phylogenetic composition of microbiota communities clustered separately by tissue (PERMANOVA, $p \geq 0.001$), with dietary impacts on phylogenetic composition only detected in the skin ($p = 0.006$, unweighted UniFrac). Gill and skin microbiota showed much higher interconnectivity than gut microbes according to covariance networks, which were also used to identify tissue-specific keystone bacteria according to measures of node centrality. Predicted metagenomic functions showed concordance with bacterial composition (Procrustes correlation = 0.4003, $p < 0.001$) and 966 KEGG orthologs were differentially abundant across mucosal tissues. When summarized as higher level MetaCyc pathways, functions were not as discriminatory as phylogenetic composition (Procrustes correlation = 0.1031, $p = 0.223$), however, 54

pathways involved in key metabolic functions were detected as differential among tissues. Analysis of systemic and mucosal immune biomarkers identified multiple tissue specific differences with dietary impacts also detected within all tissues (gut, gill, skin, and peripheral blood leukocytes). Multivariate analyses showed tissue, diet, and tissue-diet interactions to be significant (PERMANOVA, $p \leq 0.032$) in explaining gene expression profiles. Mapping of the host expression profiles to microbiota profiles indicated a high correlation in sample ordinations (Procrustes correlation = 0.818, $p \leq 0.001$), suggesting host mucosal immune regulation and microbiota composition have coadapted in a tissue-specific manner. Following the feeding trail, a 10-day common garden challenge with infective copepodid sea lice *Lepeophtheirus salmonis* showed function feed diets to have no impact on sea lice resistance. Results provide a high-resolution map of Atlantic salmon mucosal immune expression, microbiota, and their interactions across the gut, gill and skin. Insights from this study on the effects of dietary inclusion of functional feed ingredients on mucosal health will inform future decisions on nutritional supplementation in the aquaculture, as the industry continues to search for sustainable ways to bolster mucosal immunity against threats of disease.

Introduction

Mucosal surfaces lie at the interface between organism and environment and serve as the first line of defense against pathogens, pollutants, and other stressors. The importance of mucosal tissues has generated substantial research interest and concurrent scientific discovery related to teleost mucosal immunity over the last two decades. We now know the complexity of the systemic immune system pales in comparison to mucosal immune systems, which can be subdivided into distinct mucosa-associated lymphoid tissues (MALT). In teleost fish the MALT include the gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT) and gill-associated lymphoid tissue (GiALT) (Salinas et al. 2011). The MALT represent tissue-specific centers from which the immune system regulates environmental microorganisms through both innate and adaptive immune effectors. The innate arm of mucosal immunity is the first response against microbial invasion, particularly in fish. Innate mucosal immunity includes host secretions that directly interact with microbes such as mucins, antimicrobial peptides, and complement components; phagocytic cells that

engulf pathogens; as well as pathogen recognition receptors (PRR) and local cytokine signaling that regulate inflammation (Gomez et al. 2013). Mucosal adaptive immune responses are primarily achieved by secreted or membrane bound mucosal immunoglobulins (IgT/IgM) generated by local B-cells and plasmoblasts, as well as CD4+ (T-helper - T_H), CD8+ (cytotoxic-T - T_c), and FOXP3+ (regulatory-T - T_{Reg}) T-cells (Rombout et al. 2014). The adaptive immune system further relies on cell to cell communication through the major-histocompatibility complexes (MHC-I and MHC-II) to trigger these adaptive responses. Together, these systems are responsible for providing an immunological barrier against pathogens from the external environment, which is particularly important in aquatic animals due to the intimate interaction with environmental microbes (Rombout et al. 2014; De Schryver and Vadstein 2014).

Mucosal immune tissues of fish are not only responsible for preventing pathogenic invasion but must also interact with and attempt to govern the microbiota, or commensal and beneficial microbes that continuously inhabit all mucosal surfaces. Research on the mucosal microbiota of fish has flourished in the last decade and expanded our understanding of the importance and diversity of physiological impacts that mucosal microbes have on their host. Evidence from axenic and gnotobiotic zebrafish models suggest microbiota serve a critical role in priming and maintaining the development and activity of the teleost immune system (Salinas et al 2011). A majority of research on fish mucosal microbiota has been focused on gut microbiota, with the gut microbes of over 150 teleost species, across a range of environmental conditions, characterized by next-generation sequencing, to date (Perry et al. 2020). Despite the early focus on the gut microbiota, the skin, gill, and even nasopharyngeal microbiomes of fish are now receiving more attention, though the functional attributes of these microbiota still remain greatly understudied (Legrand et al. 2019).

As the first line of defense, it is not surprising that a majority of the most financially burdensome diseases hampering aquaculture fish production begin as acute perturbations to one or more of these mucosal tissues. For example, it is imperative that commercial aquaculture begin incorporating increasing amounts of terrestrial plant-based ingredients in diets to replace fishmeal and fish oil for industry growth to remain financially and environmentally sustainable (Naylor et al. 2009); however, at high levels these ingredients induce inflammatory enteritis in the gut mucosa and dysregulate gut microbiota, particularly

in high-value carnivorous fish species (Kononova et al., 2019). In addition, many of the costliest infections in aquaculture, including infectious salmon anemia, enteric red mouth (i.e. yersiniosis), ameobic gill disease, and white-spot disease (Ichthyophthiriasis) are known to initiate virulence at the site of the gill mucosa (Koppang et al., 2015). Furthermore, ectoparasitic infections such as those from sea lice, which represent the largest disease-related production-cost impacting Atlantic salmon aquaculture (Bjørndal and Tusvik, 2020), *Ichthyophthirius*, or numerous bacterial pathoges (i.e. *Aeromonas, Flavobacterium*, and *Vibrio*) are known to afflict the skin mucosa of many aquaculture species (Ángeles Esteban, 2012). Improving our understanding of mucosal health has the potential to not only increase our ability to better manage such disease outbreaks, but may also help prevent some disease by improving our ability to generate practical and efficacious mucosal vaccines that can be easily administered orally through feed or by bath immersion (Adams 2019).

As the aquaculture industry awaits further vaccine development, functional feed ingredients are commonly tested as a means of improving mucosal health in fish. In the context of aquaculture, functional feeds are defined as dietary supplements that enhance growth, health, and physiological performance when administered above basal dietary requirements and can include micro-nutrients, immune stimulants, specific lipid sources, or pre-, pro-, and synbiotics (Martin and Krol, 2017). Mannan-oligosaccharides (MOS), which are complex carbohydrate molecules derived from yeast cell walls, are a commonly used functional feed ingredient that are thought to serve prebiotic and immune stimulant functions. The utility of MOS in improving mucosal health at the gut, skin, and gill has been shown repeatedly across multiple fish species. Specific benefits from MOS supplementation include improved histomorphology following dietary and pathogenic perturbation of the intestine, (Torrecillas et al. 2014, 2015, 2018; Guerreiro et al., 2017; Leclercq et al., 2020), skin (Leclercq et al., 2020) or gill (Zhao et al. 2015), increased production and altered proteome of skin mucus (Rodriguez-Estrada et al., 2013; Micalled et al., 2017), and modulation of gut microbiota (Dimitroglou et al., 2009, 2010). Mechanisms involved in these outcomes are thought to be predominantly based in the ability of MOS molecules to (1) stimulate PRR that lead to downstream alterations in local and systemic immunity, (2) bind to and neutralize some enteric pathogens, and (3) serve as a preferred fermentable prebiotic carbohydrate to nourish specific microbiota (Leclercq et al., 2020).

Specific oil sources have also received some attention as functional dietary ingredients in aquaculture. Differences in fatty acid profiles among various dietary lipid sources are known to influence (1) cell membrane structure, function, and fluidity, (2) the production of immunologically active eicosanoids (i.e. prostaglandins, thromboxanes, and leukotrienes), (3) oxidative stress, and (4) energy metabolism (Tocher, 2003). Oil sources high in medium-chain fatty-acids (MCFA), or those fatty-acids with a chain length of 6 to 12 carbons, have also received interest as a novel dietary energy sources because of the ease with which MCF can undergo beta-oxidation to produce energy (Luo et al., 2014); however, certain MCF have also been shown to have some functional feed attributes as well. Coconut oil is a lipid source with high levels of saturated MCFA, particularly lauric (C12:0; 40-50%) and caprylic acid (C8:0; 5-10%). *In-vitro* assessment of lauric and caprylic acid have shown them to have antimicrobial (Huang et al., 2011) and antiparasitic properties (Hirazawa et al., 2001), respectively. Nevertheless, the *in-vivo* effects of high levels of dietary coconut oil and its associated MCFA profile on fish mucosal health have yet to be explored.

In what follows, an integrative data analysis approach was taken to compare host-microbiota interactions across the skin, gut, and gill microbiota of Atlantic salmon (*Salmo salar*) while also exploring the concurrent impacts of dietary supplementation of MOS, coconut oil, or a combination of the two. The aim was to compare and identify differences in tissue-specific host mucosal immune expression and mucosal microbiota composition and function, while also highlighting dietary influences on the same endpoints, as well as on sea lice resistance.

Methods and Materials

Experimental Diets and Fish Husbandry

A twelve-week feeding trial was performed at the USDA-ARS National Cold Water Marine Aquaculture Center (NCWMAC). Four experimental diets were produced at the Bozeman Fish Technology Center (Bozeman, MT, USA) using commercial extrusion technology. A control diet (Control) was formulated to match the NCWMAC post-smolt base diet. A mannan-oligosaccharide diet (MOS) was made consisting of the control diet with wheat-flour replaced by mannan-oligosaccharides (BioMOS; Alltech, Lexington, KY) to achieve a 1% (10 g kg^{-1}) inclusion. A third diet was formulated to replace 96% of the lipids (4% fish oil) in the control diet with coconut oil (CoconutOil) and the fourth diet was a

combination of the MOS and Coco treatments (CocoMOS). The trial was conducted in a twenty-four tank system supplied with flow-through natural seawater. Each dietary treatment was administered to six replicate tanks. Twenty individually tagged Atlantic salmon with an average initial weight of 358.3 g ± 17.8 (mean ± SD) were randomly stocked to each tank. All fish were allowed to acclimate to the system for one month while receiving the control diet. Photoperiod mimicked natural settings (Aug. – Nov.) and natural seawater followed ambient temperatures. Water quality, salinity (31.1 ppt ± 0.7; mean ± SD), and temperature were monitored weekly and maintained within acceptable ranges for the duration of the study. Fish were fed using automatic feeders controlled by a continuous dynamic function to supply 110% of maximum expected daily consumption. Growth was assessed at six- and twelve-weeks using bulk tank weights.

Ethics Statement

Fish were sampled in accordance to Standard Operating Procedures: Care and Use of Research Animals. Publication 4, November 2018, USDA, ARS National Cold Water Marine Aquaculture Center, 25 Salmon Road, Franklin, ME 04634. Institutional Animal Care and Use Committee approval was obtained on June 2018.

Sample Collection

At the conclusion of the twelve-week feeding trial, samples were collected from three fish $tank^{-1}$ (n = 18 $diet^{-1}$) twelve hours after the last feeding. Fish were euthanized with tricaine methanesulfonate following AMVA recommendations (Leary, 2013). Whole blood was collected by caudal venipuncture using a heparinized syringe and held on ice until further processed. Microbiota communities of the gut, gill, and skin were sampled using Whatman OmniSwabs® (GE Healthcare; Chicago, IL, USA). Skin microbiota was sampled by swabbing the left side of fish along the lateral line, between the operculum and caudal peduncle. Gill samples were collected by swabbing between gill arches. Gut microbiota samples were collected by swabbing the mucosa of the distal intestinal tract following careful excision of the intestinal tract and removal of feces. Diet microbiota samples were collected by homogenizing each treatment diet using mortar and pestle and water microbiota was collected by run 1 L of inflow water through a 0.2 µm filter to collect water microbes. Environmental microbiota samples were collected in triplicate. All microbiota samples were flash frozen in liquid nitrogen and stored at -80°C until further processed. Host tissue was

concurrently sampled from each mucosal site for gene expression analysis. Skin tissue samples consisted of a 2 cm^2 section of skin excised from between the dorsal fin and lateral line on the left side of the fish. Gill tissue was sampled from the second gill arch on the left side of the fish and a 2-3 cm section of distal intestine was taken 3 cm anterior of the cloaca to serve as the gut tissue sample. All tissue samples were preserved in RNAlater® (Thermo Fisher Scientific; Waltham, MA, USA) and stored at -80°C until processed.

Isolation of Peripheral Blood Leukocytes (PBL)

Whole blood was processed to isolate circulating peripheral blood lymphocytes and leukocytes (PBL) through hypotonic lysis and removal of red blood cells following methods first described by Crippen et al. (2001) and recently optimized by Hu et al. (2018). Briefly, hypotonic lysis was initiated by diluting 1 mL of blood with 9 mL of prechilled water (1/10 dilution) for 20 s before returning the solution to isotonicity with 10X Dulbecco's Phosphate Buffered Saline (DPBS). Cellular debris of the lysed red blood cells was allowed to settle for 10 min before filtering the supernatant through 70 µm cell strainers. The resulting supernatant was centrifuged at 200 x g for 5 min at 4°C to pellet the PBL. Cells were gently washed twice with 1x DPBS, prior to storing in RNAlater®. All reagents used in PBL isolation were pre-sterilized (0.2 µm filtering) to reduce the risk of contaminants that may stimulate or change PBL expression profiles during sample processing.

16S rRNA Gene Library Preparation

Microbiota swabs were homogenized with 0.7 mm garnet beads and a TissueLyser (Qiagen; Hilden, Germany, EU). DNA was isolated using a QIAmp 96 PowerFecal QIAcube HT Kit and QIAcube HT (Qiagen) liquid handler following standard procedures. DNA purity and concentration were assessed on a Nanodrop 2000 (Thermo Fisher Scientific) and due to low purity among some samples, as suggested by 260/230 spectrophotometric ratios, all samples were cleaned and concentrated using a gDNA Clean and Concentrate Kit (Zymo Research; Irvine, CA, USA). Raw DNA samples were normalized by fluorometry (Quant-iT PicoGreen dsDNA kit; Thermo Fisher Scientific) and used as template for preparation of V3V4 16S rRNA gene sequencing libraries following a strategy similar to that detailed by Fadrosh et al (2014). A set of custom phased V3V4 16S rRNA target specific primers were designed to include a consensus sequencing pad (Fluidigm; San Franciso, CA, USA) and custom staggered linker-spacer regions to both the 341F and 785R primers (Klindworth et al.

2013) (Table 2.1.). First, 16S rRNA gene amplicons were prepared in duplicate 25µL PCR reactions consisting of 30 cycles of PCR using Phusion HiFi Hot Start II Mastermix (Thermo Fisher Scientific), custom V3V4 341F-785R primers (500 nM each, Ta = 60°C) and 10 ng of template DNA. Duplicate PCR products were pooled and confirmed by electrophoresis on 2% agarose gel prior to purification with magnetic DNA purification beads (0,8X; MagBio; Gaithersburg, MD, USA). Resulting PCR products were diluted two-fold and 1 µL was included as template for eight cycles of PCR in 50 µL reactions including 200 nM custom barcoding primers (Ta = 72°C), containing dual-indexes and sequencing adapters. Resulting libraries were again confirmed by electrophoresis and purified with magnetics beads (0.8X) prior to equimolar pooling according to fluorometry (Quant-iT PicoGreen dsDNA kit). The final pool was analyzed on a DNA 1000 chip (Agilent Technologies; Santa Clara, CA, USA) to confirm library size and final quantification was done using a Kapa qPCR Illumina Quantification Kit (Roche; Indianapolis, IN, USA). Sequencing was done in-house at the Hagerman Fish Culture Experiment Station on a full MiSeq (Illumina) 600-cycle v3 sequencing. Sequencing required spiking-in three custom sequencing primers (BAMF-CS1, BMF-CS2, BAMF-CS2rc) which were modified by the University of Idaho Genomics Resources Core to match the thermocycling parameters of an Illumina MiSeq by incorporation of locked-nucleic acids.

Microbiota Analyses

Raw data are publicly available on the NCBI repository (https://www.ncbi.nlm.nih.gov) under BioProject PRJNA663352. Data were demultiplexed and primers were removed with dbcAmplicons (https://github.com/msettles/dbcAmplicons). DADA2 (Callahan et al., 2016) was used to process raw reads into bacterial amplicon sequencing variants (ASV). First, reads were truncated (Forward – 275 bp, Reverse – 215 bp), quality filtered (2 expected errors threshold), and merged (≥ 400 bp). After denoising, ASV were quantified and chimeric sequences were removed. Taxonomy was applied using a Bayesian classifier trained against the Silva nr_v132 rRNA database (Quast et al., 2012). Decipher (Wright, 2016) was used for sequence alignment, prior to constructing a phylogenetic tree under a GTR model using phagorn (Schliep, 2011). Singleton ASV and those assigned to the order Chloroplast or the family Mitochondria were removed. Phyloseq

(McMurdie and Holmes, 2013) and vegan (Oksanen et al. 2013) were used for all downstream data transformations and calculation of ecological indices.

Differential abundance (DA) testing was done using DESeq2 (Love et al., 2014) to identify DA by tissue, while controlling for dietary effects. Tissue-specific dietary effects on microbiota abundance were identified by comparing all dietary treatments to the control group within each tissue. All DA testing was conducted with a significance threshold of FDR-corrected $q \geq 0.05$ and $|\log2\text{-fold change}| \geq 1.0$ after fold-change shrinkage using apeglm (Zhu et al., 2018).

Metagenomic functional potential of microbiota was inferred as KEGG orthologs (KO) and higher-level summaries as MetaCyc pathways using PICRUSt2 (Douglas et al. 2020). KO and pathways were plotted and analyzed using STAMP (Parks et al. 2014) to compare the functional potential of the microbes present on the three mucosal sites using a Tukey-Kramer post-hoc test. Functions were considered significantly different when FDR corrected q-values were less than 0.01 and effect size was greater than 0.5. Associations between inferred functions and phylogenetic composition of the microbiota were tested by asymmetric Procrustes analyses to map the axes from principle coordinates analysis (PCoA) of weighted UniFrac distances to the principal competent analysis (PCA) loadings calculated from either the KO or MetaCyc datasets.

Network reconstruction was conducted on tissue specific datasets (gut, gill, and skin) to evaluate microbial co-association patterns by mucosal site and identify site-specific keystone taxa. To reduce sparsity, individual datasets were filtered to remove ASV that accounted for less than 0.001% relative abundance. Compositional data transformation and inference of sparse inverse covariance networks was conducted using SPIEC-EASI (Kurtz et al., 2015).

RNA Extraction and Gene Expression Analysis

A reduced sample size of nine fish treatment^{-1} (n = 3 tanks diet^{-1}) were processed for gene expression analysis. Gut, gill, and skin tissue, as well as PBL, were thawed and removed from RNALater. RNA isolation followed recommended procedures for tissue samples using the RNeasy 96 HT RNA Isolation Kit automated on the QiacubeHT (Qiagen). Concentration and quality were assessed by NanoDrop 2000 ($260/280 \geq 1.8$ and $260/230 \geq 1.5$). Samples below the acceptable thresholds were cleaned using a GeneJet RNA Clean-up

Kit (Thermo Fisher Scientific). Twelve samples of each tissue were randomly selected for analysis on an RNA 6000 Nano chip (Agilent Technologies) to confirm RNA integrity (RIN ≥ 7.2). Removal of gDNA and reverse transcription were conducted in triplicate 20µL reactions with 1000 ng of input RNA each using the iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad Laboratories; Hercules, CA, USA) on a T100 thermocycler (Bio-Rad Laboratories).

Primers for qPCR were designed using Primer-BLAST (Ye et al., 2012) or taken from previous publications after confirming the primer targets in-silico (Table 2.2). Primer sets were validated by running products on a 2% agarose gel to confirm specificity and size of intended target. Primer efficiency of each primer set was estimated separately by tissue using duplicate six series five-fold serial dilutions of a pooled cDNA standard. Each assay was conducted in duplicate 10 µL reactions with 1 µL of neat cDNA, 300 nM forward and reverse primer, and SsoAdvanced Universal Sybr Green Supermix (Bio-Rad Laboratories). The qPCR assays were run on a CFX96 (Bio-Rad Laboratories) for 35 cycles following recommended cycling parameters (Ta = 60°C). Melt curve analysis was conducted after each assay to ensure specificity.

Elongation factor 1 alpha (EF-1∝), hypoxanthin-guanin phosphoribosyl transferase 1 (HPRT1), and RNA polymerase 2 subunit (RPABC2) were used as reference genes with stability confirmed by the reference gene selection tool onboard the CFX96 (Bio-Rad). Raw Ct values were efficiency corrected and normalized against the geometric mean of the reference genes using soft normalization Bayesian priors in MCMC.qPCR (Matz et al., 2013). Gene expression data were analyzed separately with a set of systemic-/adaptive-immunity related genes run on the gut, skin, gill and PBL samples, and a set of mucosal-/innate-immunity related genes analyzed across the three mucosal tissues only (Table 2.2). The two gene sets were analyzed separately following the same procedures. Efficiency corrected Ct values for all genes were modeled using fixed effects of tissue, diet, and tissue-diet interaction, while controlling for random effects of tank and individual sample under a single Bayesian model. Outlier detection was done using the full model, with those samples two standard deviations from the global sample mean removed. Gene wise p-values from contrasts of interest were extracted from the model and adjusted for multiple comparison (FRD adjusted, $q \leq 0.05$).

Sea Lice Challenge

Following the diet trial, five fish per tank were transferred to common-garden tanks in a recirculating aquaculture system for a sea lice (Salmon louse, *Lepeoptheirus salmonis*) challenge. Three common garden challenges were conducted with fish from two tanks of every dietary treatment included in each challenge (40 fish challenge^{-1}). Challenges followed the protocol outlined by Peterson et al. (2020). Briefly, a static bath challenge was conducted at a density of 100 infective copepods fish^{-1}, with water supply returned after 4 h. Infections lasted 10 – 14 days prior to counting of infective sea lice and calculating right-side lice density (Gjerde et al., 2011). In addition, surface-area (cm^2) was estimated using the formula provided by Frederick et al. (2017) to calculate lice surface-area^{-1}. Lice density and lice surface-area^{-1} were tested for differences due to dietary treatment by ANOVA, while controlling for diet-trial tank and challenge tank.

Results

Growth Performance

Ambient water temperatures decreased from 20°C at the start of the trial to 11°C by the end (Figure 2.1). At the conclusion of the 12-week trial, fish weighed 646.5 g ± 35.8 (mean ± SD), with 288.2 g ± 38.3 of growth over the trial. No difference in weight gain was detected by dietary treatment (ANOVA, $p = 0.4014$). Fish grew 311.85 ± 38.16, 278.52 ± 25.34, 280.47 ± 35.26, 282.12 ± 49.95 for the Control, MOS, Coconut Oil, and Coco+MOS groups, respectively.

Microbiota Analysis

Four gut and three gill microbiota samples were sequenced at an insufficient depth and removed from the downstream dataset. From the full dataset, including environmental (diet and water) samples a total of 8,731 unique ASVs were identified, with 7,986 ASVs identified in the fish samples alone. After sample and ASV filtering, the 209 remaining samples (excluding water and diet samples) had 56,382 ± 24,627 (mean ± SD) ASV assigned reads sample^{-1}.

Alpha Diversity

Rarefaction analysis indicated that all samples were sequenced deeply enough to reach an asymptote in bacterial richness. Alpha diversity, both observed ASV richness and Shannon diversity, were calculated by individual before removing outliers using Tukey's

method (> 1.5 * IQR) and averaging values by experimental unit (tank). A two-way ANOVA (Type II SS) run on a linear model fit to tissue, diet, and tissue-diet interactions showed tissue had a significant influence on both observed richness ($p \leq 0.001$) and Shannon diversity ($p \leq 0.001$), while diet had an influence on observed richness ($p = 0.032$) but not Shannon diversity ($p = 0.444$) (Figure 2.2 A-B). Tissue and diet were not found to have any interaction effects on alpha diversity ($p \geq 0.197$). A Tukey's post-hoc test indicated that observed richness and Shannon diversity were significantly reduced in the gut samples compared to the skin and gills (Figure 2.2 A-B). Despite the global significance of dietary effects on observed richness, a pairwise Dunnett's test comparing all diets to the control showed no significant pairwise difference ($p \geq 0.995$). To further evaluate dietary effects, observed richness within each tissue were independently tested by diet alone using one-way ANOVA, and again no tissue-specific dietary differences were detected ($p \geq 0.133$).

Beta Diversity

Comparisons of the overall microbial communities among samples were made by first calculating phylogenetically informed weighted (wUniFrac) and unweighted UniFrac (uwUniFrac) sample distances. Multivariate dispersion (homogeneity of variance) was tested individually by tissue and diet, with tissue groups identified as having unequal dispersion in both wUniFrac ($p = 0.049$) and uwUniFrac ($p < 0.001$) (Figure 2.2 C-D). Beta dispersion was not influenced by diet ($p \geq 0.467$). Adonis2 (Oksanen et al. 2013) was used to perform a PERMANOVA to test for significance of the main effects of diet and tissue, as well as interaction effects, with permutations stratified by tank to account for the nested design. Because tissue had a highly significant effect ($p \leq 0.001$) on both UniFrac distances (Figure 2.2. C-D) and no interactions were detected, pairwise-PERMANOVA (Hervé and Hervé 2020) was ran between the three tissues. A highly significant difference between all pairwise tissue combinations was detected using both distance metrics ($p \leq 0.001$; FDR adjusted). Tissue specific influences of diet on beta diversity were further tested by running a PERMANOVA separately on each tissue modeling the effects of diet alone, with the only significant shifts identified in the skin microbiota ($p = 0.006$) using unweighted distances (Figure 2.2 E). A pairwise-PERMANOVA indicated all dietary treatments altered the skin microbiota composition compared to that of the control diet ($p \leq 0.040$; FDR adjusted),

though no other pairwise comparisons were significantly different ($p \geq 0.307$; FDR adjusted).

Microbiota Composition and Differential Abundance

Bacteria from 27 different phylum were detected in the study, with the top five most abundant phyla being Proteobacteria, Bacteriodetes, Firmicutes, Actinobacteria, and Verrucomicrobia for the gut, gill, diet, and water samples (Figure 2.3 B-D). In the skin samples the same phyla were present, however, Patescibacteria replaced Verrucomicrobia as the fifth most abundant phyla (Figure 2.3 A). At the phylum level, microbiota taxonomic composition was similar among all sample types. In the diet samples 249 ASV were detected and 745 were found in the water samples. A total of 782, 2,499, and 2,847 ASV were detected in the gut, skin, and gill mucosa, respectively (Figure 2.3 E). The gill and skin mucosa had the greatest overlap in shared ASV, and the two tissues also shared over 500 ASV with the water microbiota (Figure 2.3 E). Surprisingly, the gut mucosal microbiota shared more ASV with the gill samples than any other sample type, including diets (Figure 2.3E).

Differential abundance (DA) testing at the ASV level was used to determine whether the abundance of bacteria could discriminate between mucosal tissue or dietary treatments within a tissue (Figure 2.4). Forty-six ASV were identified as DA ($q \leq 0.05$; $|\log2 \text{ fold-change}| \leq 1.0$) between the gut and gill, 57 ASV between the gut and skin, and only one between the skin and gill microbiota. Of the DA ASV identified between the skin vs. gut and gill vs. gut, 31 of those were common, and the one ASV identified as DA between skin vs. gill was also DA between the skin vs. gut. Dietary influences on microbiota abundance were tested within each tissue-specific dataset, with the gill mucosal microbiota showing the only significant dietary effect. In the gill, the MOS diet was found to increase the abundance of a single ASV in the genus *Geobacillus* (Figure 2.4) in comparison to the control diet group. No other DA was detected due to dietary treatments within each tissue.

Network Analysis

Because DA analysis identified nearly no impact of diet on microbial abundance within tissues, network reconstruction was conducted at the tissue level by combining data from all dietary treatments to detect tissue-specific patterns of microbial co-association and site-specific keystone bacteria (Figure 2.5). Centrality analysis was conducted to quantify

connectivity of nodes within networks. Multiple network centrality measures were tested on each network, with the most informative metric determined by correlation with PCA loading within CINNA (Ashtiani et al., 2019). Betweenness centrality was the only centrality measure to pass the CINNA recommended contributions-threshold among all tissue-specific datasets and was therefore used to plot, analyze, and compare gut, gill, and skin networks (Figure 2.5. A-C). The gut microbiota network indicated 1,397 covariance associations between ASV, with 1,307 positive associations (94%). The gill and skin networks showed high degrees of connectivity, with 14,824 and 14,275 network edges, of which 12,600 (85%) and 11,823 (83%) were positive, respectively. Keystone species were identified from the tissue specific networks by ranking ASV by betweenness centrality. In order, the top three ASV in terms of centrality were assigned to *Cutibacterium* (ASV231), *Photobacterium* (ASV60), f_Flavobacteriaceae (ASV 447) in the gut (Figure 2.5 A); *Escherichia/Shigella* (ASV3), *Provotella* (ASV874), *Sedimenticola* (ASV1406) in the gill (Figure 2.5 B); and *Escherichia/Shigella* (ASV3), *Aliivibrio* (ASV4), and *Aliifodinibius* (ASV39) in the skin (Figure 2.5. C).

Functional Predictions

A total of 7,124 KO functions were inferred from the ASV in the three mucosal tissues, with 966 KO showing significant differences by tissue ($q < 0.01$; effect-size > 0.5). Statistical testing of functional MetaCyc pathways found 54 of 412 inferred pathways to be significantly different between the gut, skin, and gill microbiota (Figure 2.6). Asymmetrical Procrustes analysis was conducted to determine the concordance in mapping ordinations of inferred microbial functions to bacterial phylogenetic beta diversity (Figure 2.7). Procrustes analysis between wUniFrac PCoA axes and PCA loadings from KO functions showed a significant association ($m^2 = 0.8397$, correlation = 0.4003, $p < 0.001$) though mapping of the functional ordinations showed less clear clustering of samples (Figure 2.7. A). Similar analysis conducted between the abundance weighted microbiota composition PCoA axes and PCA loadings from the predicted MetaCyc pathways showed no significant association ($m^2 = 0.9894$, correlation = 0.1031, $p = 0.223$), with a large reduction in separation between samples in the functional pathway ordinations (Figure 2.7 B).

Gene Expression

Quantitative analysis of gene expression between tissues was evaluated using contrasts between tissue-specific expression levels among fish within the control diet group (FDR adjusted $q \leq 0.05$). Tissue-specific differences in gene expression were detected for all genes assayed, with the exception of membrane Toll-like receptor 5 (mTLR5), which showed high levels of intra-tissue variability (Figure 2.8). Within a tissue, all pairwise dietary treatment comparisons were tested for significance. Within the PBL, the CocoMOS diet group had significantly reduced expression of CD4 compared to all other diets. Dietary effects on expression of FOXP3 were detected in the gut (Control vs. CocoMOS and CoconutOil vs CocoMOS) and skin (Control vs. CoconutOil). Expression of the mucosal immunoglobulin (IgT) showed dietary effects in the gut (Control vs. CoconutOil and CocoMOS vs. CoconutOil), gill (Control vs. MOS, Control vs. CoconutOil, CoconutOil vs. CocoMOS, and MOS vs. CocoMOS), and PBL (Control vs. CoconutOil and CoconutOil vs. CocoMOS). Gill expression of MHC2 was influenced by diet (Control vs. CoconutOil). Expression of IL10 at the gut (Control vs. MOS and MOS vs. CoconutOil), gill (Control vs. CocoMOS, MOS vs. CoconutOil, and. MOS vs. CocoMOS), and skin (MOS vs. CoconutOil) showed dietary effects, however, IL10 expression in the gut was near the lower limit of detection for the assay and may be less reliable. Dietary effects of IL17A expression were detected in the gut with a significant difference between Control and MOS diets. In the gut, expression of mannose binding lectin type-C (MBLc) was significantly reduced by each diet in comparison to the control diet.

Multivariate Analyses of Gene Expression Profiles

Model inferred transcript abundances of each gene were used to calculate sample-wise Manhattan distance matrices to conduct multivariate analyses of the gene expression profiles (Figure 2.9). Dispersion by tissue ($p = 0.117$) and diet ($p = 0.826$) was homogeneous for the systemic-immunity gene set (Figure 2.9 A-B), although dispersion was found to vary by tissue ($p = 0.001$), but not diet ($p = 0.459$) in the mucosal-immunity gene set (Figure 2.9 C-D). Multivariate analysis of both systemic and mucosal gene expression profiles showed highly significant effects of tissue ($p = 0.001$), diet ($p = 0.001$ and 0.032, respectively), and tissue-diet interaction ($p = 0.001$) according to PERMANOVA. Pairwise-PERMANOVA indicated a highly significant difference in expression profiles ($p \leq 0.001$) between each

tissue in both gene sets, though no pairwise differences in multivariate expression profiles were detected by diet (FDR corrected $q \geq 0.57$).

Host-Microbiota Interaction

To assess associations between host gene expression and microbiota communities, a symmetrical Procrustes analysis was used to compare PCoA axes from Manhattan distances of mucosal gene expression profiles to PCoA axes of UniFrac distances in microbiota phylogenetic composition (Figure 2.10). Because of the reduced sample size in the gene expression datasets, microbiota samples not present in the expression dataset were removed prior to ordination. Procrustes identified a highly significant concordance between tissue gene expression profiles and microbial communities using both wUniFrac ($m^2 = 0.3309$, correlation = 0.818, $p \leq 0.001$) and uwUniFrac ($m^2 = 0.2595$, correlation = 0.8605, $p \leq 0.001$). Orthogonal mapping of microbial communities to gene expression profiles showed that while tissue-specific microbial beta diversity had higher levels of dispersion than tissue-specific gene expression, the datasets were highly congruent. When accounting for abundance of microbiota, gut microbiota composition showed the most deviation from host gene expression profiles (Figure 2.10 A), though when considering microbiota presence-absence, skin and gill tissue samples showed more discordance between ordinations (Figure 2.10 B).

Sea Lice Challenge

Sea lice challenge with *L. salmonis* copepodids, following the diet trial, yielded mild levels of infection (18.9 ± 9.8 lice fish^{-1}; mean ± SD). Functional feeds showed no effect on sea lice resistance in triplicate common garden challenges. When corrected for diet trial tank and challenge tank, right side sea lice density (0.27 ± 0.17) and lice surface-area^{-1} (0.028 ± 0.017) showed no significant difference by dietary treatment (ANOVA; $p = 0.614$ and $p = 0.610$, respectively).

Discussion

The functional feed treatments (MOS, CoconutOil, or the CocoMOS) in this study showed no effects on growth performance, only minor impacts on microbiota richness, composition, and abundance, and no impact on susceptibility to salmon louse challenge. Recently, a short feeding trial (44 days) evaluating 0.4% MOS supplementation in Atlantic salmon also found no significant impact on growth performance (Leclercq et al., 2020);

however, goblet cell density and coverage, mucus production, and lysozyme activity in the gut and skin were improved, while also reducing salmon louse counts during experimental challenge. Likewise, a four week 1% MOS supplementation to juvenile red drum (*Sciaenops olivaceus*) fed a high soybean-meal diet did not significantly change growth performance, though mortality and infection rates were reduced during a challenge with an ecoparasitic dinoflagellate (*Amyloodinium ocellatum*) (Buentello et al., 2010). Conversely, Refstie and others (2010) showed that 1% and 2% MOS supplementation to 680 g Atlantic salmon did not increase resistance to salmon louse, but did significantly improve growth, feed efficiency, and resistance to sunflower- and soybean-meal induced intestinal enteritis. The contradictory results on MOS supplementation in aquaculture, including those presented here, can likely be explained by differences in host species and age, MOS dose and form, as well as husbandry and environmental conditions, as was suggested by Torrecillas and colleagues (2014) in their review on the topic. In one of the only studies to evaluate the effects of fish oil replacement with coconut oil in terms of fish performance, Luo and colleagues (2014) found coconut oil to yield similar growth rates, no differences in feed efficiency, and surprisingly small changes to plasma metabolites in another salmonid, rainbow trout (*Oncorhynchus mykiss*), despite the stark differences in fatty-acid profiles between the two oil sources. Craig and Gatlin (1996) also showed that coconut oil could replace Menhaden fish oil in juvenile red drum without adverse effects on growth performance. While a few authors have explored the effects of coconut oil on growth, the present study is the first to explore its impacts on host immunity, microbiota, and salmon louse susceptibility, and therefore more studies are needed to confirm effects of coconut oil on mucosal health in fish.

This study is the first to characterize and compare the gut, gill, and skin mucosal microbiomes of Atlantic salmon, while also observing the effects of dietary functional feed ingredients on the corresponding microbial communities. In terms of dietary impacts, a slight yet significant impact of diet on bacterial richness was detected, with the dietary treatments trending toward decreased abundance in the skin and gill, but no significant differences were detected in pairwise dietary comparisons (Figure 2.2 A). Previous studies on the impacts of MOS on microbiota of fish have been relegated to the gut, and results have been rather contradictory, as discussed by Torrecillas et al. (2014). A majority of studies on the effects of MOS on the gut microbiota have been conducted on juvenile fish and over relatively short

periods (1-2 months), while the present study was conducted on 646.5 g ± 38.3 (mean ± SD) post-smolt over a twelve-week period. It is likely that the homeostatic microbiome of larger, older fish are more stable (Bledsoe et al., 2016) and therefore less susceptible to modulation by functional feed ingredients as has been observed in some studies (Dimitoglou et al., 2010; Gonçalves and Gallardo-Escárate 2017). Gajardo et al. (2017) showed the digesta-associated microbiota of Atlantic salmon to be more diverse and prone to dietary alterations, while the mucosa-associated microbiota showed significantly less diversity and lower susceptible to dietary impacts. This may explain the relatively low diversity detected in the gut mucosa, as well as the lack of dietary effects on the mucosa-associated gut microbiota in the present study. In the skin microbiome, all dietary treatments were found to alter the phylogenetic composition compared to the control in the current study, although, no microbes were identified as differential abundant by diet within the skin (Figure 2.4). This is because the dietary shifts in skin microbiota were according to microbial presence-absence (uwUniFrac) (Figure 2.2 E) and therefore may not correspond with quantitative differences in ASV abundances, as would be detected by DA analysis. Chiarello et al. (2018) also detected diet related shifts in the skin microbiome of wild coral reef fish and suggested this could be explained by systemic dietary effects on mucus production and metabolite production, though more mechanistic studies are needed to confirm this hypothesis. The only dietary impacts on tissue-specific abundance of individual ASV was detected among the gill samples, where the MOS diet significantly increased the abundance of a single ASV in the genus *Geobacillus* (Figure 2.4). This microbe was recently detected as differential abundant in the gut microbiome of rainbow trout, as it was significantly reduced by a soybean-meal based diet compared to a traditional fishmeal diet (Blaufuss et al., 2020); however, microbes belonging to *Geobacillus* are typically thermophilic and therefore may represent transient non-function bacteria within the cold-water rearing environment of salmonids.

While few studies have compared the microbiota of all three mucosal tissues (gut, gill, and skin) in Atlantic salmon, Minich et al. (2020) recently conducted a similar study which characterized the skin, gill, and digesta microbiota of Atlantic salmon reared under different hatchery conditions (recirculating vs. flow-through). Those authors found the three tissue-specific microbiomes to be significantly different in composition, irrelevant of rearing environment. Minich et al. (2020) also found that when reared in a flow-through system, as

was done in the present study, the microbial richness of Atlantic salmon was highest in the gill, followed by the digesta, and finally the skin of Atlantic salmon. Results presented here show the highest number of unique ASV in the gill, followed by the skin, and the gut (Figure 2.3. E), with significantly higher bacterial richness and diversity in the skin and gill microbiomes compared to the gut (Figure 2.2 A-B). Also in agreement with Minich and colleagues' (2020) previous report, the present study found overall bacterial communities of the gut, gill, and skin mucosa to be significantly different from one another according to both quantitative (Figure 2.2 C) and presence-absence metrics (Figure 2.2 D), though the gill and skin communities showed greater overlap. Legrand et al. (2018) also found overlap between the gill and skin microbiota, despite also detecting tissue-specific signatures in yellowtail kingfish (*Seriola lalandi*), with the gill and skin sharing 84.9% of the total detected microbes in that study. Following this trend, microbiota DA analysis conducted in the current study showed the greatest pairwise differences between the gill and gut, followed by the skin and gut, with only one differentially abundant microbe between the gill and skin. An ASV in the genus *Corynebacterium* was the only bacteria to be identified as significantly more abundant in the gill than in either of the other two tissues (Figure 2.4). Interestingly, bacteria from this genus were recently found to be the most predicative of seasonal dietary shifts in the gut microbiome of wild three-spine stickleback (*Gasterosteus aculeatus*) (Friberg et al., 2019). However, *Corynebacterium* are also observed in the skin and gill microbiomes of yellowtail and were identified as being more abundant in healthy fish compared to fish with intestinal enteritis (Legrand et al., 2018). An *Allivibrio* ASV was detected as being more abundant in the gut microbiome compared to the gill and skin in the current research, and interestingly, microbes in this genus are pathobionts (i.e. *A. salmonicida, A. wodanis*, and *A. logei*) that were recently shown to be correlated with the loss of healthy intestinal lactic acid bacteria when in high abundance in the gut microbiome of Atlantic salmon (Godoy et al., 2015). Although, Zhao et al (2020) also found *Allivibrio* to account for 13% of all microbes detected in saltwater adapted coho salmon (*Oncorhynchus tshawytscha*) gut microbiome. Despite being detected as more abundant in the gut microbiota, *Allivibrio* (ASV4) was also found to be the microbe with the second highest level of interconnectivity in the skin microbiota (Figure 2.5). According to network reconstruction, an ASV (ASV3) in the genus *Escherichia/Shigella* was found to have the highest degree of connectivity within the gill and

skin microbiota networks and was among the fifty most highly connected ASV in the gut network as well (Figure 2.5). Microbes from the genera *Shigella* and *Escherichia* can be difficult to discriminate by DNA phylotyping, hints the combined annotation, though both are typically enteric pathobionts that are commonly detected in animal microbiomes. While showing a high degree of connectivity, this *Escherichia/Shigella* node had predominantly negative covariance interactions with other nodes, at least among the topmost connective bacteria, which has also been seen in networks constructed from the human gut microbiome (Zhu et al., 2018). The most central microbe to the gut microbiota network in the present study was from the genus *Cutibacterium*. While being a common component of the human skin microbiome, *Cutibacterium* recently were identified as core gut microbes in coho salmon, irrelevant of being reared in freshwater or saltwater environments (Zhao et al. 2020). Comparing microbial networks between tissues, the gill and skin microbiota had many more covariance associations than were found in the gut, although higher connectivity can at least partial be attributed to the greater microbial richness detected in those tissues (Figure 2.2 A-B).

Here a phylogenetic alignment based functional prediction algorithm (PICRUSt2) with a database containing over 20,000 full length 16S rRNA gene to genome mappings was used to infer metagenomic functional potential of the tissue-specific microbiota as KEGG orthologs (KO) and MetaCyc pathways. Differential analysis between the three mucosal microbiomes showed 13.5% of the detected KO and 13.1% of the higher-level pathways to be differential. In many cases functions show microbial adaptation to tissue specific sites that may also provide utility to the host. For example, the gills serve as the primary site of nitrogen excretion, primarily as ammonia (NH_4), in teleost species and metagenomic functional analysis showed more gill microbiota possessed genes required for denitrification compared to the microbes found in the gut (Figure 2.6 A). In agreement, microbes known for their ability to oxidize ammonia, nitrite, and nitrate were detected not only in the water of Atlantic salmon hatcheries but also in the fish skin, gill and digesta microbiomes (Minich et al., 2020), though just as in this study, it is possible these microbes are not highly adapted to the mucosal environment and may simply be a product of environmental transfer from the water microbiome. In addition, anaerobic fermentation capabilities of the gut microbiota in this study was highlighted by functional analysis, as the gut microbiota were shown to have a

greater ability to conduct mixed acid fermentation, while the exterior mucosal sites (gill and skin) were populated with microbiota with a higher prevalence of genes related to aerobic respiration (Figure 2.6 B-C). The abundance of the fermentation pathway in the gut microbiota suggest those bacteria are generating short chain fatty acids as fermentation byproducts, which are known to serve the host as a source of energy for enterocytes, but are also are involved in enteroendocrine signaling that modulated cellular proliferation, inflammation, and metabolism (Butt and Volkoff 2019). Furthermore, some metabolic pathways related to carbohydrate and amino acid metabolism were found to be more likely detected in gut microbes, where they likely aid in digestion of food items consumed by the host (Figure 2.6 D-G).

To determine whether microbial functions at both the KO and pathway levels maintained the tissue-specific separation that was observed in bacterial phylogenetic composition asymmetric Procrustes analysis was conducted to map PCA ordinations from microbial functional datasets to ordinations based on wUniFrac phylogenetic composition. Procrustes analysis showed a significant correlation between ASV wUniFrac composition and inferred KO functions, yet predicted MetaCyc pathways were not significantly correlated with the ASV composition. A seminal study on the mammalian gut microbiome, showed that Bray-Curtis ordinations of KO determined by shotgun metagenomics mapped well to wUniFrac with a m^2, a measure analogous to the sum of squared deviations, of 0.451 (Muegge et al. 2011). In the current study, the goodness-of-fit between KO and wUniFrac was worse ($m^2 = 0.8397$), but the correlation (0.4003) was found to be significant ($p \leq 0.001$). The Procrustes plots (Figure 2.7) suggest that microbial gene functions and pathways are conserved among the microbiota of the three mucosal tissues relative to the observed differences in phylogenetic profiles. This same phenomenon was observed by Muegge et al. (2011) as well, with those authors showing that the clear separation between omnivorous, carnivorous, and herbivorous mammal gut microbiota networks completely disappeared when gene functions were considered.

Despite the popularity of functional feeds, few studies evaluate the effects of such ingredients on mucosal immunity at the molecular level, especially across multiple distinct mucosal tissues, as was done here. Tissue specific expression profiles are dependent on the distribution of cell types present in the sampled tissues and therefore, comparisons of gene

expression, particularly that of certain cell markers like CD4, CD8, FOXP3, IgT, IgM, across tissues may be used as a proxy for the relative distribution of cell types by tissue. The CocoMOS diet induced a reduction of CD4 and FOXP3 expression in the circulating PBL and gut (Figure 2.8 A), respectively, suggesting the combination of the two functional feed ingredients altered helper and regulatory T-cell distribution or transcriptional activity in these tissues. According to expression levels, CD4+ T_H cells were higher in all mucosal tissues compared to that of the circulating PBL, while CD8 transcripts derived from cytotoxic T-cells were more abundant in the gut and PBL compared to the skin and gill (Figure 2.8 A). The PBL showed the highest level of expression of immunoglobulins (IgM and IgT), suggesting the circulating peripheral B-cells were transcriptional active, but it should be noted that this sample type consisted of purified lymphocytes and leukocytes, so the high expression of these markers was expected. First identified in 2005, IgT has been shown to be a teleostean analog to the mammalian mucosal antibody IgA that is responsible for adaptive humoral regulation of pathogens and commensal microbiota on the mucosal surfaces of fish, while IgM responses are more focused to the serum (Zhang et al., 2010). As such, the relatively high expression of IgT in the PBL was somewhat surprising (Figure 2.8 A), although Hu et al. (2018) also identified high levels of IgT expression in rainbow trout PBL while optimizing the isolation technique utilized in this study. Expression of the major histocompatibility complexes (1 and 2) showed very little intra-individual variation, with the only dietary influence resulting in a reduction of MHC2 in the gills when fish were fed the CoconutOil diet (Figure 2.8 A). This reduction in MHC2 may suggest a reduced activity or abundance of antigen presenting cells in the gills when fed the CoconutOil diet. Alkaline phosphatase (ALP) expressed in the gut participates in the regulation of microbes, control of intralumenal pH, and detoxification of inflammatory molecules generate by the host or microbiota. Intestinal ALP activity has been shown to respond to the introduction of pathogens and should therefore be responsive to major shifts in microbiota, though ALP activity is highest and most responsive to perturbations within the proximal intestine of fish (Lallès 2020). This likely explains the low expression of ALP in the gut compared to the gill and skin tissue in this study, as gut samples were taken from the distal portion of the intestine. Somewhat surprisingly, mannose binding lectin protein c (MBLc) expression was reduced in the gut by all treatment diets compared to the control in the present study. When

produced by intestinal epithelial cells, MBLc is a binding lectin that functions as an opsonin in the lectin-complement pathways and is known to interact with mannose on the surface of various microbes (Choteau et al., 2015). With the addition of a 1% yeast-derived mannose supplement to the MOS and CocoMOS diets, it was expected that the increased number of target antigens would also lead to an increase in MBLc expression, at least in the gut, though the opposite pattern was observed (Figure 2.8 B). In a mouse model, Choteau et al. (2015) found that a daily gavage of alpha-mannoside residues for one-week saturated intestinal MBL receptors and lead to a reduced ability to immunological control a fungal pathogen *Candida albicans* during challenge, although the mannan in that study was at much higher concentrations then in the present study. Taken together, this suggests perhaps the treatment diets used here induced attenuation of intestinal MBLc expression after chronic activation and saturation of the lectin complement pathway, although, this would not explain the observation of reduced expression of intestinal MBLc with the CoconutOil diet (Figure 2.8 B). While assessing tissue specific expression of MBLc, Peterson et al. (2015) showed the liver to be the tissue with by far the highest levels of transcription in channel catfish (*Ictalarus punctatus*), with relatively low expression in the intestine and no detectable expression in the gills. In agreement, results presented here showed similar levels of expression for MBLc in the gut and skin tissue, but gill expression was near the lower limit of detection (Figure 2.8 B). In terms of the cytokine signaling, interleukin 10 (IL10), IL17a, and IL1β mRNA expression were measured in this study to assess regulation of inflammation across diets and tissues. The only dietary influences on the tested cytokines was in IL10, which showed dietary influences on expression in each tissue, as well as a reduction in IL17A in gut samples taken from MOS fed fish (Figure 2.8 B). In juvenile Atlantic cod (*Gadus morhua*) a 0.1% MOS inclusion was found to alter only interferon-gamma expression within the anterior intestine, while also measuring IL1β, IL8, and IL10 across two other segments of the intestine as well (Lokesh et al. 2012). In the present study, IL1β, IL17A, and IL10 expression in the gut was significantly lower than that of the gill or skin. Of all the tissues, skin showed the highest expression of the anti-inflammatory cytokines, while gill tissue showed higher levels of IL1β expression than skin or gut. IL1β and IL17A are primarily expressed by activated macrophages and T_{H17} cells respectively, and are proinflammatory cytokines that, at least in mammals, are thought to be released following

pathogen detection, while more anti-inflammatory responses, such as those resulting from TGFβ, IL8, or IL10, are thought to be activated following interactions with commensal microbes (Gomez et al. 2013). Under this paradigm, with the highest expression of anti-inflammatory cytokines, IL10 and IL17a, the skin could be assumed to exhibit the most stable host-microbiota interactions; however, skin microbiota was the only mucosal microbiota community to be susceptible to significant dietary alteration (Figure 2.2 E). This contradiction highlights the need for a better understanding on the interplay between mucosal cytokine expression and mucosal microbe regulation in fish.

Multivariate analysis of gene expression data was used to agglomerate gene specific expression results into an overall expression profile enabling better comparisons of transcriptional regulation profiles across tissues. Ordinations showed significant separation in mRNA expression profiles between all assayed tissues across both systemic and mucosal immunity gene sets, while also detecting significant effects of diet and tissue by diet interactions (Figure 2.9 A-D). Friberg et al. (2019) recently used similar multivariate techniques to show separation in immune responses related to seasonal diet shifts in three-spine stickleback. Those authors also detected repeatable tissue-specific (liver, gill, fin, and spleen) differences in immune expression, and found a significant Mantel correlation between splenic immune expression profiles and both gill and gut microbiome compositions (Friberg et al., 2019). In the current study, tissue-specific mucosal immunity gene expression profiles were correlated with mucosal microbiota using both abundance weighted and presence-absence phylogenetic composition metrics (Figure 2.10). The high correlation between host gene expression profiles and microbiota composition across the three mucosal tissues suggests tissue-specific coadaptation has occurred between host mucosal immune regulation and microbial ecology.

In summary, this is the first study to characterize and compare the host gene expression profile, as well as, microbiota composition and function across the gut, skin, and gill mucosa, while assessing concurrent impacts of dietary functional feed ingredients in a teleost species, specifically Atlantic salmon. Dietary MOS supplementation and coconut oil replacement had no impacts on growth performance or sea lice performance, while moderate yet important differences in mucosal and systemic gene expression profiles and microbiota composition were detected. All tissue specific microbiomes clustered separately, though gill

and skin microbiota showed more similarity in both composition, covariance structure, and function. Multivariate ordination of tissue-specific gene expression profiles based on eight genes with key roles in mucosal immunity showed a high degree of congruency with ordinations of mucosal microbiota composition. Taken together, these findings show that while mucosal gene expression can be moderately impacted through nutritional intervention, immunological signatures of each mucosal tissue remain. In addition, strong evidence was also provided for a tissue-specific co-adaptation of host and microbiota, suggesting host and microbiota are interdependent upon one another.

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Table 2.1. Custom PCR1 primers used in the generation of V3V4 16S rRNA gene sequencing libraries. Blue highlighted primer sequences are Fluidigm consensus sequence pads, red regions are custom linker-spacer nucleotides added to increase read diversity, and the black sequences are the target specific V3V4 primers from Klindworth et al. (2013).

Primer Set	Name	Primer Sequence
Forward Primer Pool	CS1-341F	ACACTGACGACATGGTTCTACACCTACGGGNGGCWGCAG
	CS1-341F_LS1	ACACTGACGACATGGTTCTACAAGCCTACGGGNGGCWGCAG
	CS1-341F_LS2	ACACTGACGACATGGTTCTACATAGCCTACGGGNGGCWGCAG
	CS1-341F_LS3	ACACTGACGACATGGTTCTACAGTAGCCTACGGGNGGCWGCAG
	CS1-341F_LS4	ACACTGACGACATGGTTCTACACGTAGCCTACGGGNGGCWGCAG
	CS1-341F_LS5	ACACTGACGACATGGTTCTACAACGTAGCCTACGGGNGGCWGCAG
Reverse Primer Pool	CS2-785R	TACGGTAGCAGAGACTTGGTCTGACTACHVGGGTATCTAATCC
	CS2-785R_LS1	TACGGTAGCAGAGACTTGGTCTATGACTACHVGGGTATCTAATCC
	CS2-785R_LS2	TACGGTAGCAGAGACTTGGTCTCATGACTACHVGGGTATCTAATCC
	CS2-785R_LS3	TACGGTAGCAGAGACTTGGTCTTCATGACTACHVGGGTATCTAATCC
	CS2-785R_LS4	TACGGTAGCAGAGACTTGGTCTGTCATGACTACHVGGGTATCTAATCC
	CS2-785R_LS5	TACGGTAGCAGAGACTTGGTCTAGTCATGACTACHVGGGTATCTAATCC

Table 2.2. Primer sequences used for RT-qPCR. Those primers with listed "References" were taken from previously published literature (after confirming specificity in-silico), and all other primers were designed using NCBI Primer-BLAST using the listed accession as the target. NCBI accessions are taken from RefSeq where possible, with those accessions denoted by * coming from the GenBank nucleotide repository.

Category	Full Target Name	Symbol	NCBI Accession	Primer Sequence	Reference
Reference Genes	Elongation Factor 1 α - paralog A	EF-1α	NM_001123629	F: GCAGTGGCAGTGTGATTTCG R: GTAGATCAGATGGCCGGTGG	
	Hypoxanthin-guanine	HPRT1	XM_014212854	F: CCGCCTCAAGAGCTACTGTAAT R: GTCTGGAACCTCAAACCCTATG	Sahlmann et al., 2013

	phosphoribosyl transferase				
	RNA polymerase subunit	RPABC 2	XM_014193251	F: CCAATACATGACCAAATATGAAAGG R: ATGATGATGGGGATCTTCCTGC	
Mucosal Genes	Alkaline Phosphatase	ALP	XM_014133547	F: CTAGTTTGGGTCGTGGTATGT R: TGAGGGCATTCTTCAAAGTA	Skugor et al., 2008
	Lysozyme	LYZ	NM_001146413	F: TGCTGGGTTTGTGTGTTTGAC R: CCATAGCGAGAGGCCATGTT	
	Complement protein C3	C3	XM_014186867	F: TCAGGAAATGGTGAGGCGAC R: CGTCCTCGTGGCTTGTTTTG	
	Mannose-binding protein C precursor	MBLc	NM_001141497	F: CCTGCAGGAGCTACTGGTATT R: GCAGGGGTTCCAGGTCTAAG	
	Interleukin 1β	IL-1b	NM_001123582	F: ACAAGTGCTGGGTCCTGATG R: TAGGGCTACAGGTCTGGCTT	
	Interleukin 10	IL-10	XM_014168417	F: TATAGAGGGCTTCCCCGTCAG R: GAATGCCTTCGTCCAACAGG	
	Interleukin 17	IL17a	XM_014193546	F: TGGTTGTGTGCTGTGTGTCTATGC R: TTTCCCTCTGATTCCTCTGTGGG	Mutoloki et al. 2010
	Membrane Toll-like Receptor 5	mTLR5	* HQ664667	F: TTCAACTTCCTCACATACCTCCAA R: GTCAGGAGAGGCCAGGAATTG	
Systemic Genes	Cluster differentiation 8α	CD8a	NM_001123583	F: CACTGTATGCCACTGCAACC R: CTGCCATTCTCGGCTGTCTT	
	Cluster differentiation 4	CD4	NM_001146408	F: CGGACAAGGGCCAAGATGAT R: GCTGCCTGTGGTACAGTGAT	
	Forkhead-box P3	FoxP3	NM_001198847	F: AGCTGGCACAGCAGGAGTAT R: CGGGACAAGATCTGGGAGTA	Zhang et al., 2011
	Immunoglobulin M	IgM	* Y12456 *Y12457	F: TGTAAAGAGAGCAGACTGGGACAG R: GAGACGGGTGCTGCAGATATTC	Austbø et al., 2014
	Immunoglobulin T	IgT	* GQ907004 * GQ907003	F: CTGACGGTGACTCTGAACCC R: GCTGTTCAGGTTGCCCTTTG	
	Major histocompatibility complex 1	MHC1	XM_014177344	F: CCAACTGGAATGACCCCAACA R: CCAAATGACGACCCCAACAAC	Tadiso et al., 2011

	Major histocompatibility complex 2	MHC2	* EF451156	F: CTCACTGAGCCCATGGTGTAT R: GAGTCCTGCCAAGGCTAAGATG

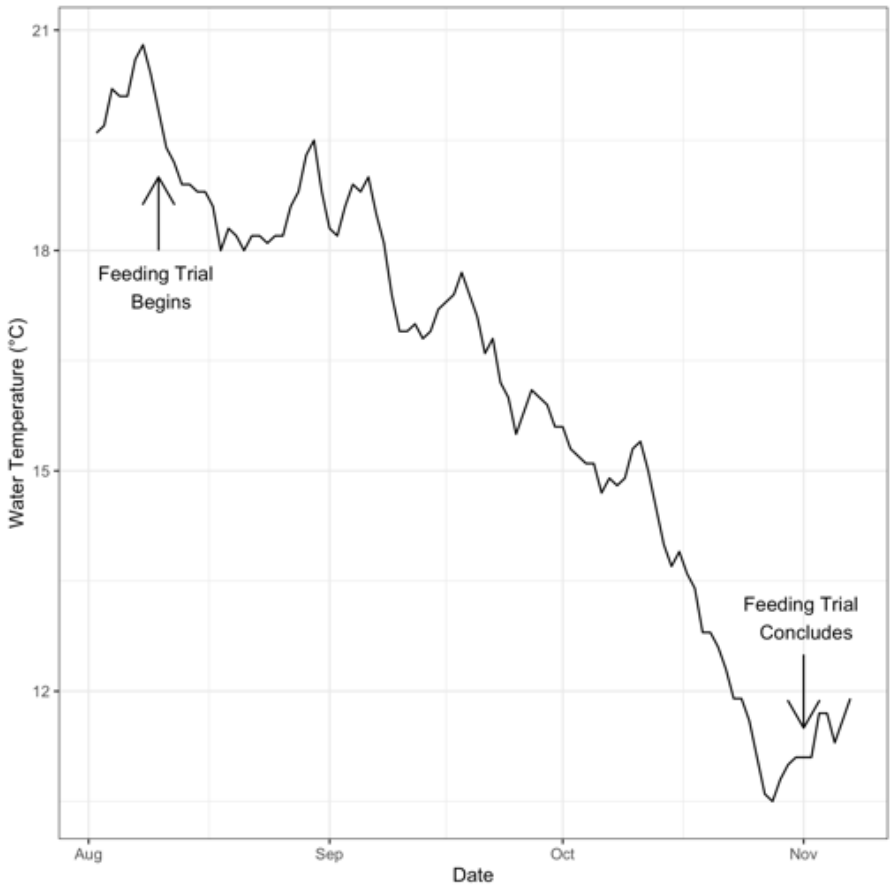

Figure 2.1. Water temperatures observed in the seawater flow-through rearing system throughout the trial.

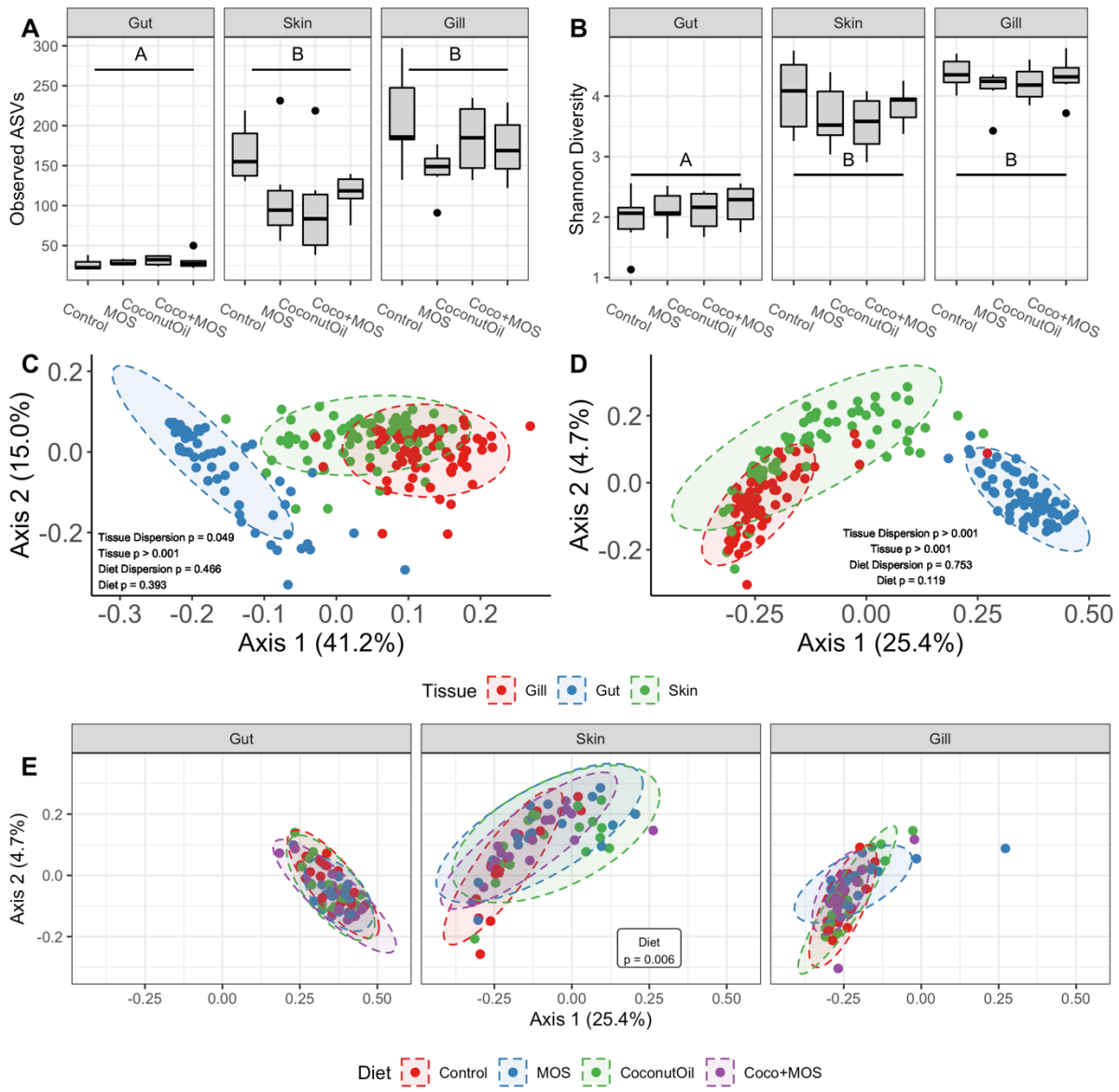

Figure 2.2. Microbiota composition detected across the gut, gill, and skin mucosa of Atlantic salmon fed diets supplemented with functional ingredients. Fish were fed either a control diet (Control), a 1% mannan oligosaccharide supplemented diet (MOS), a diet with 96% lipid replacement using coconut oil (CoconutOil) and a combination of the two treatments (Coco+MOS). Alpha diversity (A-B) was tested by two-way ANOVA with tissue and diet having an impact on richness (A) and only tissue effecting diversity (B). Beta diversity by tissue is displayed using principle coordinates analysis (PCoA) of weighted (C) and unweighted (D) UniFrac distances. Within tissue dietary effects were only detected in the skin microbiota with all dietary treatments significantly shifting the microbial community relative to the control diet according to unweighted UniFrac (E).

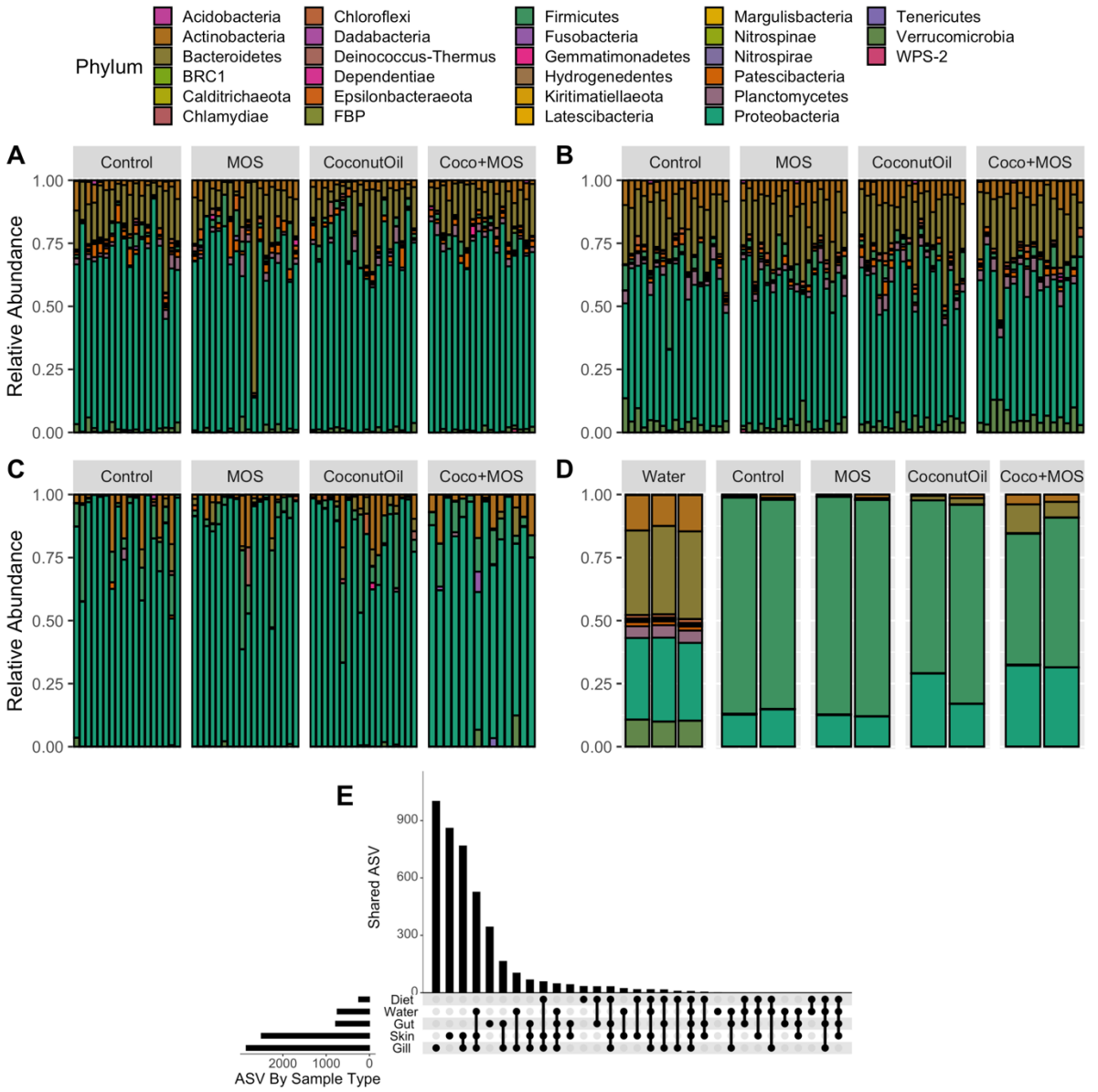

Figure 2.3. Microbiota composition by sample type. Phylum level microbiota composition across dietary treatment are listed for the skin (A), gill (B), and gut (C) mucosa of Atlantic salmon, as well as the environmental samples (water and diet) (D). An upset plot (E) shows the total number of ASV observed by sample type as well as the overlap (Shared ASV) between sample types.

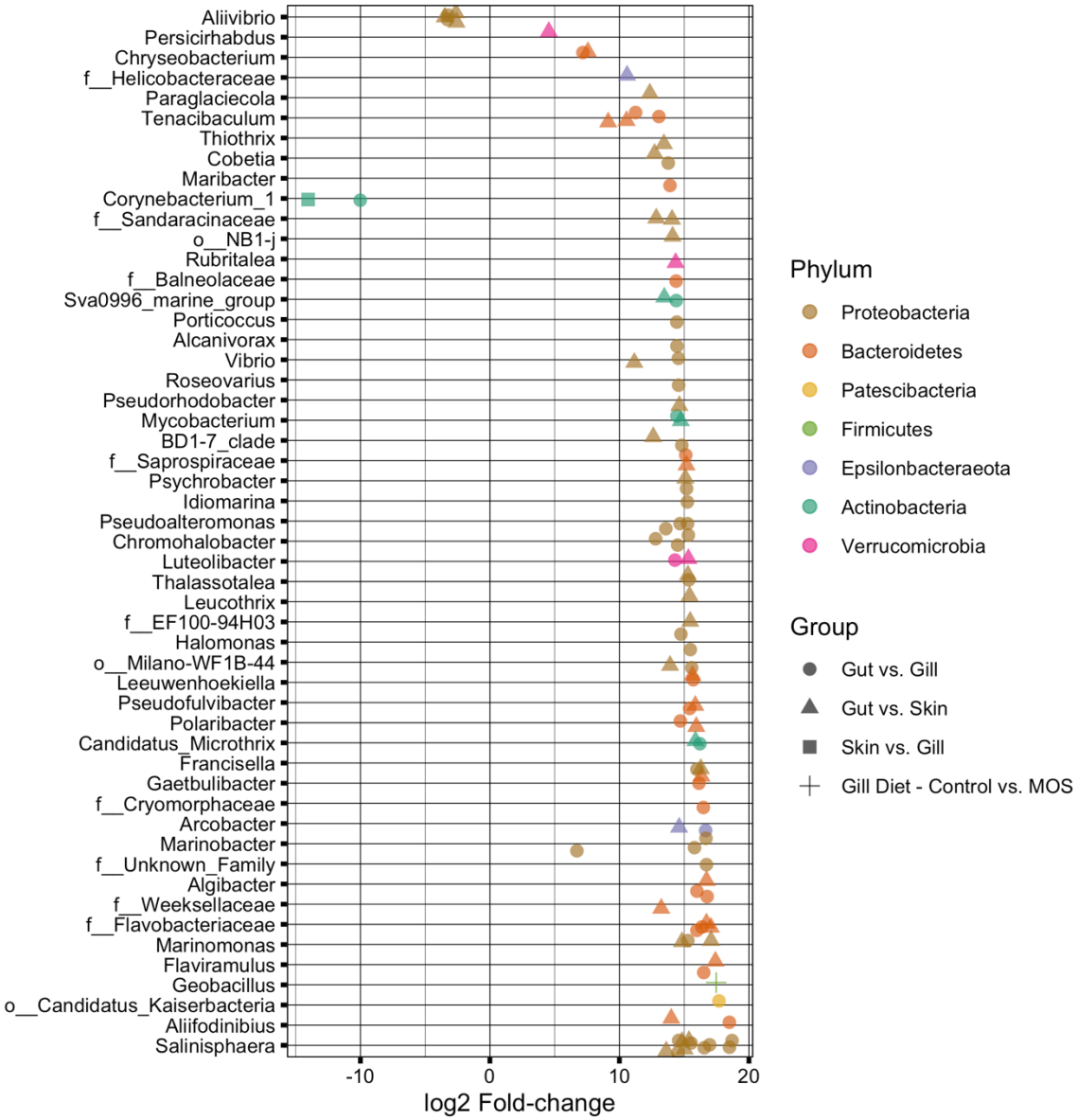

Figure 2.4. Differential abundance (DA) testing of bacterial amplicon sequence variants (ASV) detected in the gut, gill, and skin mucosa of Atlantic salmon fed diets supplemented with functional ingredients. Pairwise DA testing was conducted between mucosal tissues while controlling for dietary treatment. Dietary effects were assessed by comparisons of each treatment diet to the control diet separately within each tissue. Only those groups with significant DA (FDR corrected $q \leq 0.05$ log2-fold change$| \geq 1$ following fold-change shrinkage) listed. Dietary effects were only detected within the gill (Control vs. MOS diets). Genera of the bacteria identified as DA are shown on the y-axis and points are colored by phylum. The group from which the significant pairwise DA was detected is indicated by the shape of points. f_ - family level taxonomy; o_ - order level taxonomy.

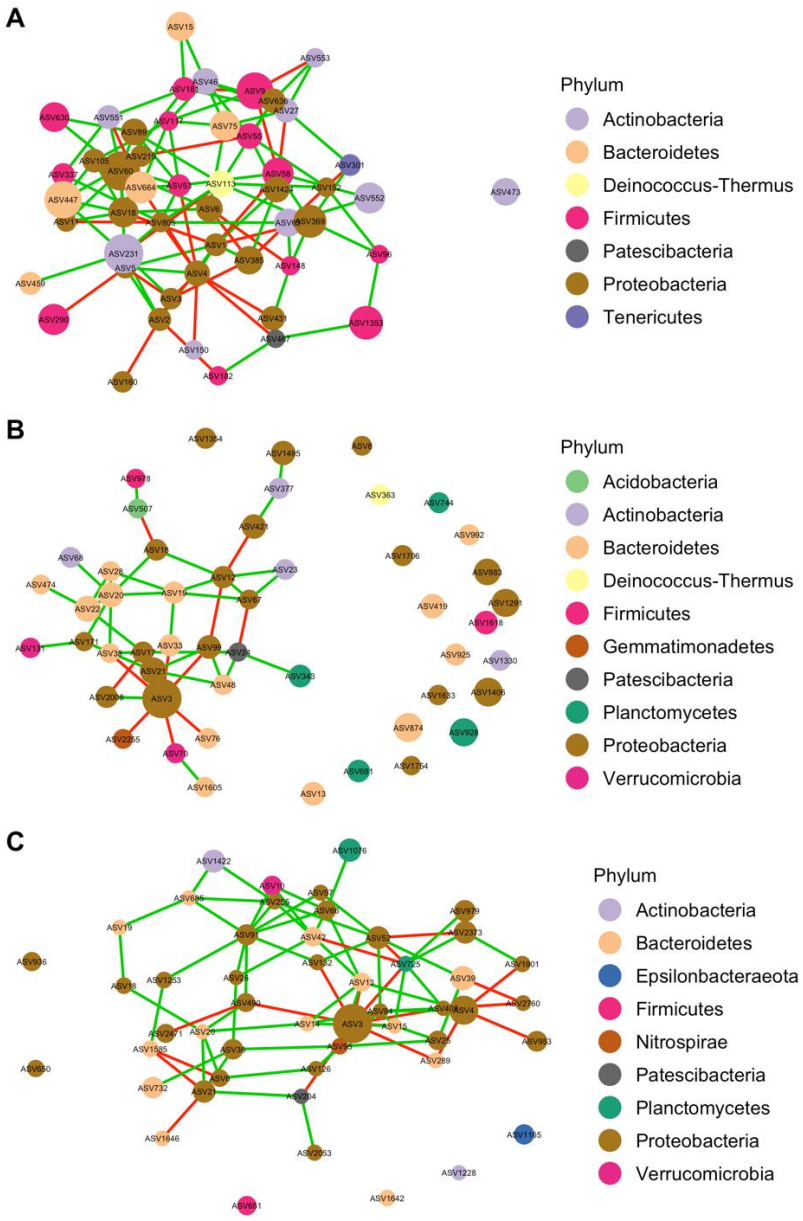

Figure 2.5. Sparse inverse co-variance networks reconstructed from the microbiota detected on mucosal tissues of Atlantic salmon. Displayed networks were filtered to display only the top 50 nodes (ASVs) according to measures of betweenness centrality for the gut (A), gill (B), and skin (C) microbiota. Nodes are labeled by ASV, size indicates the level of centrality, and color indicates the phylum for each node. Edges indicate a positive (green) or negative (red) covariance between nodes. Nodes which lack edges are not connected to any of the displayed top 50 nodes taken from the full network.

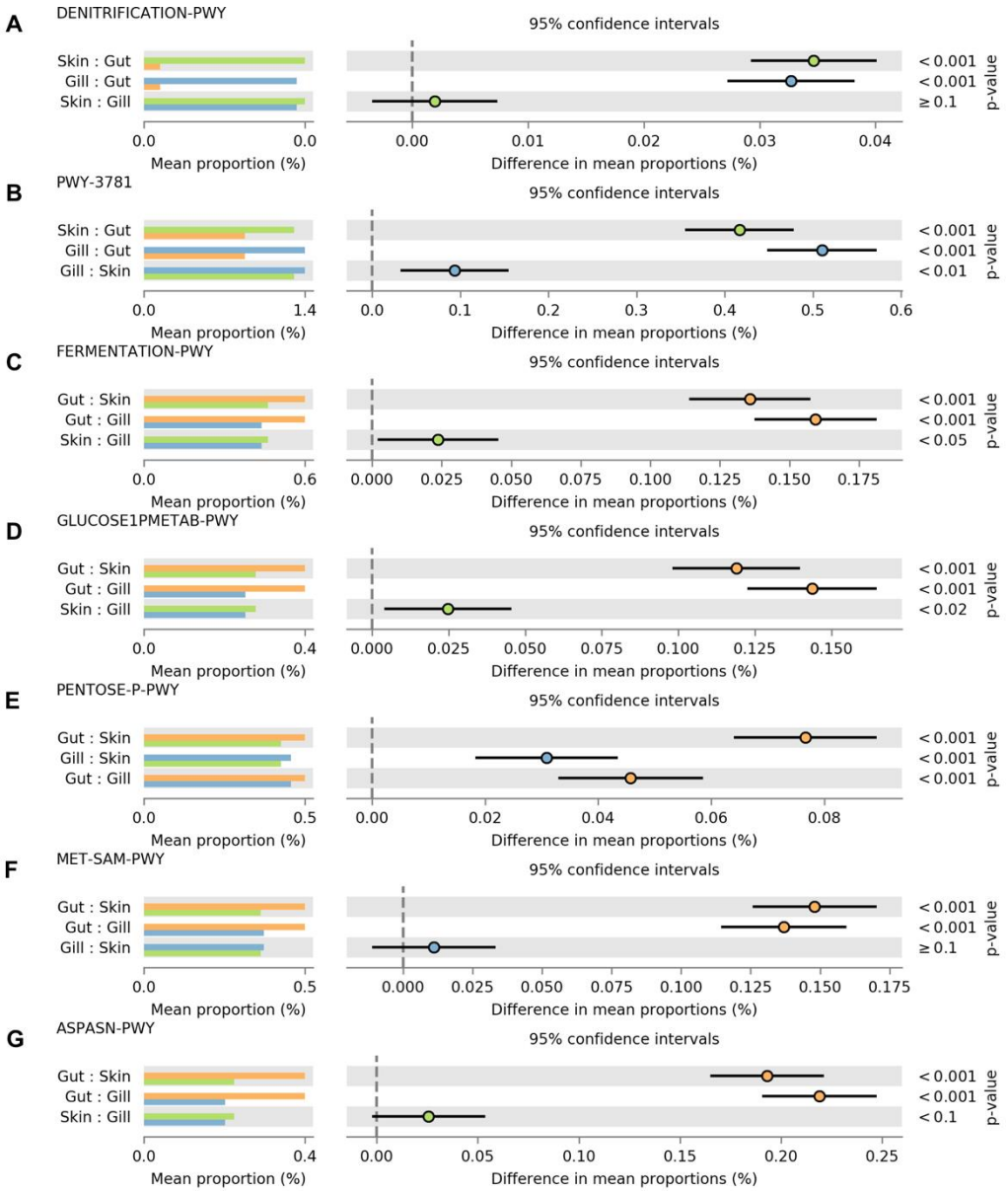

Figure 2.6. Post-hoc plots showing tissue specific differences in microbiota functional MetaCyc pathways. Seven of the 54 pathways identified as significantly different by tissue are shown. P-values are derived from Tukey-Kramer post-hoc test with FDR correction. Pathways listed are (A) nitrate reduction I (denitrification), (B) aerobic respiration I (cytochrome c), (C) mixed acid fermentation, (D) glucose and glucose-1-phosphate degradation, (E) pentose phosphate pathway, (F) superpathway of S-adenosyl-L-methionine biosynthesis, and (G) superpathway of L-aspartate and L-asparagine biosynthesis.

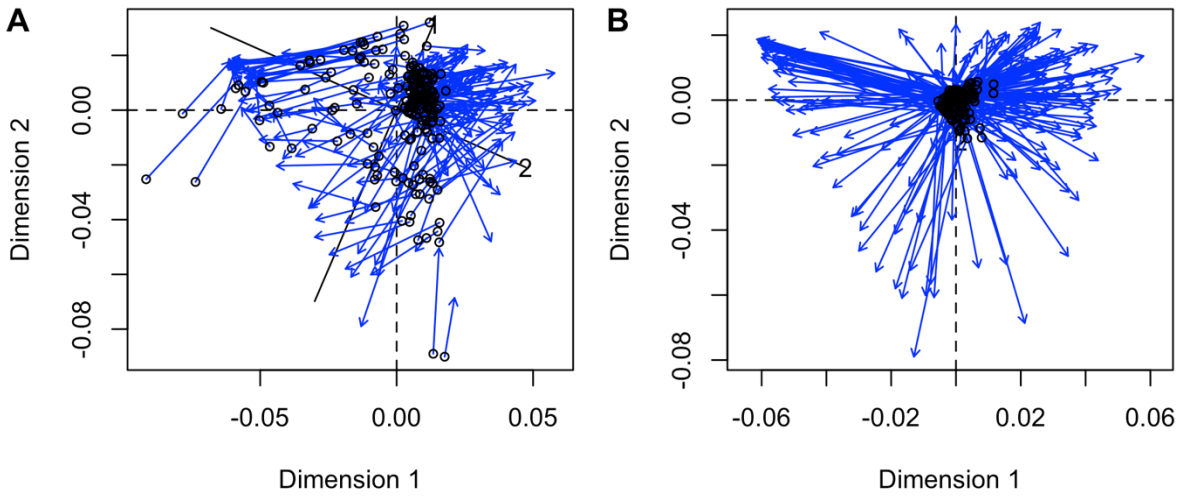

Figure 2.7. Procrustes analysis between microbiota composition and function. Comparison between microbial composition and function were made by comparing weighted UniFrac PCoA axes to PCA loadings taken from the predicted KEGG functional orthologs (A) or MetaCyc functional pathways (B). Points indicate sample ordination position according to function with lines pointing to the Procrustes mapping of microbial composition. A significant association was found with KEGG functions ($m^2 = 0.8397$, correlation = 0.4003, $p < 0.001$), but not with MetaCyc pathways ($m^2 = 0.9894$, correlation = 0.1031, $p = 0.223$).

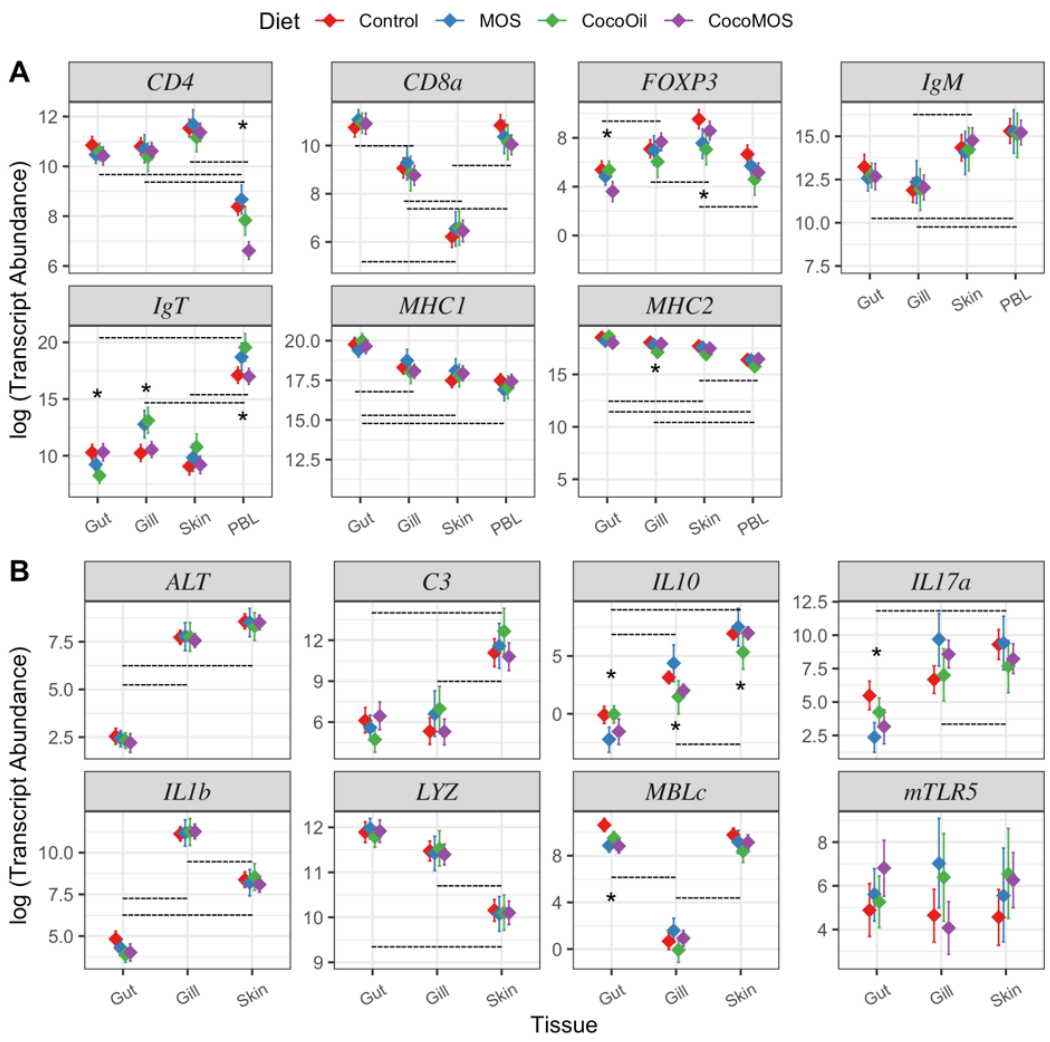

Figure 2.8. Results of RT-qPCR analysis conducted on multiple tissues (x-axis) of Atlantic salmon receiving different dietary treatments (colors). A set of systemic-/adaptive-immunity genes (A) were assayed in the gut, gill, and skin tissue, as well as peripheral blood lymphocytes (PBL), while a set of mucosal-/innate-immunity markers (B) were assayed only in the three mucosal tissues. Log(transcript abundances) were inferred for each gene using a global Bayesian model. Dashed lines indicate a significant (FDR corrected $q \leq 0.05$) pairwise difference between tissues among fish receiving the control diet. Significant pairwise differences in expression between two or more dietary treatments within a specific tissue are denoted by *.

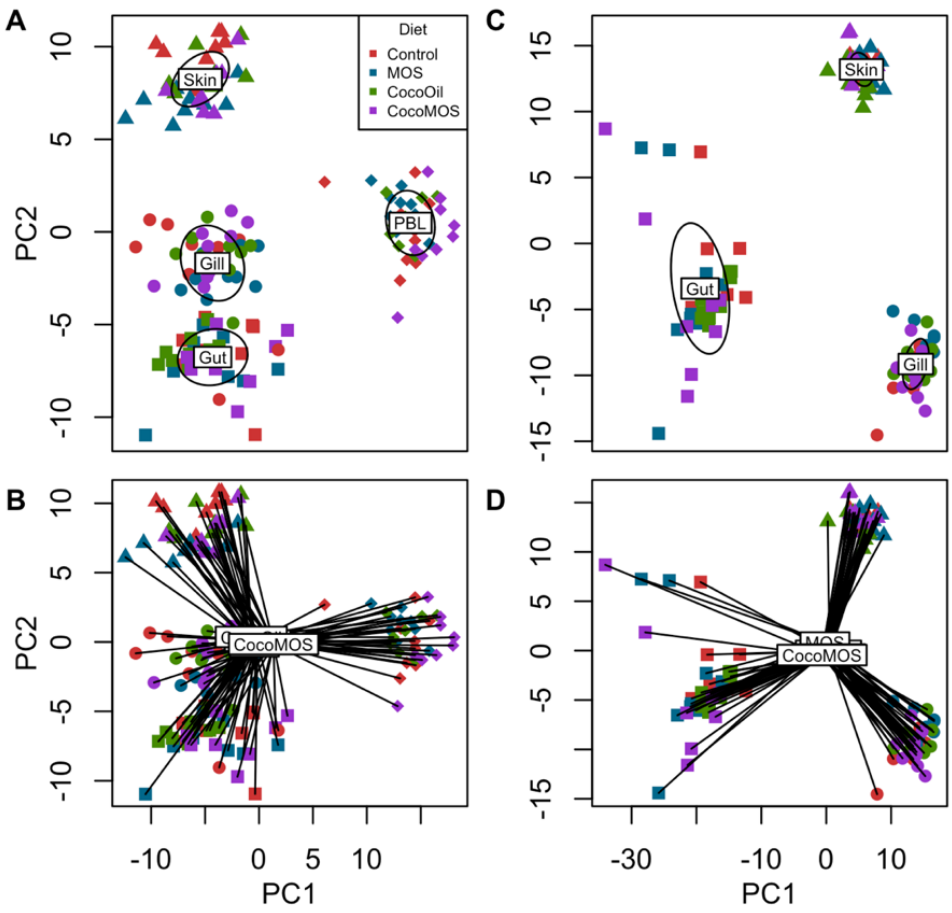

Figure 2.9. Multivariate analysis of RT-qPCR conducted on multiple tissues (shape) of Atlantic salmon receiving different dietary treatments (colors). Plots display results of principle coordinates analysis (PCoA) conducted on Manhattan distances calculated from normalized data generated for a systemic-immunity gene set (A-B) and a mucosal-immunity gene set (C-D). Multivariate centroids are labelled by tissue (A and C) and diet (B and D). PERMANOVA indicated a significant influence ($p \leq 0.05$) of tissue, diet, and tissue-diet interaction in the expression profile for both gene sets.

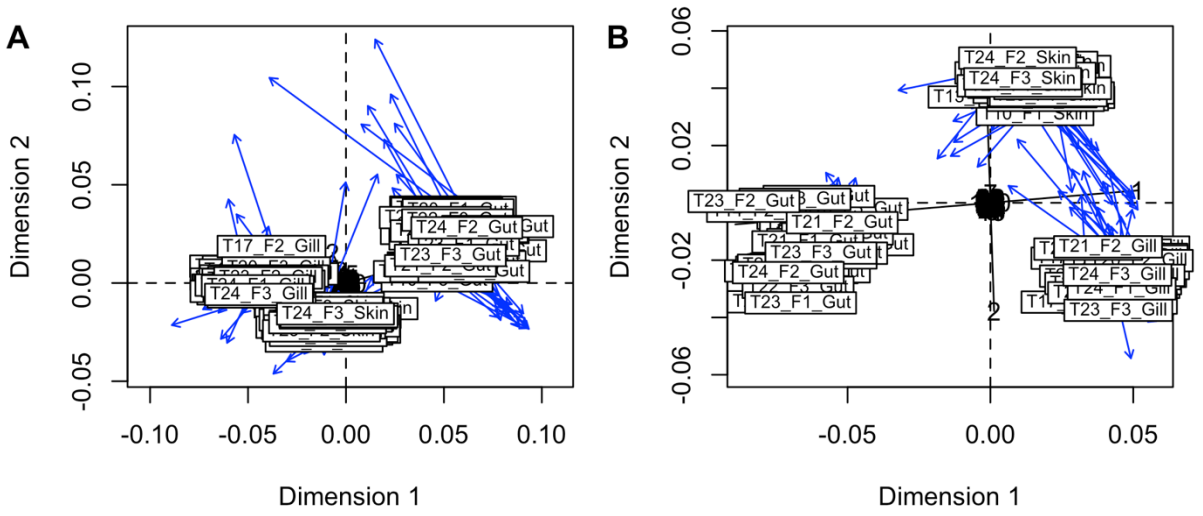

Figure 2.10. Procrustes analysis comparing mucosal microbiota composition to host mucosal gene expression profiles in the skin, gut, and gill of Atlantic salmon. PCoA axes from weighted (A) and unweighted (B) UniFrac distances were mapped to PCoA axes of Manhattan distances calculated from mucosal gene expression profiles. Sample IDs show the ordination of samples according to gene expression profiles and lines indicating the corresponding scaled and rotated sample mapping to microbiota compositional data. Significant association were detected according to both weighted (m^2 = 0.3309, correlation = 0.818, $p \leq 0.001$) and unweighted UniFrac (m^2 = 0.2595, correlation = 0.8605, $p \leq 0.001$).

Chapter 3: Signatures of selection for performance on plant-based diet are outweighed by parallel ontogenetic shifts in intestinal transcription and microbiota during early life stages of rainbow trout *Oncorhynchus mykiss*

Abstract

Over eight generations (16 years) of selection, researchers at the USDA and University of Idaho have developed a strain of rainbow trout that exhibits superior growth on an economically and environmentally sustainable all plant protein, high-soy diet, and is resistant to the development of soybean meal-induced enteritis. To better characterize the physiological mechanism behind the superior performance of the select strain, homeostatic intestinal transcription and microbiota composition of the select strain were compared to that of a commercial control line of trout. Samples were collected at early life stages known to be critical in the development of host-microbe interactions in the gut of rainbow trout (20- and 65-days post hatch). All female fish of both strains were reared in the same environment starting from eggs. Intestinal samples from 5 fish per group (2 trout strains; 2 developmental stages; 20 samples total) were used to generate mRNA-seq libraries, while fifteen fish per group were used for gut microbiota analysis. RNAseq data was quantified at the transcript and gene level prior to testing for differential transcript usage and differential gene expression between the trout strains and developmental stages. In total, 74 genes were shown to be differential transcribed (36.5% and 43.2% by strain and timepoint alone, respectively). By trout strain 118 genes were differentially expressed, but 2,413 genes were differentially expressed by developmental timepoint. Gene ontology analysis showed ontogenetically differential genes to be particularly enriched for functions involved in oxygen carrying capacity, tricarboxylic acid cycle, innate immune function and cellular proliferation and dedifferentiation. Gut microbiota followed a similar pattern to intestinal gene expression, with more significant differences observed by developmental timepoint than by host strain. Signatures of selection were apparent in both overall phylogenetic composition and in a higher abundance of *Escherichia/Shigella* bacteria in the select strain. However, separation in microbial communities by developmental timepoint was much greater, as was the number of differentially abundant microbes. A highly significant correlation in intestinal gene expression profile and gut microbiota phylogenetic composition was detected (Procrustes

analysis; $m^2 = 0.19$, correlation = 0.90, $p = 0.001$), suggesting the host and microbiota undergo ontogenetic development in concert. These findings serve to provide early life-stage biomarkers of selection for performance on plant-based diets and further characterizes intestinal ontogenetic development in rainbow trout.

Introduction

The projected explosion of world human population towards 9.7 billion by 2050 is predicted by the United Nations Food and Agriculture Organization to require at least a 60% increase in concurrent food production (Hunter et al. 2017). Further intensifying this crisis, currently more than 85% of the world's wild fisheries are already harvested at or above their recommended quotas. Aquaculture production is often touted as part of the solution to these problems, as the industry has grown by more than 527% over the last three decades (1990-2018), to now supply over half of the worlds foodfish (FAO 2020). However, increased seafood demand can only be met by escalating gains in aquaculture production, while also focusing on increasing future sustainability of aqua feeds. Historically, aquaculture feedstuffs have been sourced from fisheries (fishmeal and fish oil) because of the optimal nutrient density and desirable amino and fatty acid profiles, particularly for high value carnivorous finfish (Glencross et al., 2020). However, over the last decade the aquaculture industry has pushed toward greater utilization of terrestrial plant-based feed ingredients to add flexibility to dietary formulations and increase financial and environmental sustainability of finfish diets. Soybean based products are the most widely produced and utilized alternative raw feedstuffs in aquaculture and have been implemented with substantial success in many aquaculture species (Glencross et al., 2020). However, in aquaculture the use of plant-based ingredients, such as soy, is often met with drawbacks.

While there are nutritional shortcomings in such ingredients, including limiting essential amino acid profiles, the most challenging consequence of high inclusion of dietary soy is the presences of biologically active antinutritional factors (ANF). Particularly in high value salmonids, such as Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss,* an increase in plant feedstuffs and corresponding exposure to various ANF has led to an increase in diseases related to gut function and immune dysregulation (Krogdahl et al., 2010). Similarly, high inclusion of sustainable plant-based ingredients often produces gut microbiota dysbiosis, or an imbalance in intestinal microbes which is maladaptive to host

performance (Kononova et al. 2019). The negative effects of plant-based feedstuffs in aquaculture can be mitigated, to some degree, through microbial fermentation, enzyme supplementation, or bio-mechanical processing of the raw ingredient (Krogdahl et al., 2010; Kononova et al., 2019). However, optimal outcomes are more likely to be achieved through genetic screening and selection for fish that perform well on these ingredients (Overturf et al., 2004), particularly since it has been estimated that only 10% of aquaculture stocks currently have undergone any genetic improvement (Gjedrem et al 2012).

After 16 years (8 generations), a strain of rainbow trout has been developed at the University of Idaho Hagerman Fish Culture Experiment station through a selection program managed in collaboration with the USDA Agriculture Research Service (ARS-UI select strain). The strain was founded by introgression of nine domesticated rainbow trout strains and selection has been strictly applied according to growth performance on an all plant protein diet every generation (Abernathy et al., 2017). The ARS-UI selected trout strain has repeatedly been shown to exhibit superior growth rates on an environmentally and economically sustainable all plant protein, high-soy diet (Overturf et al., 2013; Blaufuss et al., 2020) with growth rates nearly double that of the founding populations (Abernathy et al., 2017). The select strain shows resistance to the development of soy-induced intestinal enteritis (Venold et al. 2012) and differences in IL17 signaling, a cytokine pathway involved in adaptive oral tolerance to feed antigens, have repeatedly been observed in the select strain compared to a control strain when both are fed plant-based diets (Abernathy et al., 2017; Blaufuss et al., 2020). The select strain has also been shown to exhibit differences in central (liver) and peripheral (muscle) metabolism as well as innate immunity at the transcript level (Abernathy et al., 2017). Furthermore, differences in gut bacteria have been detected in the select strain compared to commercial trout, when fed either fishmeal or plant-protein based diets (Blaufuss et al., 2020; unpublished data).

To date, the characterization of the select trout strain performance has been primarily focused on strain differences during plant-based feeding at later life stages. Albeit, rainbow trout fry are still commonly fed a starter diet rich in fishmeal for approximately the first five to six weeks of exogenous feeding in order to provide optimal nutrition during critical stages of development. Previous research on the intestinal immune expression and gut microbiota in rainbow trout suggested that early life stages (26 and 49 days post first feeding) were

important in the development and trajectory of the host immune system and microbiota composition (Ingerslev et al., 2014). Therefore, what follows is a resolute analysis of host intestinal transcript usage and gene expression paralleled by analysis of gut microbial ecology across the experimental factors of host genetics (commercial vs. select strain) and developmental timepoint (one week post first feeding - 20dph; six weeks post first feeding - 65 dph) in rainbow trout. The aim is to determine if genetic selection for superior growth on plant-protein diets has altered homeostatic intestinal gene expression and gut microbiota during critical early stages of fish development, prior to the introduction of plant-based diets.

Methods and Materials

Study Design, Fish Husbandry, and Sampling

The study design and all sampling procedures were approved by the University of Idaho Institute of Animal Care and Use Committee (IACUC-2017-80) prior to the study. All-female cohorts of the ARS-UI selected strain of rainbow trout (select) and a common commercial strain (commercial) were reared alongside one another starting from eggs. Following industry standard practices, fertilized eggs were treated for 15 minutes with a 100 ppm iodine solution at the time of collection and were incubated in Heath trays with a daily 800 ppm formalin treatment to inhibit fungal growth during incubation. Upon hatching, trout fry were stocked separately into flow-through troughs supplied with 15°C spring water at equal densities. From first feeding through the conclusion of the study, fish were fed a commercial trout fry starter diet (Skretting USA; Tooele, UT) with formulation remaining consistent while pellet sizes increased with fish growth. At 20 days post-hatch (dph) and 65 dph, fifteen fish from each strain were sampled. Because of the high metabolic rate and continuous need for feed at these early stages, fish were fed up to four hours prior to the sampling. Fish were euthanized with an overdose of tricaine-methanesulfonate immediately prior to sample collection. Fish length and weight were recorded prior to dissection. The exterior of fish was briefly rinsed in 75% ethanol and all dissection tools were sterilized between individuals to limit contamination. A dissecting microscope was used to assist in the isolation of the intestinal samples. Intestinal mucosa and digesta contents were collected for microbiota analysis, while intestinal tissue was placed in 1 mL of Qiazol (Qiagen; Hilden, Germany) to be used for downstream RNA sequencing. Environmental microbiota were characterized in triplicate by sampling homogenized diets used throughout the study and by

collecting water microbiota from 1 L of inflow water at each sampling timepoint using 0.2 µm Supor® filters (Pall Corporation; New York, USA). Samples were flash frozen and stored at -80°C until further processed.

DNA Isolation and Microbiota 16S V3V4 rRNA Gene Sequencing

A DNeasy PowerSoil Pro 96 HT kit (Qiagen) was used to isolate DNA from intestinal contents. Samples were homogenized using a TissueLyzer (Qiagen) and isolations were automated on a QIAcube HT (Qiagen). Samples were inspected for quality and quantity using a NanoDrop spectrophotometer (ThermoFisher Scientific; Waltham, MA). Due to minor contamination, as made evident by reduced 260/230 values, all samples were cleaned using a Genomic DNA Clean and Concentrate 96 kit (Zymo Research; Irvine, CA). Samples were quantified by fluorometry (Quant-iT PicoGreen; ThermoFisher) and 10 ng μL^{-1} were used as input template for V3V4 16S rRNA gene sequencing library preparation. A custom two-step PCR dual-barcoding strategy was used to generate amplicon libraries. Universal target specific 16S rRNA V3V4 341F-785R primers (500 nM each, Ta = 60°C) were utilized in the first round of PCR with 30 rounds of amplification using Phusion Green Hot Start II HiFi Master Mix (ThermoFisher). Amplicon products were confirmed by 1% agarose gel electrophoresis and amplicons were cleaned using HighPrep PCR clean-up beads (Magbio Genomics; Gaithersburg, MD) at 0.8X ratio prior to a two-fold dilution. Custom dual-indexes developed by University of Idaho Genomics Resources Core (UI-GRC) were add to the sample libraries in 8 rounds of PCR. Final libraries were again verified by electrophoresis and cleaned (0.8X bead ratio). Libraries were quantified by fluorometry and pooled at equimolar concentrations. Size distribution was confirmed using a DNA 1000 kit (Agilent). The final sequencing pool was quantified using Kapa Library Quantification Kit (Kapa Biosystems; Wilmington, MA) before sequencing on a MiSeq with 15% PhiX spike-in using a 600-cycle V3 kit (Illumina; San Diego, CA) at the Hagerman Fish Culture Experiment Station. Sequencing of the custom amplicons required the use of custom Fluidigm sequencing primers (BAMF-CS1, BAMF-CS2, and BAMF-CS2rc) modified by the University of Idaho Genome Resources Core lab.

Raw 16S rRNA gene reads are publicly available on the NCBI repository (https://www.ncbi.nlm.nih.gov) under BioProject Accession PRJNA659058. Raw sequencing reads were demultiplexed and primers were removed with dbcAmplicons

(https://github.com/msettles/dbcAmplicons). DADA2 (Callahan et al. 2016) was used to trim (Forward – 275 bp, Reverse – 215 bp) and error filter (2 expected errors) sequences prior to denoising reads into chimera checked amplicon sequence variants (ASV). Taxonomy was applied to ASV using the Silva v138 database (Quast et al., 2012). A phylogenetic tree was constructed for all microbiota detected in the fish gut samples to enable the calculation of phylogenetically informed distance metrics (UniFrac) using a GTR model with help from the packages Decipher (Wright, 2016) and phangorn (Schliep, 2011). The packages phyloseq (McMurdie and Holmes, 2013) and vegan (Oksanen et al. 2013) were used to filter and analyze microbiota data. All ASV assigned to the order Chloroplast or the family Mitochondria as well as singletons were removed.

Within sample diversity (alpha diversity) was assessed using the metrics observed ASV (obsASV) richness and Shannon diversity. Alpha diversity of the environmental (diet and water) microbiota and gut microbiota were compared by Mann-Whitney U test. A two-way ANOVA was used to test alpha diversity in fish gut samples. A log transformation was applied to obsASV prior to ANOVA to meet assumptions of normality. Between sample diversity (beta diversity) was tested by permutational multivariate analysis of variance (PERMANOVA) of weighted (wUniFrac) and unweighted (uwUniFrac) UniFrac sample distance matrices. DESeq2 (Love et al., 2014) was used to test for differential abundance (DA) between experimental groups at the ASV and genus level. Log-fold change shrinkage was applied using the apeglm (Zhu et al., 2018). DA was considered when $|\text{log-fold change}| \geq 1.5$ and FDR corrected p-value ≤ 0.5. A log-ratio test was used to test for significant interaction effects microbial abundance (FDR ≤ 0.05). All statistical tests were conducted modeling the effects of fish strain (commercial and select), developmental timepoint (20 dph and 65 dph), and strain by time interaction effects.

mRNA Sequencing and Analysis

Intestinal samples were thawed and homogenized on a TissueLyser using a 5 mm steel bead. RNA was purified using a RNeasy 96 kit (Qiagen) with an on-column DNase I treatment. Quantity and quality were inspected on a NanoDrop 2000 and by fluorometry (Quant-iT RiboGreen; ThermoFisher). RNA integrity numbers (RIN) were estimated using an RNA 6000 Nano kit (Agilent; Santa Clara, CA) to measure sample purity. All samples were of good quality (260/280 – 2.10 ± 0.04; 260/230 – 2.16 ± 0.20; and RIN – 8.7 ± 0.8).

Based on quality and concentration, the five best samples from each experimental group were selected for gene expression by sequencing (n = 5). Intact poly(A) tailed mRNA was isolated from 800 ng of input total RNA using the NEBNext Poly(A) Magnetic mRNA Isolation module (NEB; Ipswich, MA). Unique dual-indexed stranded RNA sequencing libraries were then generated using the Ultra II Direction RNA Library Prep Kit (NEB). Final cleaned libraries were assessed for quality using a DNA 1000 kit (Agilent), prior to pooling at equimolar concentrations according to quantification from a Kapa Library Quantification Kit (Kapa Biosystems). Sequencing was performed on a NextSeq 500 (Illumina) using 150-cycle v2.5 high-output kits (Illumina) to generate paired-end (PE) 75 bp reads at the Hagerman Fish Culture Experiment Station. All samples were sequenced in duplicate as part of a larger pool of samples to avoid introducing batch effects across sequencing lanes.

Transcript quantification was achieved by selective alignment with GC bias correction using Salmon v.1.2.1 (Patro et al., 2017). Reads were mapped to a decoy-aware reference transcriptome index constructed using all transcripts in the NCBI Omyk_1.0 genome repository. The transcriptome index was made decoy-aware against the Omyk_1.0 genome to reduce the occurrence of spurious mapping of reads. For gene level analyses, transcripts were summarized at the gene level using the Omyk_1.0 genome GTF annotation.

Differential transcript usage (DTU) analysis was conducted using DRIMseq (Nowicka and Robinson 2016; Love et al., 2018) using scaled transcripts per million counts as input. The dataset was first filtered at the transcript and gene level to remove transcripts with an expression lower than ten or those not detected in five or more samples. Genes which were not expressed across all samples and which did not have an expression above ten were also removed prior to conducting DTU analysis. Gene and transcript p-values were combined and FDR adjusted using a two-stage statistical framework in stageR (Van den Berge et al., 2017) to identify DTU and differentially transcribed genes (DTG) under the study design at an overall false discovery rate (OFDR) of 5%.

Differential gene expression (DGE) analysis, including data transformation, normalization and outlier gene detection, was conducted using DESeq2 (Love et al., 2014). DGE analysis was conducted using default parameters with the same model, statistical contrasts, and significance thresholds as were described above for microbiota DA testing. A variance stabilizing transformation (VST) was applied to the data, prior to conducting

multivariate analysis. A PERMANOVA was performed modeling the effects of fish strain (commercial vs. select), developmental timepoint (20 dph vs. 65 dph), and a strain by time interaction term based on a sample-wise Euclidean distance matrix calculated on VST transformed data. Symmetrical Procrustes analysis was then used to map principle coordinate analysis (PCoA) ordination of gene-expression Euclidean distances to a sample matched PCoA ordination of microbiota phylogenetic composition, according to wUniFac or uwUniFrac distances.

Gene ontology (GO) analysis was conducted to summarize genes identified by DGE analysis. First, the entire reference rainbow trout transcriptome was annotated by Blastx-fast (E-value < 1E-5) against the NCBI non-redundant protein database (nr) filtered to include only entries from related and reference species (*Oncorhynchus mykiss, Salmo trutta, Salmo salar, Oncorhynchus nerka, Oncorhynchus tshawytscha, Oncorhynchus kisutch, Salvelinus alpinus, Esox lucius,* and *Myripristis murdjan, Danio rerio* and *Homo sapien*) to reduce computational burden. Blast2GO (Gotz et al 2008) was then used to map and annotate GO terms, EMBL-EBI InterPro IDs, as well as KEGG enzyme codes and pathway maps, where possible. Annex augmentation was conducted to increase the number of annotations and confirm GO terms. Enrichment analysis was conducted by Fisher's Exact test using the full rainbow trout transcriptome as the reference set with a significance threshold of FDR ≥ 0.05.

Results

At 20 dph, fish from the commercial strain of rainbow trout were significantly larger in weight (paired Student's T-test; $p \leq 0.001$), yet by 65 dph the fish from the select strain weighed significantly more than the commercial fish ($p = 0.0089$) (Figure 3.1). Fish length followed the same pattern, with the commercial fish (32.73 mm ± 1.58; mean ± SD) being significantly ($p = 0.0011$) larger than select strain fish (29.47 mm ± 1.88) at 20 dph. Although, by 65 dph the select strain fish (94.74 mm ± 3.15) were significantly greater in length ($p = 0.0054$) than the commercial fish (89.07 mm ± 4.42). All fish remained healthy throughout the study and no signs of disease were detected in either population.

Gut Microbiota

After quality filtering, removing singleton ASV and those assigned to Chloroplast or Mitochondria, a total of 1.28 million reads were processed into ASV for microbiota analysis, when including environmental samples. From all samples, a total of 8,389 unique ASV were

detected. From the sixty gut microbiota samples, a total of 966,988 processed and filtered reads were retained (16,116 ± 7,259; mean ± SD) and assigned to 2,351 unique ASV.

In terms of microbiota alpha diversity, Mann-Whitney U Tests showed richness (obsASV) and Shannon diversity to both be significantly ($p \leq 0.001$) different between each sample type (water, diet, and fish gut). Water samples showed the greatest richness (1,230 ± 31) and diversity (5.95 ± 0.47). Diet samples were intermediate in richness (162 ± 31) (Figure 3.2. A) and showed the lowest Shannon diversity (2.25 ± 0.38), while gut samples had the lowest obsASV (88 ± 56) (Figure 3.2. A) and intermediate diversity (3.26 ± 0.45). A two-way ANOVA conducted on fish gut samples alone showed no significant effects of fish strain, developmental timepoint, or interaction effects on microbiota richness ($p \geq 0.1649$) (Figure 3.2. A) or diversity ($p \geq 0.3432$).

Bray-Curtis dissimilarities showed microbiota composition to cluster separately by sample type (PERMANOVA; $p = 0.001$), though differences in sample size and multivariate dispersion ($p \leq 0.001$) must be considered in the comparison across sample types (Figure 3.2 B). The microbiota composition of fish gut samples was compared using abundance-weighted (wUniFrac) and presence-absence (uwUniFrac) based UniFrac phylogenetic distances. A significant difference in multivariate dispersion by developmental timepoint was detected according to wUniFrac distances (Table 3.1; Figure 3.2 C), though no other differences in dispersion were detected (Table 3.1). Two-way PERMANOVA identified significant effects of both trout strain and developmental timepoint using wUniFrac (Figure 3.2 C) and uwUniFrac (Figure 3.2 D), and significant interaction effects were also detected based on uwUniFrac distance (Table 3.1).

When including all samples, bacteria from 33 phyla were detected with 30, 17, and 27 phyla being represented in the water, diet, and gut, respectively. Proteobacteria was the most abundant phyla among both the water and gut microbiota and was the third most abundant phyla in the diet samples (Figure 3.2 E). Dietary samples were highest in microbes from the phylum Cyanobacteria, most of which are from the genus *Arthrospira_PCC-7345*, which was also abundant in the gut of fish (Figure 3.2 E and F). Bacteria from the phyla Firmicutes, including those in the genera *Streptococcus, Lactobacillus, Bacillus,* and *Enterococcus,* were the second most abundant in the diet and gut samples both (Figure 3.2 E and F). At the phylum level, Bacteroidota (i.e. Bacteroidetes) was the second most abundant in the gut

samples but was not among the topmost abundant phyla in the environmental microbiomes (Figure 3.2 E). At the genus level, gut samples were dominated by *Aeromonas* and *Streptococcus*, which represented 20.1% ± 1.9 and 18.3% ± 1.9 (mean ± SEM) of the total relative abundance respectively (Figure 3.2 F).

Differential abundance (DA) testing was undertaken to identify specific gut microbiota which show significantly different abundance according to host genetics (commercial vs. select strain) or developmental timepoint (20 dph vs. 65 dph), while controlling for covariates. At the ASV level, 47 ASV had an FDR ≤ 0.05 when testing by fish strain, although only three of those crossed the corrected log2 fold-change threshold (≥ 1.5). ASV assigned to the genera *Escherichia/Shigella* and *Acinetobacter* were found to be significantly enriched in the select strain of rainbow trout (Figure 3.2 G). Testing by developmental timepoint identified 106 ASV with an FDR ≤ 0.05, and of those 37 passed the fold-change threshold to be considered DA. One ASV each, was identified in the genera *Streptococcus* and *Acinetobacter* which were indicative of the trout gut microbiota at 20 dph, while 35 other ASV from various genera were enriched at 65 dph (Figure 3.2 H). A single ASV, belonging to the genera *Acinetobacter* was identified as showing interaction effects between fish strain and developmental timepoint as well. Of those ASV identified by DA testing, 65.8% (27 out of 41 total) were from the phyla Proteobacteria. When merging ASV at the genus level, only *Escherichia/Shigella* met the significance threshold in testing by fish genetics and were found to be enriched in the select strain. Ten genera where identified as significantly enriched among the 65 dph trout gut microbiota compared to 20 dph, consisting of *Streptococcus, Myroides, Enterococcus, Proteus, Leptotrichia, Providencia, Dysgonomonas, Conchiformibius, Morganella,* and *Klebsiella*. No bacterial genera showed interaction effects in DA testing.

Intestinal Gene Expression

Raw RNAseq data is publicly available on the NCBI Gene Expression Omnibus (GEO) repository under accession GSE156372. From the twenty RNA sequencing libraries, 251.4 million raw PE (2 x 75) reads were generated, with 12.6 ± 2.2 million (mean ± sd) reads per library. On average 74.3% ± 3.0 of the raw reads were mapped to the reference Omyk_1.0 transcriptome index, with 9.3 ± 1.6 million mapped reads per sample. One sample from a select trout collected at 20 dph was identified as being a clear outlier according to

multivariate data exploration and was removed prior to downstream analysis. Of the 79,244 unique transcripts present in the reference rainbow trout transcriptome index, a total of 68,572 (87%) were detected in the unfiltered dataset, corresponding to 44,522 genes.

After filtering, 11,556 transcripts corresponding to 4,721 genes were tested for DTU and DTG. Of those genes, 70% (3,351) had only two isoforms, although eleven genes encompassed eight or more transcripts (max of 13). The number of transcripts and genes identified participating in DTU was similar across experimental factors, though ontogenetic development did show greater DTU than did differences in trout genetics (Figure 3.3 A). Sixty-seven transcripts from 41 genes were found to display DTU at the overall false discovery threshold (≤ 0.05), based on timepoint (Figure 3.4. A-C). Testing for DTU by fish strain identified 57 transcripts from 34 genes (Figure 3.4 D-F), with 23 transcripts from 14 genes detected as showing strain by timepoint interactions on DTU (Figure 3.4. G-I). All genes and transcripts identified by DTU analysis are listed by experimental factor in Table 3.2.

Raw transcript counts were summarized at the gene level and normalized by library size before applying a variance stabilizing transformation for downstream analysis. A Euclidean sample distance matrix was generated using VST transformed gene countsto compare intestinal gene expression profiles by experimental group (Figure 1.5 A). PERMANVOA showed a highly significant impact ($p \leq 0.05$) of developmental timepoint on expression profiles, but no strain or interaction effects were detected (Table 3.1). Gene expression profiles across the experimental factors showed a high congruency (Procrustes; $m^2 = 0.19$, correlation = 0.90, $p = 0.001$) with sample ordinations of gut microbial ecology (Figure 3.5 B).

Differential expression analysis was used to determine if the expression level of genes could discriminate between experimental factors. According to FDR corrected p-values (≤ 0.05) 485 genes were identified as differentially expressed across the two rainbow trout strains, although only 118 of those also met the apeglm corrected log2 fold-change threshold ($\geq |1.5|$) (Table 3.3). Based on complete-linkage hierarchical clustering the top forty most differential genes according to absolute log2 fold change by trout strain were shown to differentiate samples well with only one commercial fish at 65 dph overlapping with the selected trout strain cluster (Figure 3.6). Interaction effects were identified as having

significant effects on the expression of 22 genes (Table 3.4), but developmental timepoint unquestionably had to the largest influence on gene expression with 2,413 DEG (Figure 3.3. C). For each experimental factor, genes identified by DGE analysis were utilized for gene ontology (GO) enrichment analysis. Genes differentially expressed by fish strain and interaction effects showed no significant GO enrichment (Fischer's Exact Test; FDR ≤ 0.05); however, genes which were differentially expressed by developmental stage showed enrichment for 372 GO terms. Of these enriched GO terms, 67% were in the category Biological Process (Table 3.5), 25% were Molecular Function (Figure 3.7) and 8% were of Cellular Component.

Discussion

The superior growth performance of the select strain was confirmed by the results of this study. At the first sampling timepoint, which occurred after only one week of exogenous feeding, commercial strain trout fry were larger in size. Although, by 65 dph the select strain surpassed the commercial fish in size (Figure 1). Anecdotally, the commercial trout eggs were larger in size than that of the select strain, possibly suggesting maternal effects influenced the larger size of the commercial fish at the first sampling timepoint. Springate and Bromage (1985) observed that, in rainbow trout, larger eggs produced larger fry; however, those authors suggested that genetics and environment overcame such factors to regulate growth after four weeks of exogenous feeding. Environmental factors were held constant in this study, suggesting genetic factors alone explain the superior growth of the select strain between 20 dph and 65 dph. Regardless, the select strain has been reported to exhibit more rapid growth than control or commercial strains of trout multiple times (Overturf et al., 2013; Blaufuss et al., 2020; Lee et al., 2020), particularly when fed plant-based diets, though this is the first example of the strain's superior growth at an early life stage.

Here gut microbiota richness was stable from seven days post first-feeding (dpff) (20 dph) through 65 dph and was not influenced by host genetics (Figure 3.2 A). Similarly, Ingerslev et al. (2014) found no difference in 16S rRNA gene abundance or Shannon diversity in the gut of rainbow trout at 26 and 49 dpff; although, those authors also showed that bacterial load and diversity was elevated at those timepoints compared to one day prior to exogenous feeding. Together, this suggests initiation of exogenous feeding in trout

inoculates new environmental bacteria to the gut microbiota to bolster diversity and richness, though the host appears to regulate the microbiota to relatively stable levels of alpha diversity, at least through most of the rest of the juvenile period. Both the alpha diversity and microbial composition of the environmental microbiota (diet and water) was shown to be stable in this study and may help also partially explain the stability of gut alpha diversity (Figure 3.2 A-B).

Unfortunately, many studies on the microbiota of fish fail to simultaneously characterize the microbiota of environmental samples. As one of the first, if not the only, study to characterize environmental microbiota alongside the gut microbiota of rainbow trout fry, results offer new insight into the role that environmental microbiota play on early colonization dynamics. At 20 dph, trout gut microbes more closely resembled that of water and diet microbiota than at 65 dph, suggesting environmental microbiota have larger influences near the beginning of first feeding in trout (Figure 3.2B). This is validated by the previously discussed results on alpha diversity and is in line with Ingerslev and others (2014) who speculated that the trout gut microbiota development is highly influenced by water and diet microbiota, just before and after first feeding, respectively.

The most abundant bacteria detected in the diet were from the genus *Arthrospira_PCC-7345* (Figure 3.2 F). Generally called spirulina, these filamentous cyanobacteria are commonly used as a dietary supplement or feedstuff, and are likely transient non-functional DNA artifacts of dietary origin in gut samples, where they were also abundant (Figure 3.2 F). Similarly, the lactic acid bacteria *Streptococcus* and *Lactobacillus* observed to be abundant in the trout gut in this study may also have been non-functional DNA artifacts (Figure 3.2 F), though these microbes are frequently detected in the fish gut and are commonly used as probiotics (Ringo et al., 2018). Because the sequencing approach used in this study cannot distinguish viability from presence of DNA, discerning whether these ASV are DNA artifacts or viable, functioning microbiota is not possible. Interestingly, very few of the most abundant microbiota from the culture water were also detected at high abundance in the gut of trout fry, with only the genus *Acinetobacter* showing high abundance across both sample types (Figure 3.2 F); however, the natural spring-fed water in this study had very high bacterial richness (Figure 3.2 A) so there was considerable diversity to selectively colonize the trout gut. It should also be noted, the version of bacterial taxonomy

applied to ASV in this study (Silva v.138) was recently updated with changes in both nomenclature and reference phylogenetics. For example, certain phyla (Actinobacteria, Bacteroidetes, Fusobacteria, etc.) have been renamed with the addition of the suffix -ota (i.e. Actinobacteriota, Bacteroidota, Fusobacteriota, etc.) (Figure 3.2 E) (Whitman et al., 2018). Additionally, ASV previously annotated to the phylum Tenericutes that have been seen as dominate in the gut of rainbow trout (Lyons et al. 2016) have now been reclassified within the class Bacilli in the phylum Firmicutes, which were also abundant in the gut samples here (Figure 3.2 E). This highlights the importance of considering methodological or database differences when comparing results from various microbiota studies.

For the aquaculture industry to fully capitalize upon the wealth of new information generated on the finfish microbiome, we must accurately delineate sources of natural variance to better understand teleost-associated microbiota dynamics. Changes in gut microbiota across intrinsic factors such as ontogenetic development (Ingerslev et al., 2014) and host genetics (Brown et al., 2019; Chapagain et al., 2019), as well as extrinsic factors like diet (Ingerslev et al., 2014; Blaufuss et al., 2019), rearing environment (Lyons et al., 2016), disease status (Brown et al., 2019) and husbandry related stress (Uren Webster et al., 2020) have all been characterized in rainbow trout. Understanding these factors which inherently govern the biology of finish microbiota will enable more efficacious strategies to manage and improve host-microbiota interactions. Here, both host genetics and ontogeny had significant effects on the trout gut microbial communities, and ontogenetic shifts in gut microbiota composition followed similar trajectories (Figure 1.2 B-D) irrelevant of host genetics. Chapagain and colleagues (2019) conducted a similar study to test for host genetic differences in the gut microbiota of fast- and slow-growing selected strains of rainbow trout. Their results showed that while growth performance did significantly differ between the strains, no differences in overall gut bacterial communities were detected (Bray-Curtis dissimilarity PERMANOVA) and only ten operational taxonomic units (OTU) were identified as indicative between the two strains. In that study, the fast-growing strain of trout showed an enrichment of eight OTUs from the phyla Firmicutes (Chapagain et al., 2019). In the results presented here, a significant difference in overall bacterial community due to host strain was detected by PERMANOVA (Table 3.1). Although, samples showed less separation by fish strain than by developmental timepoint (Figure 3.2 B-D) and only three

ASV from the phyla Proteobacteria were enriched in the faster growing selected strain (Figure 3.2 G).

While effects of host genetics were observed on the gut bacteria, by far the biggest changes in bacterial ecological dynamics in this study were seen between development timepoints (Figure 3.2 B-D). A temporal change in the distribution of sample-wise gut microbiota composition was apparent using both weighted and unweighted phylogenetic composition (Figure 3.2 C-D; Table 3.1) and the most differentially abundant ASV were detected according to time (Figure 3.2 H). This suggests ontogenetic changes in host regulation of gut microbiota outweigh differences in microbiota which can be attributed to host genetics. In agreement, a study on gut microbiota colonization dynamics of three phylogenetically distinct teleost species, Yan et al. (2016) showed that ontogenetic shifts in gut microbiota overshadowed host genetic differences as the microbiota of the three species became less distinguishable throughout development. Similar effects of early ontogenesis on gut microbiota has also been repeatedly observed in channel catfish (Bledsoe et al., 2016; Burgos et al., 2018). Of the ASV shown to be temporally differential in abundance, an ASV in *Acinteobacter* was the only microbe enriched in the trout fry gut at 20 dph. These free-living saprophytic bacteria are commonly detected in the environment (soil and water) and are also a small part of the normal skin microbiota in one quarter of humans; although *Acinetobacter* are also found in the gut of humans and animals, as opportunistic pathogens with relatively low virulence (Bergogne-Bérézin 2014). An ASV from the genus *Streptococcus* was also enriched in the trout gut at 20 dph in this study (Figure 3.2 H), but similar ASV from this genus were enriched at 65 dph suggesting there was a temporal shift in the bacterial species or strains in the genus. Though it should be noted that when merging ASV at the genus level, no bacterial genera showed enrichment in the 20 dph trout fry gut. The abundance of some 35 other ASV was shown to be a signature of the 65 dph trout gut microbiome (Figure 3.2 H), including some from known pathobiont genera such as *Aeromonas, Klebsiella,* and *Shewanella*. Although, these microbes are commonly detected in the gut of healthy teleost, as Dimitroglou et al. (2009) identified nearly all the same microbes in the gut of healthy juvenile and subadult rainbow trout using culture-based techniques.

Only a single *Acinetobacter* ASV showed significant strain by time interaction effects on individual microbiota abundance; however, a qualitative index of overall microbial

composition (uwUniFrac) did show interacting effects of host genetics and ontogeny on the gut bacteria (Table 1.1). These interacting effects highlight the need for an integrated understanding of the microbiome that reflects the interplay among the multiple biological factors regulating bacterial dynamics, instead of simply considering such factors individually. Often studies on fish microbiota focus on one axis of variation (i.e. diet, time, genetics, etc.), though research will have more industry application through the thoughtful combination of sources of variance, as are experienced across aquaculture production facilities.

To this author's knowledge, no study to date, has conducted DTU analysis using non-targeted RNAseq data in an aquaculture species, particularly in the intestine of selected and non-selected strains of rainbow trout. While a majority of genes are stably expressed by a dominant isoform with a significantly higher expression compared to that of other isoforms, it is now known that DTU can be an adaptive response to certain environmental and biological factors (Aanes et al., 2013). Isoform switching, or DTU, is controlled by *cis*- and *trans*-acting regulatory elements that alter start/stop codon usage, exon-skipping, or splicing within a gene and in some genes is highly controlled by epigenetics (Luco et al., 2010). Effects of DTU can range from no detectable effect on phenotype to modulated translational efficiency or even altered protein function depending on the type of transcriptional change and whether it occurs in 3'-UTR or protein coding regions (Aanes et al., 2013). A number of studies in rainbow trout have identified single gene products with differential isoform expression across various experimental factors. For instance, Kobayashi et al. (2002) found that an essential gene involved in germ cell progression, *vasa,* was differentially transcribed with either the full-length transcript or a shortened transcript that translated to a protein lacking the N-terminus, *vasa-s*, depending on biological sex and stage of germ cell differentiation. Similarly, transcription of interferon 1 in rainbow trout has been shown to fluctuate between at least two dominant isoforms whose relative expression are changed by viral infection or DNA vaccination (Purcell et al, 2009), although the targeted characterization of isoforms can also be complicated by gene duplication and presence of pseudo-genes. The ancestral teleost-specific genome duplication that occurred approximately 300 million years ago and the successive stepwise rediploidization within the evolutionary history of rainbow trout (Berthelot et al., 2014) is likely to further complicate the transcriptional landscape and produce more opportunity for adaptive DTU.

Here, a relatively small number of genes were found to participate in DTU according rainbow trout strain genetics and developmental time (Figure 3.3 A-B; Table 3.2). This is at least partly due to the abundance filtering applied prior to DTU analysis that removed nearly 90% of the detected genes; however, filtering has been shown to be an important step to reduce false-discoveries in RNAseq based DTU analysis, particularly when using smaller sample sizes and moderate sequencing depth (Love et al., 2018). The genes involved in DTU in this study were involved in a range of biological functions including proteosome function (PSMB9a; Figure 3.4 B), protein and lipid glycosylation (PGM3; Table 3.2), cell cycle regulation (CDK10; Figure 3.4 F) and RNA processing (PRPF4; Figure 3.4 G). The importance of some of these DTG, suggests DTU is adaptively regulated. While evaluating transcript usage in zebrafish embryos pre- and post-activation of zygotic transcription, Aanes et al. (2014) found thousands of genes involved in DTU, with some loci showing severe "switch" like changes in isoform expression during zygotic transcription. Similar isoform switches were observed for some genes in this study like NOC2L (Figure 3.4A), a gene involved in epigenetics through inhibition of histone acetyltransferases, though just as seen by Aanes et al. (2014), most changes in transcript usage here showed relatively subtle changes in complex patterns of isoform usage (Figure 3.4 E and H). The DTG in the present study serve as candidates for further evaluation to define the role that DTU may play on ontogenetic or strain differences in rainbow trout, with further analyses required to determine whether these DTG relate to meaningful biological change.

Differential gene expression analysis was implemented here to determine if genetic selection for performance on plant-based diet, as was applied to the select strain, results in detectable differences in homeostatic gene expression profiles at critical early stages of development. While the number of differentially expressed genes according to trout strain were minor compared to that of developmental timepoint (Figure 3.3 C), even a small subset of DEG clearly separated samples by fish strain (Figure 3.6). When conducting whole body DGE analysis in three genetic lines of rainbow trout selected for varying levels of susceptibility to *Flavobacterium psychrophilum* infection, Marancik et al. (2015) identified only 21 genes to be DE in three strains when controlling for infection, compared to the 118 genes found to be DE in this study according to trout strain alone. The DEG by fish strain did not show significant enrichments in GO terms, although multiple immune related genes such

as *CD4-related protein* (LOC100136286), *tumor necrosis factor receptor superfamily member 5* (TNR5), *T-cell surface glycoprotein CD5-like* (LOC110534451), *NLR family CARD domain-containing protein 3-like* (LOC110485298), *leucine-rich repeat and immunoglobulin-like domain-containing nogo receptor-interactin protein 1* (LOC110513592) and *proteosome subunit beta type-8-like* (LOC110496229) were upregulated in select trout. Genes related to nutrient metabolism, nuclear function, and cell structure were also enriched in the select trout intestines as well (Table 3.3). Further analysis will be needed to confirm the utility of these DEG as biomarkers for the selected strains performance. Despite the DGE by trout strain, there was no significant difference in overall gene expression profile according to fish genetics (Table 3.1; Figure 3.5 A).

Temporal changes in intestinal gene expression were the most profound effects detected in this study with 2,413 DEG (Figure 3.3) and significant shifts to multivariate expression profiles (Table 3.1; Figure 3.5 A). Gene ontology analysis showed the genes DE by developmental timepoint were particularly enriched for biological processes involved in amino acid (i.e. serine, glycine, and sulfur-containing amino acids), lipid (long-chain fatty acid metabolism, prostaglandins, and inositol), energy (i.e. pyruvate, tricarboxylic acid cycle, and oxygen transport), and cholesterol (i.e. biosynthesis, mevalonate pathway) metabolism. As well as immune function (i.e acute-phase response, innate immune response, complement activation, antigen processing, mucopolysaccharide metabolism, immunoglobulin production, and B-cell receptor signaling pathway) and cellular differentiation and proliferation (kinetochore assembly, apoptosis, microtubule-movement, DNA monophosphate biosynthesis, cytokinesis, cell dedifferentiation, mitotic spindle assembly, and cell-matrix adhesion) (Table 3.5). Alternatively, biological processes related to basal cellular function, such as ribosomal processes, as well as transcriptional and translational showed a significant under-enrichment, or stability, across developmental timepoint according to GO analysis (Table 3.5). In a similar fashion, molecular functions identified by GO enrichment analysis predominately related to innate immune function, energy and nutrient metabolism, and enzyme activity (Figure 3.7). Few studies have conducted RNAseq to characterize intestinal transcription during early points of development and exogenous feeding, although Ingerslev et al. (2014) used a targeted panel of ten innate and adaptive immune markers to measure gene expression by qPCR at 26 and 49 dpff. In that study,

significant ontogenetic shifts were detected for every gene measured except for CD4 and FOXP3b at 26 dpff compared to one-day prior to first feeding. This agrees with the number of immune related GO terms detected in the study (Figure 3.7).

The non-targeted quantification of host intestinal mRNA transcription alongside gut bacteria characterization, as was done here, allows simultaneous comparison of both host and microbiota response to the experimental factors under investigation. Results showed that host genetics and ontogenetic development had similar effects on both intestinal transcription and gut microbiota communities (Figure 3.2 C-D; Figure 3.5 A). Sample-wise host intestinal gene expression profile and gut microbiota composition (Figure 5.3 B) showed a highly significant correlation (90%; $p = 0.001$) and while separation by trout strain was somewhat muddled in the Procrustes plot, temporal patterns in host transcription and microbiota show similar patterns (Figure 3.5). Merging the characterization of host phenotypes or transcription with microbiota studies helps to strengthen the application of microbiome knowledge in aquaculture. It is difficult to discern whether host transcriptional changes drive the detected shifts in gut microbiota across developmental ontogeny, or whether the inverse is true. Regardless, the results here show intestinal gene expression and gut microbiota are interconnected in rainbow trout fry and appear to progress ontogenetically in concert, at least during early stages of development.

In summary, an integrative analysis of intestinal transcription and gut microbiota characterization was used to evaluate homeostatic differences in a superior performing strain of rainbow trout selected for its growth performance on an all plant diet. Superior growth performance of the selected trout, even at early life stages, was confirmed. Some differences in gut microbiota were attributable to trout genetics, but the biggest differences in microbiota composition and abundance were due to difference in developmental timepoint (20 vs. 65 dph). Similar patterns were observed in host intestinal gene transcription. While the 74 genes that showed evidence for DTU were somewhat evenly distributed across independent variables, ontogenetic shifts in gene expression greatly outweighed the number of DEG between the selected and commercial trout strains. Gene ontology analysis of temporally regulated genes showed an enrichment in biological processes related to tricarboxylic acid cycle, innate immune function, protein refolding, as well as cellular proliferation and dedifferentiation. Intestinal gene expression profiles and gut microbiota communities showed

a high degree of congruency in their response to experimental factors, particularly across ontogeny. Results from this study provide a list of candidate gut bacteria as well as intestinal transcripts and genes that are indicative of the select strain and may correlate with the superior growth and immune performance of the selected trout strain, though further validation of these markers is required to confirm their functional roles. In addition, the interconnected relationship between host transcription and microbiota in the intestine of rainbow trout was definitively shown in this study.

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Table 3.1. Summary of multivariate statistical analysis on intestinal RNA sequencing and microbiota in two strains of rainbow trout (Commercial vs. Select).

Test	Factor	Microbiota wUniFrac (n = 15)	Microbiota uwUniFrac (n = 15)	RNAseq Euclidean (n = 5)
Dispersion	Fish Strain	0.053	0.797	0.551
	Timepoint	< *0.001*	0.391	0.867
PERMANOVA	Fish Strain	*0.001*	*0.030*	0.063
	Timepoint	*0.001*	*0.001*	*0.001*
	Strain by Time Interaction	0.136	*0.041*	0.249

Table 3.2. List of intestinal genes and transcripts detected by differential transcript usage analysis between two strains of rainbow trout (Commercial vs. Select) at 20 days post hatch (dph) and 65 dph. Significance was assessed using a two-stage statistical testing framework with an overall false discovery rate threshold of ≤ 0.05. Gene and transcript IDs are from NCBI.

Factor	Gene ID	Transcript ID
Timepoint	cdk10	XM_021595131.1
Strain, Timepoint, and Interaction	ddx24	XM_021584908.1
Strain, Timepoint, and Interaction	ddx24	XM_021584910.1
Interaction	LOC100136336	XM_021557396.1
Strain and Interaction	LOC110485518	XM_021556607.1
Strain and Interaction	LOC110485518	XM_021556608.1
Strain, Timepoint, and Interaction	LOC110485968	XM_021557241.1
Strain, Timepoint, and Interaction	LOC110485968	XR_002468016.1
Timepoint	LOC110486150	XM_021557539.1
Timepoint	LOC110486150	XM_021557540.1
Timepoint	LOC110486267	XM_021557724.1
Timepoint	LOC110486267	XM_021557725.1
Strain, Timepoint, and Interaction	LOC110486362	XM_021557890.1
Strain	LOC110486552	XR_002468081.1
Strain	LOC110486552	XR_002468082.1
Strain	LOC110487047	XM_021558957.1
Strain	LOC110488171	XR_002468362.1
Strain	LOC110488171	XR_002468364.1
Strain	LOC110488288	XM_021560458.1
Timepoint and Interaction	LOC110488571	XM_021560834.1

Timepoint and Interaction	LOC110488571	XM_021560835.1
Strain	LOC110488728	XM_021561055.1
Strain	LOC110488728	XM_021561057.1
Timepoint	LOC110489723	XM_021562663.1
Strain	LOC110489823	XM_021562754.1
Strain	LOC110489823	XM_021562756.1
Timepoint	LOC110489999	XM_021563073.1
Timepoint	LOC110489999	XM_021563074.1
Interaction	LOC110491208	XM_021564562.1
Interaction	LOC110491208	XM_021564569.1
Strain	LOC110493834	XR_002469195.1
Strain	LOC110494624	XM_021569855.1
Strain	LOC110494624	XM_021569856.1
Strain	LOC110494801	XR_002469317.1
Strain	LOC110494801	XR_002469318.1
Timepoint	LOC110495070	XM_021570427.1
Timepoint	LOC110495070	XR_002469375.1
Timepoint	LOC110495758	XM_021571167.1
Timepoint	LOC110495758	XM_021571169.1
Strain, Timepoint, and Interaction	LOC110496203	XM_021571966.1
Strain, Timepoint, and Interaction	LOC110496203	XM_021571968.1
Strain	LOC110496207	XM_021571975.1
Strain	LOC110499747	XM_021576807.1
Timepoint	LOC110500246	XM_021577517.1
Timepoint	LOC110500246	XM_021577554.1
Strain	LOC110500400	XM_021577756.1
Timepoint	LOC110501349	XM_021578865.1
Interaction	LOC110501349	XM_021578865.1
Timepoint	LOC110505032	XM_021583972.1
Timepoint	LOC110505032	XM_021583973.1
Timepoint	LOC110505739	XM_021585152.1
Timepoint	LOC110506586	XM_021586312.1
Timepoint	LOC110506586	XM_021586313.1
Interaction	LOC110507842	XM_021588201.1
Strain	LOC110507953	XM_021588376.1
Strain	LOC110507953	XM_021588377.1

Timepoint	LOC110508323	XM_021588758.1
Timepoint	LOC110508323	XM_021588767.1
Timepoint	LOC110509090	XM_021590049.1
Timepoint	LOC110509090	XM_021590052.1
Timepoint	LOC110509965	XM_021591210.1
Timepoint	LOC110512929	XR_002471932.1
Timepoint	LOC110512929	XR_002471933.1
Strain	LOC110512933	XM_021593026.1
Strain	LOC110512933	XM_021593027.1
Timepoint	LOC110518458	XM_021596107.1
Timepoint	LOC110518458	XM_021596120.1
Timepoint	LOC110520290	XM_021597488.1
Strain	LOC110521168	XM_021598602.1
Strain	LOC110521215	XM_021598650.1
Strain	LOC110521215	XM_021598651.1
Strain	LOC110522342	XM_021600667.1
Strain	LOC110522342	XM_021600669.1
Strain	LOC110522428	XM_021600807.1
Timepoint	LOC110523849	XM_021602912.1
Timepoint	LOC110524019	XM_021603302.1
Timepoint	LOC110524259	XM_021603744.1
Timepoint	LOC110527512	XM_021608833.1
Timepoint and Interaction	LOC110528208	XM_021610103.1
Timepoint and Interaction	LOC110528208	XM_021610191.1
Timepoint	LOC110530711	XM_021613966.1
Strain	LOC110531556	XM_021614791.1
Strain	LOC110531556	XM_021614792.1
Interaction	LOC110532354	XM_021616190.1
Strain	LOC110532711	XR_002474876.1
Strain	LOC110532750	XM_021616874.1
Strain	LOC110532750	XM_021616875.1
Strain	LOC110532762	XM_021616890.1
Timepoint	LOC110532762	XM_021616890.1
Timepoint	LOC110533398	XM_021617552.1
Timepoint	LOC110533398	XM_021617553.1
Timepoint	LOC110534269	XM_021619027.1

Timepoint	LOC110534269	XM_021619028.1
Timepoint	LOC110537380	XM_021623382.1
Timepoint	LOC110537380	XM_021623383.1
Timepoint	LOC110537790	XM_021624180.1
Timepoint	LOC110537790	XM_021624189.1
Timepoint	LOC110538037	XM_021624595.1
Timepoint	LOC110538037	XM_021624604.1
Timepoint	LOC110538446	XM_021625277.1
Timepoint	LOC110538446	XM_021625278.1
Timepoint	LOC110538565	XM_021625457.1
Timepoint	LOC110538565	XM_021625458.1
Strain	mrpl38	XM_021564686.1
Strain	mrpl38	XM_021564687.1
Strain	ndkb	NM_001165224.1
Strain	ndkb	XM_021556167.1
Strain	noc2l	XM_021615951.1
Strain	noc2l	XM_021615952.1
Timepoint	pgm3	XM_021600641.1
Timepoint	pgm3	XM_021600642.1
Strain	pldn	XM_021585492.1
Strain	pldn	XM_021585493.1
Strain	prdx6	XM_021598382.1
Strain	prdx6	XM_021598383.1
Interaction	prpf4	XM_021590713.1
Interaction	prpf4	XM_021590714.1
Strain	psmb9a	NM_001124258.1
Strain	psmb9a	XM_021570528.1
Timepoint	rcc1l	XM_021617657.1
Strain, Timepoint, and Interaction	rft1	XM_021609957.1
Strain, Timepoint, and Interaction	rft1	XR_002474201.1
Timepoint	spryd4	XM_021566620.1

Table 3.3. Differentially expressed genes in the intestine of rainbow trout according to fish strain (Commercial vs. Select). Genes with a negative log2 fold-change are overexpressed in the commercial strain of trout, while positive fold-changes indicate genes more highly expressed in the selected strain. Gene IDs and descriptions are taken from NCBI.

Gene ID	Description	Log2 Fold Change	FDR Adj. P-Value
LOC100135947	myosin light chain 1	-5.53	1.18E-05
LOC110499747	fish-egg lectin-like	-5.29	2.49E-07
LOC110525460	UDP-glucuronosyltransferase 2A2 pseudogene	-2.98	2.38E-04
LOC110514868	cuticle collagen sqt-1-like	-2.96	2.84E-02
LOC110488715	glyoxylate reductase/hydroxypyruvate reductase-like	-2.92	3.63E-05
LOC110500972	cytidine monophosphate-N-acetylneuraminic acid hydroxylase-like	-2.89	4.39E-03
upp2	uridine phosphorylase 2	-2.85	2.44E-06
nefl	neurofilament light	-2.69	2.50E-03
LOC110495159	collagen alpha-6(VI) chain-like	-2.64	5.17E-03
LOC110537581	hemicentin-1-like	-2.52	1.20E-03
LOC110526342	uncharacterized LOC110526342	-2.31	1.69E-03
LOC110488086	collagen alpha-1(XXVIII) chain-like	-2.24	1.98E-03
LOC110494169	nuclear receptor subfamily 4 group A member 1-like	-2.01	3.20E-03
LOC110532751	plexin-B1-like	-1.86	3.93E-04
LOC110533149	sodium/hydrogen exchanger 9B2-like	-1.85	7.98E-03
LOC110508698	sickle tail protein-like	-1.82	2.65E-03
LOC110518470	sperm-associated antigen 5-like	-1.76	1.63E-03
LOC110491867	uncharacterized	-1.75	9.74E-03
ryr3	ryanodine receptor 3	-1.68	7.96E-04
LOC110508898	myocardin-like	-1.68	8.41E-04
LOC110503971	roundabout homolog 2-like	-1.67	1.63E-03
LOC110534323	sodium/hydrogen exchanger 9B2-like	-1.65	3.79E-06
LOC110498052	protein CLN8-like	-1.60	8.49E-05
LOC110535597	insulin-induced gene 1 protein-like	-1.59	1.98E-03
LOC110529682	uncharacterized LOC110529682	-1.58	1.69E-02
LOC110504465	dual specificity protein phosphatase 1-like	-1.55	4.62E-04
LOC110528579	acid-sensing ion channel 1-like	-1.55	1.33E-04
LOC110489570	pleckstrin homology-like domain family B member 2	-1.54	4.11E-02
LOC110533389	early growth response protein 1-like	-1.54	4.54E-04
LOC110520099	Golgi reassembly-stacking protein 2-like	-1.52	2.23E-02
LOC110520863	ephrin-A1-like	-1.50	7.10E-05
LOC110524767	myomegalin-like	1.50	2.76E-02
LOC110530640	rho GTPase-activating protein 45-like	1.51	2.53E-05
LOC110487064	galectin-3-binding protein A-like	1.51	2.70E-02
LOC110522335	histidine triad nucleotide-binding protein 3-like	1.52	4.57E-02
LOC110501933	trafficking protein particle complex subunit 10-like	1.52	1.63E-03
LOC110485617	uncharacterized LOC110485617	1.53	1.28E-05
LOC110485824	rhomboid-related protein 3-like	1.53	8.40E-03
LOC100136286	CD4-related protein	1.53	6.34E-03
LOC110490500	poly [ADP-ribose] polymerase 12-like	1.54	1.73E-03
LOC110509332	voltage-dependent N-type calcium channel subunit alpha-1B-like	1.55	4.61E-02
LOC110536323	gamma-glutamyl hydrolase-like	1.55	1.78E-03
LOC110505795	dapper homolog 1-like	1.55	6.58E-03
LOC110535224	allograft inflammatory factor 1-like	1.58	4.62E-04
LOC110487416	uncharacterized LOC110487416	1.58	4.82E-04

LOC110489359	sodium-dependent neutral amino acid transporter B(0)AT1-like	1.58	4.62E-04
piwil1	piwi like RNA-mediated gene silencing 1	1.60	7.47E-03
LOC110500454	protein inturned-like	1.60	4.34E-02
LOC110515983	uncharacterized LOC110515983	1.63	3.34E-10
LOC110517169	28S ribosomal protein S29, mitochondrial-like	1.64	7.83E-05
LOC110486897	uncharacterized LOC110486897	1.64	2.69E-03
LOC110517082	ATP-binding cassette sub-family G member 2-like	1.67	1.07E-03
tnr5	Tumor necrosis factor receptor superfamily member 5	1.68	1.41E-07
LOC110530885	poly(U)-specific endoribonuclease-C-like	1.76	7.10E-05
LOC110533195	ATP-binding cassette sub-family G member 2-like	1.76	9.35E-04
LOC110510226	uncharacterized LOC110510226	1.76	1.01E-03
LOC110498357	fibroblast growth factor 23-like	1.77	2.50E-03
LOC110494588	leucine-rich repeat neuronal protein 1-like	1.78	2.45E-02
LOC110535953	uncharacterized LOC110535953	1.80	2.15E-02
LOC110531552	poly [ADP-ribose] polymerase 14-like	1.82	4.71E-05
LOC110490499	poly [ADP-ribose] polymerase 12-like	1.88	2.17E-02
LOC110504691	DNA damage-inducible transcript 4-like protein	1.88	2.26E-02
LOC110522607	protein BANP-like	1.89	2.59E-02
LOC110535952	tripartite motif-containing protein 35-like	1.95	2.69E-04
LOC110486715	tubulin beta-4B chain-like	2.00	1.14E-02
LOC110534451	T-cell surface glycoprotein CD5-like	2.03	4.75E-03
LOC110538482	microtubule-associated serine/threonine-protein kinase 1-like	2.09	4.62E-04
LOC110537233	fibroblast growth factor receptor homolog 1-like	2.10	9.59E-03
LOC110485298	NLR family CARD domain-containing protein 3-like	2.12	1.56E-02
LOC110534426	E3 ubiquitin-protein ligase TRIM39-like	2.13	3.50E-03
LOC110499480	cystatin-like	2.14	3.25E-02
mast1	microtubule associated serine/threonine kinase 1	2.16	3.69E-03
LOC110519912	regucalcin-like	2.16	8.38E-03
LOC110501563	uncharacterized LOC110501563	2.17	2.51E-02
LOC110527219	carboxypeptidase O-like	2.21	1.07E-07
LOC110526299	embryonic polyadenylate-binding protein 2-B-like	2.32	1.76E-02
LOC110505108	carcinoembryonic antigen-related cell adhesion molecule 6-like	2.33	4.69E-04
LOC110517874	dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3B-like	2.37	1.46E-02
LOC110506833	uncharacterized LOC110506833	2.43	5.29E-03
LOC110486522	lysine-rich arabinogalactan protein 18-like	2.44	3.63E-05
LOC110520624	SLIT and NTRK-like protein 5	2.46	1.53E-06
LOC110511935	uncharacterized LOC110511935	2.51	1.34E-06
LOC110499513	cofilin-2-like	2.56	3.63E-03
LOC110486599	uncharacterized LOC110486599	2.63	6.73E-03
LOC110494817	glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic-like	2.72	4.75E-03
LOC110496123	uncharacterized LOC110496123	2.72	2.08E-02
LOC110506891	hereditary hemochromatosis protein homolog	2.74	7.28E-03
LOC110538122	U3 small nucleolar RNA-associated protein 18 homolog	2.77	1.53E-06
LOC110520308	cleavage and polyadenylation specificity factor subunit 5	2.78	2.22E-03
LOC110539101	tryptase-2-like	2.80	7.98E-03
col15a1	collagen type XV alpha 1 chain	2.87	3.96E-04
LOC110508214	inhibin beta C chain-like	3.02	1.51E-02
LOC110535619	zinc-binding protein A33-like	3.04	1.15E-03
LOC110514348	fucolectin-6-like	3.15	4.72E-02
LOC110505715	B2 bradykinin receptor-like	3.40	2.30E-07
LOC110513113	uncharacterized LOC110513113	3.49	3.41E-02
LOC110510414	28S ribosomal protein S21, mitochondrial-like	3.55	4.91E-05
LOC110516983	GTPase IMAP family member 4-like	3.63	4.61E-02

LOC110533448	glutamate receptor ionotropic, kainate 4-like	3.81	6.34E-03
LOC110505338	uncharacterized LOC110505338	3.88	3.83E-02
LOC110535798	annexin A1-like	3.89	4.24E-03
LOC110501547	glutaminase kidney isoform, mitochondrial-like	4.02	1.54E-05
LOC110513560	uncharacterized LOC110513560	4.09	2.01E-04
LOC110515470	endonuclease domain-containing 1 protein-like	4.11	8.40E-03
LOC110515468	endonuclease domain-containing 1 protein-like	4.14	9.45E-03
LOC110485437	uncharacterized LOC110485437	4.14	1.68E-02
LOC110514582	heterogeneous nuclear ribonucleoprotein R-like	4.28	2.69E-04
LOC110530755	NACHT, LRR and PYD domains-containing protein 3-like	4.42	1.01E-04
LOC110493924	uncharacterized LOC110493924	4.54	7.47E-04
LOC110518721	basement membrane-specific heparan sulfate proteoglycan core protein-like	4.78	9.40E-03
LOC110531554	poly [ADP-ribose] polymerase 14-like	4.81	1.53E-06
LOC110513592	leucine-rich repeat and immunoglobulin-like domain-containing nogo receptor-interacting protein 1	4.93	4.42E-03
LOC110514975	SWI/SNF complex subunit SMARCC1-like	5.19	1.39E-02
LOC110506238	triadin-like	6.44	4.50E-04
LOC110491340	alpha-N-acetylgalactosamine-specific lectin-like	6.77	1.53E-06
LOC110532536	kynurenine 3-monooxygenase-like	6.78	1.43E-02
LOC110536347	vasotocin-neurophysin VT 2	6.85	2.25E-02
LOC110496229	proteasome subunit beta type-8-like	8.17	3.79E-06

Table 3.4. Genes with significant interaction effects on expression level in the intestine of two strains of rainbow trout (Commercial vs. Select) across two developmental timepoints (20 and 65 days post hatch). Interactions effects were detected using a Wald test with p-values adjusted using FDR.

Gene ID	Description	Wald Statistic	FDR Adj. P-Value
LOC110488353	BOLA class I histocompatibility antigen, alpha chain BL3-7-like	27.25	0.0029
LOC110518778	pyruvate carboxylase, mitochondrial-like	27.71	0.0029
prr13	proline-rich protein 13	23.52	0.0134
LOC101268925	long-chain-fatty-acid--CoA ligase 4	22.40	0.0180
LOC100136198	14-3-3G1 protein	19.86	0.0384
LOC110492468	solute carrier family 12 member 5-like	18.62	0.0384
LOC110497198	UPF0462 protein C4orf33 homolog	20.46	0.0384
LOC110498281	fibulin-5-like	19.68	0.0384
LOC110501949	guanylate-binding protein 1-like	18.66	0.0384
LOC110505108	carcinoembryonic antigen-related cell adhesion molecule 6-like	18.52	0.0384
LOC110505715	B2 bradykinin receptor-like	19.15	0.0384
LOC110528811	secretory phospholipase A2 receptor-like	18.63	0.0384
LOC110535619	zinc-binding protein A33-like	18.78	0.0384
LOC110536323	gamma-glutamyl hydrolase-like	18.41	0.0384

LOC110537950	ferritin, middle subunit-like	18.57	0.0384
LOC110503971	roundabout homolog 2-like	17.89	0.0420
LOC110528824	insulin receptor substrate 2-like	17.79	0.0420
LOC110536419	uncharacterized LOC110536419	18.05	0.0420
LOC110538933	nuclear body protein SP140-like protein	17.97	0.0420
LOC110490108	max-binding protein MNT-like	17.50	0.0467
LOC110515983	uncharacterized LOC110515983	17.29	0.0487
LOC110518815	tumor protein p63-regulated gene 1-like protein	17.23	0.0487

Table 3.5. Enriched biological process gene ontologies based on genes identified as differentially abundant in the intestine of rainbow trout between 20 days post hatch (dph) and 65 dph. Differential expression analysis between 20 dph and 65 dph detected 2,413 significant genes, while controlling for differences in fish strain (commercial and select). Fisher's exact test was used to conduct enrichment analysis with differential genes used as the test set, and the remainder of the annotated rainbow trout transcriptome serving as the reference set. Significantly enriched biological process ontologies are listed, after reducing to only the most specific GO terms. Red terms were over enriched in the test gene set, and green terms were underrepresented in the differential expression gene set.

GO ID	GO Name	FDR
GO:0015671	oxygen transport	1.04E-08
GO:0006508	proteolysis	1.51E-05
GO:0006563	L-serine metabolic process	2.13E-05
GO:0006544	glycine metabolic process	3.80E-05
GO:0006953	acute-phase response	4.22E-05
GO:0006695	cholesterol biosynthetic process	7.57E-05
GO:0006566	threonine metabolic process	7.83E-05
GO:0045087	innate immune response	9.22E-05
GO:0000915	actomyosin contractile ring assembly	1.42E-04
GO:0019287	isopentenyl diphosphate biosynthetic process, mevalonate pathway	9.97E-04
GO:0051382	kinetochore assembly	0.00121
GO:0006601	creatine biosynthetic process	0.0020632
GO:2000042	negative regulation of double-strand break repair via homologous recombination	0.0020632
GO:0070098	chemokine-mediated signaling pathway	0.0046421
GO:0006099	tricarboxylic acid cycle	0.0051781
GO:0009070	serine family amino acid biosynthetic process	0.0054637
GO:0006958	complement activation, classical pathway	0.0058380
GO:0002474	antigen processing and presentation of peptide antigen via MHC class I	0.0065014
GO:0006090	pyruvate metabolic process	0.0089864
GO:0042981	regulation of apoptotic process	0.0121760
GO:0007018	microtubule-based movement	0.0135228
GO:0006555	methionine metabolic process	0.0149070
GO:0009157	deoxyribonucleoside monophosphate biosynthetic process	0.0169916
GO:0000097	sulfur amino acid biosynthetic process	0.0175601
GO:0032465	regulation of cytokinesis	0.0206198
GO:0016114	terpenoid biosynthetic process	0.0208863
GO:1903510	mucopolysaccharide metabolic process	0.0230525
GO:0042026	protein refolding	0.0237493
GO:1902850	microtubule cytoskeleton organization involved in mitosis	0.0282966

GO:0072348	sulfur compound transport	0.0291091
GO:0001676	long-chain fatty acid metabolic process	0.0301921
GO:0043697	cell dedifferentiation	0.0362618
GO:0071466	cellular response to xenobiotic stimulus	0.0362618
GO:0002639	positive regulation of immunoglobulin production	0.0362618
GO:0015838	amino-acid betaine transport	0.0362618
GO:0050853	B cell receptor signaling pathway	0.0362618
GO:0006230	TMP biosynthetic process	0.0362618
GO:0006826	iron ion transport	0.0369583
GO:0007094	mitotic spindle assembly checkpoint	0.0369583
GO:0001516	prostaglandin biosynthetic process	0.0423462
GO:0006020	inositol metabolic process	0.0435040
GO:0007160	cell-matrix adhesion	0.0445093
GO:1901475	pyruvate transmembrane transport	0.0494007
GO:0045806	negative regulation of endocytosis	0.0494007
GO:0042254	ribosome biogenesis	2.96E-05
GO:0006357	regulation of transcription by RNA polymerase II	0.0025149
GO:0006417	regulation of translation	0.0071074
GO:0034470	ncRNA processing	0.0086962
GO:0015031	protein transport	0.0168350

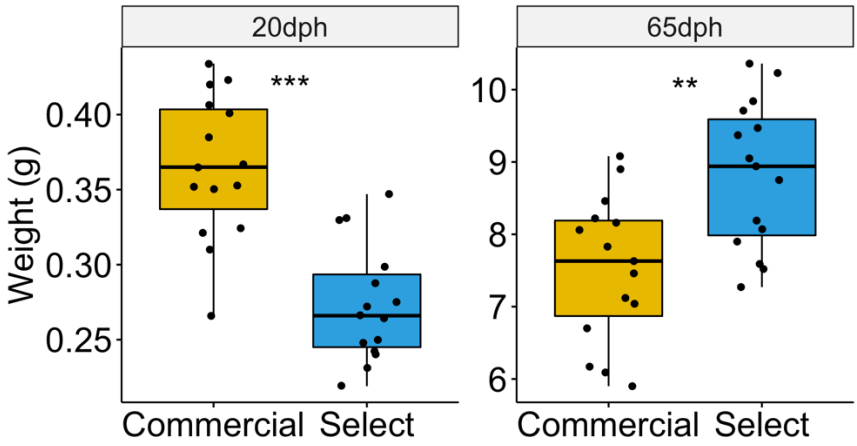

Figure 3.1. Fish weight (g) of a commercial and selected strain of rainbow trout at 20 days post hatch (dph) and 65 dph. A t-test was used to compare fish weight by strain at each timepoint. Significant differences were detected at both 20 dph ($p \leq 0.001$) and 65 dph ($p = 0.0089$).

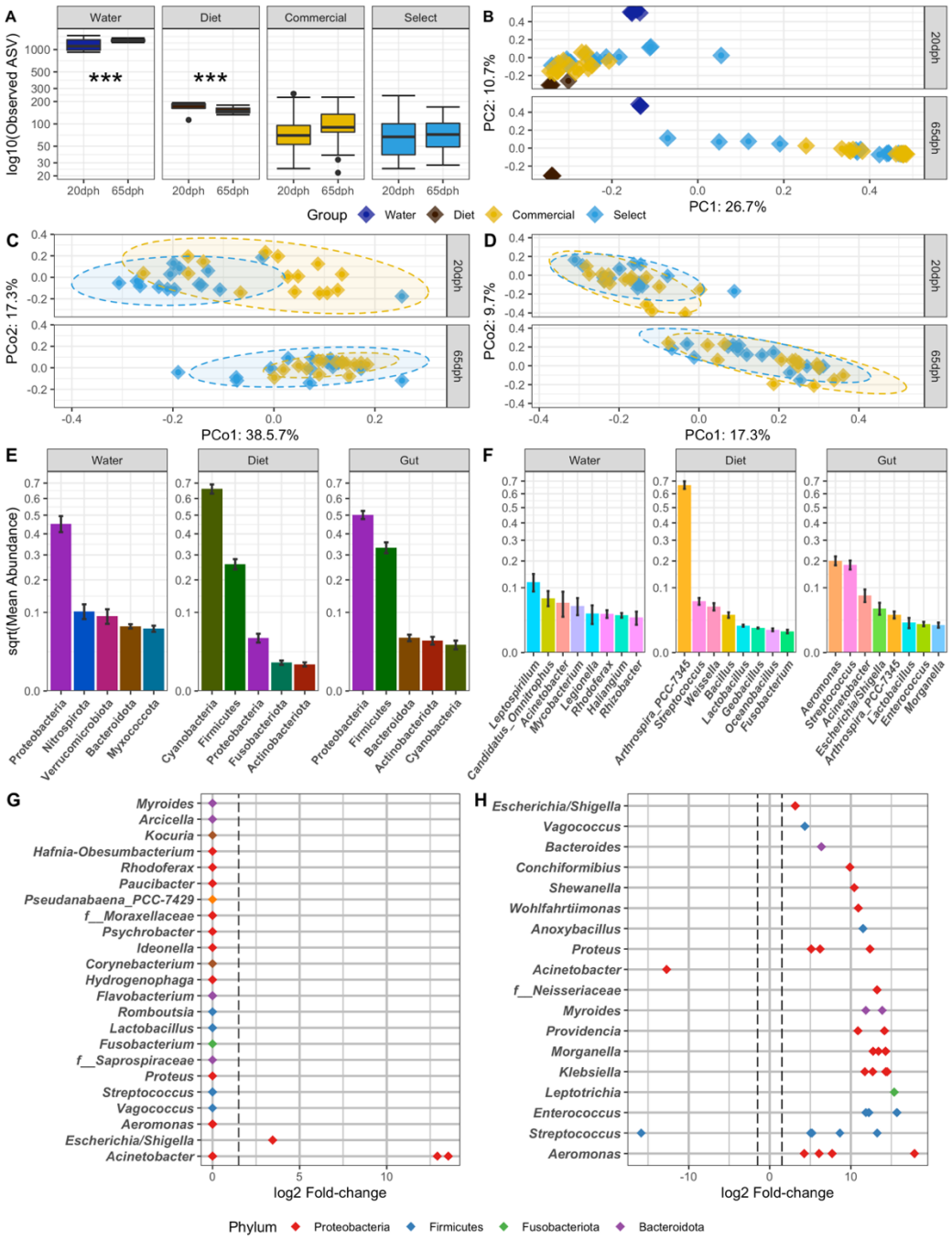

Figure 3.2. Results on the intestinal microbiota of a commercial and selected strain of rainbow trout at 20 days post hatch (dph) and 65 dph. Microbiota richness is displayed on a log10 scale and *** indicates a highly significant ($p \leq 0.001$) difference between sample type (water, diet, and gut) (A). Bray-Curtis dissimilarity ordination of microbiota composition by sample type (B). A wUniFrac (C) and uwUniFrac (D) ordination of gut microbiota composition by fish strain and timepoint. The top five phyla in terms of mean relative abundance within each sample-type are shown with error-bars displaying SEM plotted on a square-root axis (E), with a comparable plot showing the top eight genera (F). Differential abundance analysis was conducted at the ASV level using a full two-way model in DESeq2 (FDR ≤ 0.05; $|\log2 \text{ Fold-change}| \geq 1.5$). All ASV with FDR ≤ 0.05 are shown for fish strain (G) while only those with both FDR ≤ 0.05 and $|\log2 \text{ Fold-change}| \geq 1.5$ are listed for timepoint (H). Genus level (*f_* - family level) taxonomy is listed on the y-axis (G-H). Positive fold changes indicate enrichment in the select strain (G) and at 65 dph (H).

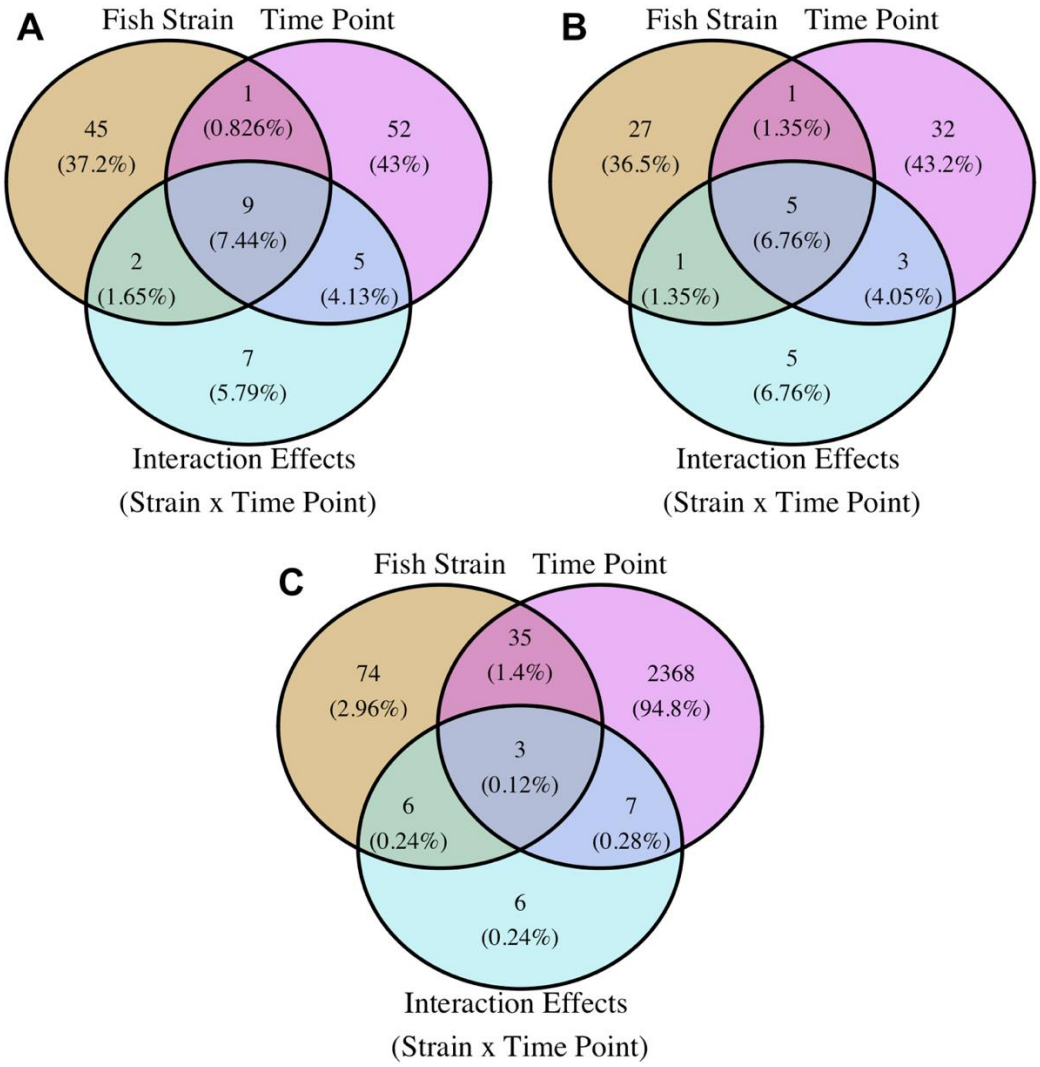

Figure 3.3. Venn diagrams summarizing the distribution of treatment effects on differential transcript usage (A), differentially transcribed genes (B), and differentially expressed genes (C) in intestinal samples from two strains (Commercial and Select) of rainbow trout at 20 days post hatch (dph) and 65 dph.

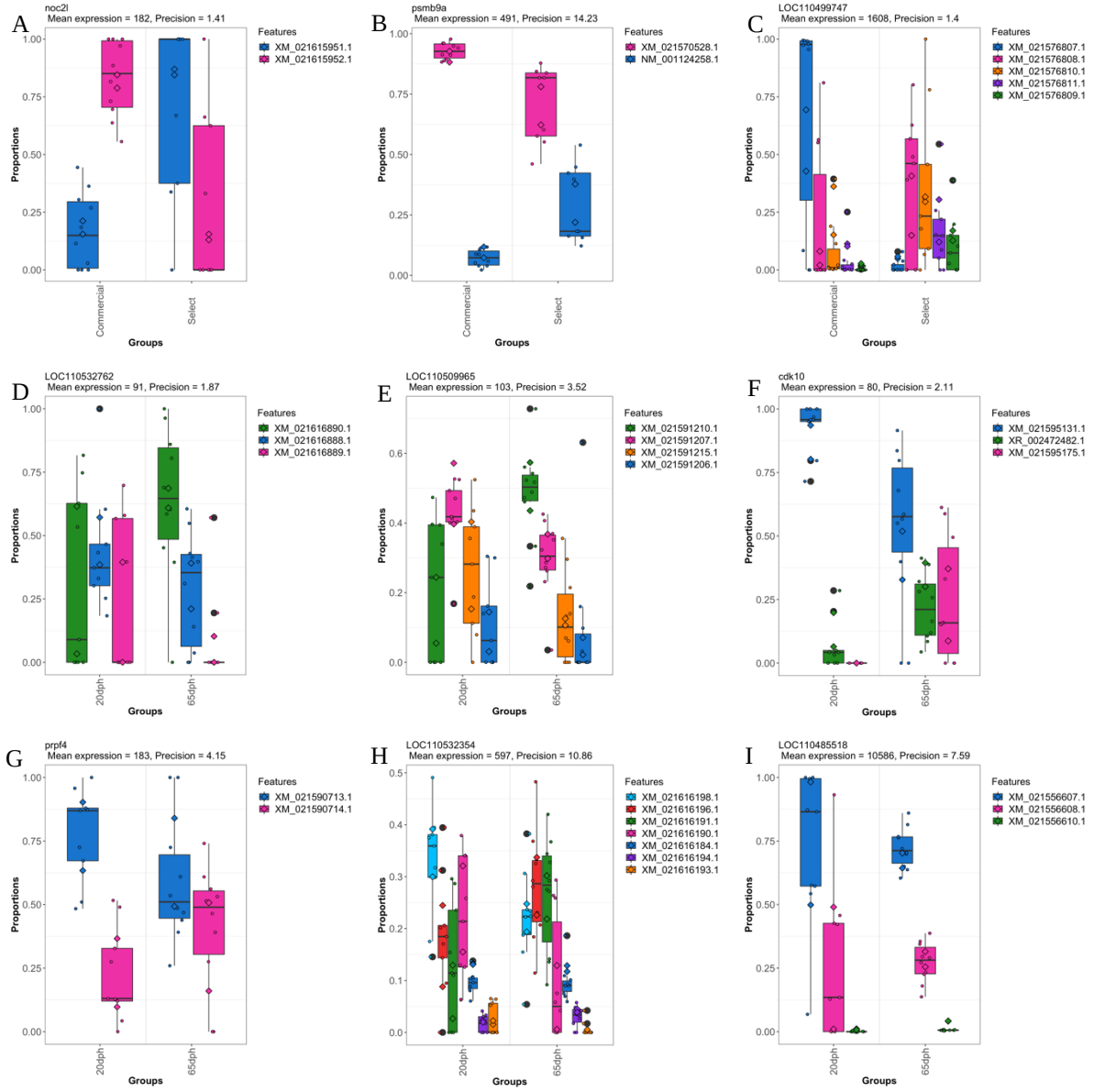

Figure 3.4. Differential transcript usage by fish strain (A-C), developmental timepoint (D-F), and strain by time interactions (G-I) among two strains of rainbow trout at 20 days post hatch (dph) and 65 dph. Differential transcript usage analysis was conducted using a two-stage statistical framework with only those transcript-gene combinations with an overall false discovery rate of ≤ 0.05. Not all significantly differentially transcribed genes are presented.

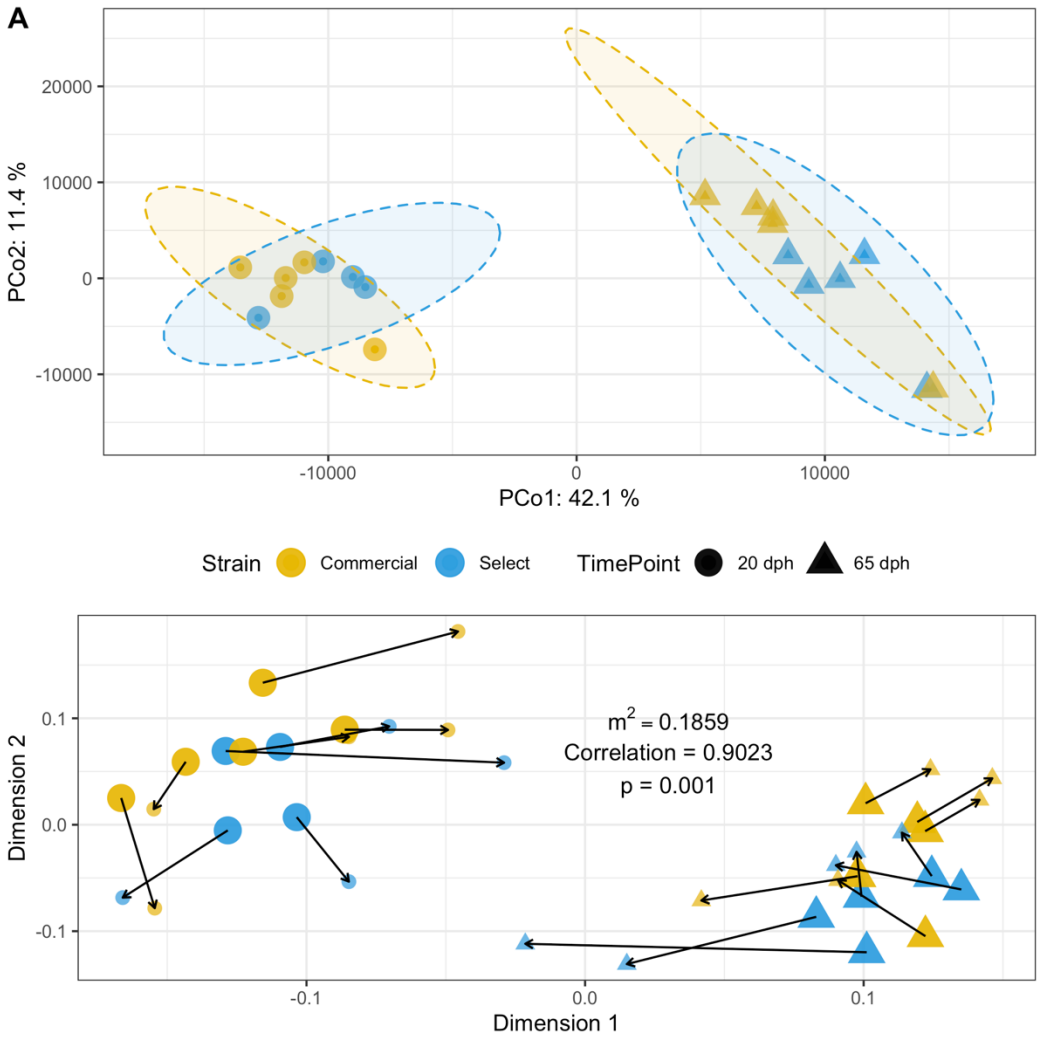

Figure 3.5. Multivariate analysis of intestinal gene expression in two strains (Commercial and Select) of rainbow trout at 20 days post hatch (dph) and 65 dph. Principle coordinates analysis (PCoA) plot based on sample-wise Euclidean distances from gene expression (RNAseq) data following a variance stabilizing transformation (A). Procrustes plot displaying the scaled and rotated mapping of gene expression Euclidean PCoA ordinations (large points) to microbiota uwUniFrac PCoA ordinations (small points) (B).

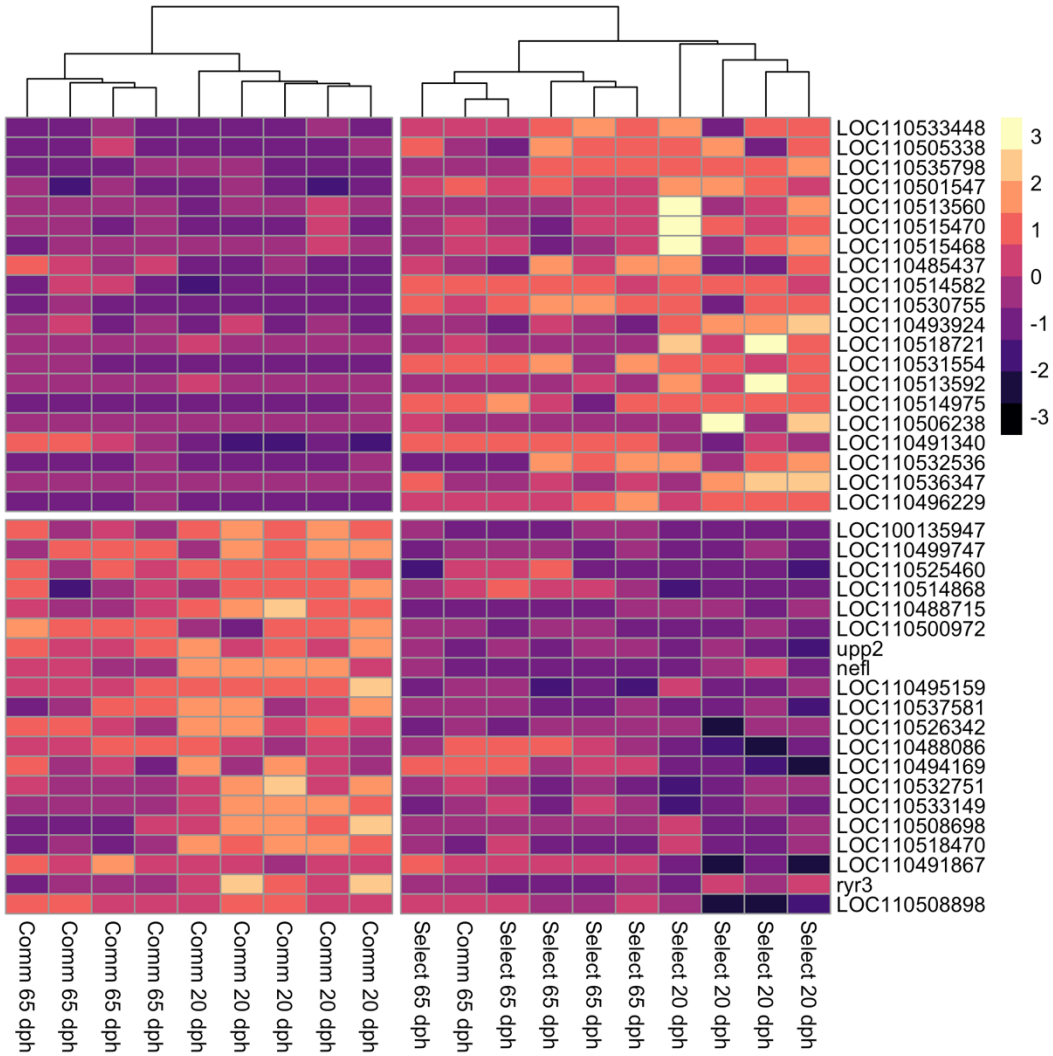

Figure 3.6. Heatmap showing the top forty differentially expressed genes in the intestines between a commercial and select strain of rainbow trout. Data were normalized and variance stabilizing transformation was applied prior to scaling row values for aesthetic plotting. The top twenty genes are listed in increasing order of positive log2 fold change, indicating over expression in the select strain of trout, while bottom twenty genes are listed in descending order of negative log2 fold change, representing genes over expressed in the commercial trout strain. Samples are ordered according to complete linkage hierarchical clustering (top dendrogram). Darker cells represent low expression, while lighter colors correspond to high expression.

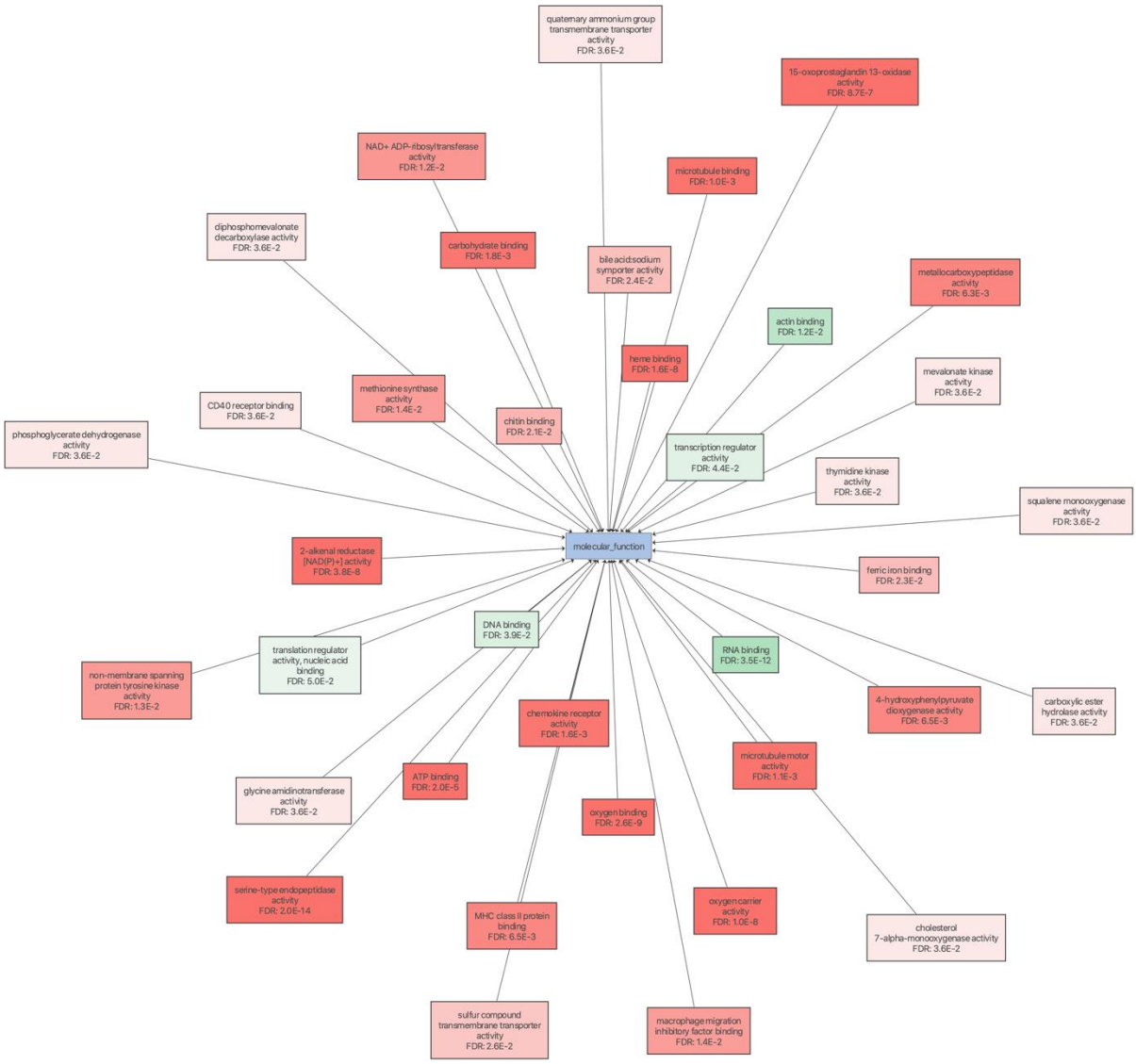

Figure 3.7. Relational map of enriched molecular function gene ontologies based on genes identified as differentially abundant in the intestine of rainbow trout between 20 days post hatch (dph) and 65 dph. Only those molecular function ontologies with multiple test sequences (≥ 2) that were also identified as significantly enriched (FDR ≤ 0.05) by Fisher's Exact Test are shown. Results were reduced to only the most specific GO term. Colors correspond to FDR significance with darker red indicating highly over enriched ontologies and darker green corresponding to highly under enriched ontologies. White ontologies are at the limit of the FDR significant threshold. "DNA binding", "transcription regulator activity", and "translation regulator activity, nucleic acid binding" ontologies are under enriched and all other white terms are over enriched.

Chapter 4: Tracking intestinal transcriptomic response and gut microbiota during viral (IHNV) or bacterial (*Flavobacterium psychrophilum*) infection in two strains of rainbow trout with underlying differences in disease resistance

Abstract

Losses to disease cost the aquaculture industry over 10 billion USD annually and hamper economically and environmentally sustainable growth of the industry to meet demands of a growing world population. Rainbow trout *(Oncorhynchus mykiss*) production, which accounts for a significant portion of finfish aquaculture in various global markets, is especially hampered by losses due to infectious hematopoietic necrosis virus (IHNV) and *Flavobacterium psychrophilum* (*Fp*), the etiological agents responsible for Infectious Hematopoietic Necrosis (IHN) disease and Rainbow Trout Fry Syndrome/Bacterial Coldwater Disease, respectively. Despite the impact of these diseases and other viral and bacterial diseases, little is known about the intestinal transcriptomic and gut microbiota dynamics during or following infection. This research describes the analysis of intestinal transcriptomic responses (RNA sequencing) and gut microbial ecology (16S rRNA gene sequencing) during early (4-5 days post challenge) and late (20-21 days post challenge) stages of an experimental IHNV or *Fp* disease challenges, using a commercial and selected strain of rainbow trout with proven differences in disease resistance. IHNV infections caused similar cumulative percent mortality (CPM) in the commercial (52.9% ± 7.4) and select strain (51.1% ± 3.90), while the *Fp* challenge showed significant differences (Kaplan-Meier Log Rank Test, $p \leq 0.001$) between commercial (94.8% ± 5.6) and select (70.4% ± 4.6) strain. Serum IHNV neutralization titers showed no difference by trout strain but were upregulated in infected fish compared to sham controls. Serum lysozyme activity during both challenges was higher ($p \leq 0.05$) in the select strain at both stages of infection, although serum alternative complement activity (ACH50) was upregulated in the commercial strain following the IHNV challenge. Differential transcript usage (DTU) analysis of intestinal transcriptomic data identified 544 and 610 differential transcribed genes across the experimental factors of trout strain, disease status (sham vs. infected), and disease stage (early vs. late). After summarizing transcript counts at the gene level, multivariate analysis of

gene expression profiles showed highly significant ($p \leq 0.005$) effects of host strain, disease status, and disease stage, as well as evidence of some interacting effects, in both disease challenges. Differential gene expression (DGE) analysis in both disease challenge datasets identified thousands of genes as potential biomarkers for differences between trout strains, infected and uninfected fish, and late and early stages of infection. Gene ontology (GO) enrichment analysis of DTU and DGE results by Fisher's Exact Test identified adaptive changes in transcript usage and gene expression. In the IHNV challenge, functions related to genomic repair, transcriptional regulation, cell differentiation, viral RNA replication, as well as, chemokine, cytokine, and interferon signaling were all enriched, as were many other GO terms, in the sham vs. infected comparisons. Similar enrichments were seen in the *Fp* challenge with functions related to chemokine, cytokine, and interferon signaling enriched, although functions related to cytoskeleton reorganization and maintenance, metalloendopeptidase activity, and fever generation and many other GO terms were also detected in the *Fp* infected fish. Results on gut microbiota showed that IHNV infections slightly reduced bacteria richness (observed ASV) and diversity (Shannon's Index) but not significantly, although *Fp* infections significantly reduced both richness and diversity during early stages of the disease. Multivariate analysis of the gut microbiota profiles using abundance weighted (wUniFrac) and unweighted (uwUniFrac) phylogenetic distances showed only significant effects of strain by stage interaction in the IHNV challenge based on wUniFrac, while uwUniFrac showed significant effects of disease status, stage main effects, as well as interacting effects. Gut microbiota profiles in the *Fp* challenge showed more significant influences of experimental factors, with all main effects and interactions, with the exception of the full interaction term, found to have significant effects on either weighted or unweighted UniFrac profiles. Differential abundance analysis at the bacterial genus level showed somewhat similar patterns to DGE group comparisons, with hundreds of potential biomarker genera identified across the experimental factors in both pathogen challenges. These results are the first report of parallel characterization of mortality, circulating immune performance, transcriptome wide intestinal response, and gut microbiota during viral or bacterial disease challenges in genetically distinct strains of rainbow trout. By combining the experimental factors of trout genetics, disease status, disease stage, and pathogen type (viral and bacterial), results from this study have widespread implications on selective breeding and

fish health, while also bolstering our basic understanding of host-microbiota-pathogen interactions in metazoans.

Introduction

Aquaculture production of rainbow trout *(Oncorhynchus mykiss*) has experienced exponential growth since the 1950s, with over 800,000 metric tons produced globally in 2016 (FAO 2020). However, during the last half decade the growth of trout production has substantially slowed. While market factors are responsible for some of the slowed growth in production, losses due to disease have also constrained the expansion of rainbow trout aquaculture. It has been estimated that 1 in 10 animals reared in aquaculture are lost due to infectious disease and these losses cost to the global aquaculture industry in excess of 10 billion USD annually (Adams 2020). Environmentally and economically sustainable growth of the aquaculture industry hinges on our ability to manage fish health and better mitigate disease related losses.

Despite the cost of disease in aquaculture and the many studies related to pathogenesis, particularly in salmonids, the intestinal response to pathogens at the molecular level has been poorly studied in teleost fish. A few studies have conducted characterization of specific immune transcripts in the intestine during or after infection (Evenhuis and Cleveland 2012), though transcriptome wide characterizations of intestinal response to disease are rarely conducted. While the intestine is not the primary target tissue for many of the pathogens that most negatively affect aquaculture, it is a multifunctional organ that serves central and peripheral roles that are critical for obtaining nutrition, regulating commensal microbiota, and defending against environmental pathogens and antigens. As such, a systems level understanding of the responses to virulent pathogens that occur in the gut throughout the progression of disease could improve our ability to control or mitigate the negative impacts of disease. Our understanding of the importance of the homeostatic gut microbiota in salmonids and their physiological relevance to the host has greatly increased over the last decade, yet few studies have tracked the intestinal microbiota of fish during or after viral or bacterial infection (Brown et al., 2019; Dong et al., 2019). There is therefore a paucity of knowledge on the response of the gut microbiota and host intestinal transcriptome to systemic infection in salmonids, and whether there are lingering effects of infection on host transcription or microbial ecology in individuals that survive or recover from disease.

In rainbow trout production, many of the costliest diseases are caused by viral and bacterial pathogens derived from the water supply or production facilities. This trend follows in the Hagerman Valley, the epicenter of trout production in the US, where two of the pathogens of biggest concern are the virus Infection Hematopoietic Necrosis Virus and the bacterium *Flavobacterium psychrophilum*. Infectious hematopoietic necrosis virus (IHNV) is an enveloped negative-sense single-stranded RNA virus in the family Rhabdoviridae and is the etiologic agent behind infectious hematopoietic necrosis (IHN) disease in a range of wild and cultured fish species, particularly salmonids (Dixon et al. 2016). While the virus has been shown to produce mortalities between 3 - 18ºC, virulence appears to be highest between 8 - 15ºC and therefore IHNV is of greatest concern in cold water systems (Bootland and Leong 2011). As an OIE (World Organization For Animal Health) listed pathogen, IHNV is considered one of the most serious viruses impacting aquaculture (Bootland and Leong 2011). First described in sockeye and kokanee salmon *Oncorhynchus nerka* cultured at hatcheries in the Pacific Northwest (Rucker et al. 1953), IHNV was thought to be endemic to wild salmonids in Japan and the Pacific Northwest, but is now particularly prevalent in major rainbow trout *Oncorhynchus mykiss* producing regions in North America, Europe, and Asia (Winton 1991). While vertical transmission may occur, horizontal transmission is most common due to the shedding of high viral loads in the feces, urine, mucus, and sexual fluids of infected individuals (Bootland and Leong 2011). Acute disease can cause rapid epizootic events (up to 90% mortality) with mortalities preceding even the earliest onset of symptoms, though lethargy, sporadic whirling, exophthalmia, petechial hemorrhaging, pale gills, and opaque mucoidal feces are commonly observed in moribund individuals. As the name implies, IHNV has a tropism for hematopoietic tissues, such as the kidney and spleen, though the virus must first gain entry via the gills, skin, fin base, and alimentary canal (Bootland and Leong 2011). While typically most virulent in salmonid fry and juveniles up to two months of age, IHNV has also shown clinical signs of disease in trout as large as 500 g and broodstock may show susceptibility associated with spawning stress (LaPatra 1998). Multiple isolates of IHNV have been collected from various geographical locations and are known to have varied levels of virulence, just as different host species and populations are known to have varying levels of resistance (LaPatra 1998). As the epicenter for trout production in the US, Hagerman, ID has been a center for research on IHNV and an IHNV

isolate commonly used for disease challenge due to its reproducible virulence in rainbow trout, isolate 220-90, was first collected in the Hagerman Valley (Ammayappan et al., 2010).

Flavobacterium psychrophilum is a Gram-negative rod bacterium capable of gliding motility, which causes disease in a wide range of fish species, but most severely infect salmonids (Nematollahi et al., 2003). Initially referred to as *Cytophaga pyschrophila* or *Flexibacter pychrophilus*, this pathogen was first recovered from an epizootic of coho salmon *Oncorhynchus kisutch* in the Pacific Northwest in 1948 with clinical symptoms that included erosion of the caudle-peduncle and fin rays, skin lesions, mild exophthalmia, abdominal distention with ascites, and pale gills (Starliper 2011). These symptoms are now considered classical signs of rainbow trout fry syndrome (RTFS) and bacterial coldwater disease (CWD), both diseases caused by *F. psychrophilum*, with the difference being that RTFS typically involves acute infections in sac-fry and fingerling trout, while CWD is usually applied to infections in juvenile and older fish. Viable, replicating bacteria can be found free-living in the aquatic environment if sufficient nutrients and environmental conditions exist, though severely infected and dead fish often serve as an acute disease vector and vertical transmission can also occur. As suggested by the species name, *F. psychrophilum* thrives in water temperatures 4-16ºC, but is often most virulent in systems with temperatures below 10ºC (Starliper 2011). With an ability to survive outside of the host in aquatic ecosytsms, it is likely that this pathogen can remain in an aquatic system as an opportunistic pathobiont, which is confirmed by observation that microbes from the genera *Flavobacterium* are commonly detected as part of the fish skin and gill microbiota (Nematollahi et al., 2003). This pathogen typically begins disease by gaining entry into the host through skin abrasions (Madetoja et al., 2000). Although, while the pH of the gastric environment has been shown to be very adverse conditions for *F. psychrophilum* (Sera et al., 1972), there is also evidence that *F. psychrophilum* can rapidly multiple once reaching the intestine (Faruk 2000). Once infection is initiated, proteases, adhesin proteins, LPS endotoxin, and other enzymes that lyse host cells and subvert the immune system are among the virulence factors used by *F. psychrophilum* to cause disease with mortally infected fish typically succumbing to septicemia (Starliper 2011; Nematollahi et al., 2003).

Here two distinct strains of rainbow trout, a commercial reference strain and a selected strain, are utilized to evaluate the effects of viral or bacterial disease on intestinal

responses in hosts with disparity is disease susceptibility. The select strain, which has been selected for 16 generations by the USDA-ARS and University of Idaho (ARS-UI) strictly for growth performance on a plant-based diet, has been shown to also have superior resistance to experimental disease challenge (*e.g.* IHNV, *F. psychrophilum*, and *F. columnare*) (Overturf et al., 2010; Overturf et al., 2003; unpublished data). In what follows is an analysis of intestinal transcriptomic response and gut microbial ecology during early and late stages of viral (IHNV) or bacterial (*Fp*) disease challenges, across two genetically and phenotypically distinct strains of rainbow trout.

Methods and Materials

Study Design and Disease Challenges

Eggs were collected from female ARS-UI broodstock and were fertilized with milt from ARS-UI neomales to generate all female cohorts. Four ARS-UI trout families were spawned and combined. All female, freshly fertilized eggs were also obtained from a commercial supplier on the same day. Eggs were reared in the same environment following standard procedures at the Hagerman Fish Culture Experiment Station (Hagerman, ID, USA). At 25 days post hatch (dph), 500 trout fry from both the common commercial strain (Commercial), as well the ARS-UI selected strain (Select) were brought to the Aquatic Animal Laboratory (AAL) at the University of Idaho (Moscow, ID, USA) for disease challenge. Each strain was held in a single 160 L tank supplied with dechlorinated municipal water operating on partial recirculation and feed to satiation daily, six days a week. Fish were acclimated to the system for two months until reaching an average size of approximately four grams (3.93 g ± 0.90; mean ± SD) when fish from both strains were subjected to a disease challenge.

Disease challenges were conducted using a factorial design with the two fish strains challenged by either a viral (Infectious Hematopoietic Necrosis Virus) or bacterial (*Flavobacterium psychrophilum*) salmonid pathogen (three challenge tanks and one mock group^{-1}) (Figure 4.1). For the viral challenge, virulent IHNV (isolate 220-90) was harvested from epithelioma papulosum cyprinid (EPC) cells following standard methods (LaPatra et al., 1989 and 1994). The virus was delivered by 1 h static bath immersion at a final concentration of 0.5 x 10^5 plaque forming units (PFUs) mL^{-1}, while viral mock challenged groups were treated with a 1 h static bath containing an equal concentration of sterile MEM

media. For the bacterial challenge, a virulent strain of *F. psychrophilum* (isolate 259-93) (Crump et al., 2001) was cultured for 72 h in tryptone-yeast extract salt broth (TYES), harvested, and diluted to the desired concentration in TYES according to spectrophotometric optical density (525 nm). The *Fp* inoculum was further quantified by triplicate 6 x 6 drop plate assay (Chen et al. 2003) to determine colony forming units (CFU) mL^{-1}. Bacteria challenged fish were injected intramuscularly (IM) with 50 µL of TYES (0.154 OD; 525 nm), which corresponded to 6.27 x 10^6 colony forming units (CFUs) mL^{-1} of *Fp* (3.14 x 10^5 CFU $fish^{-1}$). Mock challenged *Fp* groups were injected with an equivalent volume of sterile TYES sham. All fish were sedated with 200 mg L^{-1} buffered tricaine methanesulfonate (MS-222) during pathogen delivery.

In both challenges, fish were stocked at a density of 45 fish $tank^{-1}$ (Figure 4.1) in a flow through aquaculture system supplied with dechlorinated water. Water temperatures were recorded daily and maintained near 15°C (IHNV challenge - 14.5°C ± 0.463; *Fp* – 14.5°C ± 0.502). Fish in both challenges were observed regularly, with mortalities recorded and feed offered daily (1% body-weight day^{-1}) with a commercial fishmeal-based trout fry feed (47% protein and 18% fat). The IHNV and *Fp* disease challenges were concluded at 20 and 21 days post challenge (dpc), respectively (Figure 4.1). Tank served as the experimental unit for disease challenges and total mortality, cumulative percent mortality (CPM), and Kaplan-Meier survival estimates were calculated. Paired t-tests were used to test total mortalities and CPM for differences by trout strain, while Kaplan-Meier survival estimates were tested log-rank test ($p \leq 0.05$).

At early (4- and 5-dpc) and late (20 and 21 dpc) timepoints in the progression of IHNV and *Fp*, five fish $tank^{-1}$ were sampled from each group (Figure 4.1). Fish sampled during the early sampling timepoints were excluded from CPM calculations, leaving 40 $tank^{-1}$ for survival analyses. Fish were randomly selected from each tank at the early timepoint and effort was taken to not disturb remaining unsampled fish. Sampled fish were euthanized by overdose with MS-222 and whole blood was immediately collected from the caudal vein by capillary action following tail ablation. Fish were briefly rinsed in 70% ethanol to reduce contamination. The intestine was then excised, and distal gut contents were collected for microbiota analysis and distal intestinal tissue was preserved in Qiazol (Qiagen; Hilden, Germany) for RNA sequencing (RNAseq) (Figure 4.1). At the time of sampling, water

microbiota samples were also taken by filtering (0.2 µm)1 L of water sample from the challenge systems inflowing water supply, in triplicate. Microbiota and RNAseq samples were flash frozen and stored at -80°C.

Immune Assays

Whole blood collected at the time of sampling was held at 4°C for 12 h before centrifugation at 15,000 G for 5 min to isolate serum. Sera was pooled by tank and stored at -80 °C. Complement-dependent IHNV plaque neutralization titers in sera of viral challenged groups was determined by the UI-AAL following the procedures detailed by LaPatra et al. (1993). Circulating lysozyme activity in pooled "early" and "late" sera samples from all groups was measured using the EnzChek Lysozyme Assay Kit (ThermoFisher; Waltham, MA, US).

Alternative complement was assayed using methods adapted from previously described protocols (Welker et al., 2007; Sunyer and Tort 1995). Briefly, competent rabbit red blood cells (RRBC) were washed (300 x g at 4°C for 5 min) 5x in PBS+ (PBS, 0.1% gelatin, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.2). Cells were then eluted in PBS+ to a final concentration of 5 x 10^7 cells mL^{-1} based on quantification by flow cytometry (Attune NxT; ThermoFisher). Serial dilutions of experimental sera samples (1/5 – 1/625) were mixed with RRBC in round bottom plates, alongside positive (100% lysis) and negative controls (0% lysis) and were incubated on a slow rocker platform for 1 h at room temperature. Hemolytic activity was stopped by chilling samples on ice for 5 min prior to centrifugation at 800 x g for 10 min at 4°C to pellet unlysed cells. Hemolysis in the supernatant was measured by optical density at 410 nm (OD 410) using a plate reader (M200 Pro; Tecan, Switzerland). Any hemolysis of host blood cells that may have occurred during sera collection was corrected by subtracting background OD 410 from untreated experimental serum dilutions. Hemolytic activity was determined by linear regression to determine alternative complement hemolytic 50 activity (ACH50), corresponding to the serum dilution required to yield 50% lysis of RRBC. Due to a shortage of sera at the early sampling timepoint, ACH50 was only assayed at the "late" timepoint. All immune assays were tested for differences by fish strain using a paired t-test.

Intestinal RNA Isolation, Sequencing, and Analysis

After thawing, intestinal tissue samples were homogenized by TissueLyser using a sterile 5 mm steel bead. Isolation of RNA was conducting using a RNeasy 96 kit (Qiagen) with on-column DNase I treatment. Spectrophotometry (NanoDrop 2000; ThermoFisher) and fluorometry (Quant-iT RiboGreen; ThermoFisher) were used to determine RNA quality and quantity. RNA integrity was assessed and RNA integrity numbers (RIN) were calculated using an RNA 6000 Nano kit (Agilent; Santa Clara, CA). All samples were of good quality (260 nm/280 nm ≤ 1.8; 260 nm/230 nm – 2.0; and RIN ≤ 8.0). From the five samples $tank^{-1}$ originally collected, the best samples from each experimental group were used to produce two pools of RNA $tank^{-1}$ (two fish $pool^{-1}$) for RNAseq (Figure 4.1). This resulted in two pooled samples for each sham group and six pooled samples for each infected experimental group. Using these pooled samples, poly(A) tailed mRNA was selected using the NEBNext Poly(A) Magnetic mRNA Isolation module (NEB; Ipswich, MA) and 800 ng of input total RNA. The Ultra II Directional RNA Library Prep Kit (NEB) was then used to create unique dual-indexed stranded RNA sequencing libraries. Final cleaned libraries were checked for size distribution and absence of adapter-dimer using a DNA 1000 kit (Agilent). Libraries were pooled at equimolar concentrations using a Kapa Library Quantification Kit (Kapa Biosystems). Sequencing was performed on a NextSeq 500 (Illumina; San Diego, CA, US) at the Hagerman Fish Culture Experiment Station using a 150-cycle v2.5 high-output kits (Illumina) to generate paired-end (PE) 75 bp reads. All samples were sequenced in duplicate as part of a larger pool of samples to avoid introducing batch effects across sequencing lanes.

Transcript quantification was achieved by selective alignment with GC bias correction using Salmon v.1.2.1 (Patro et al., 2017). Reads were mapped to a decoy-aware reference transcriptome index constructed using all transcripts in the NCBI Omyk_1.0 genome repository. The transcriptome index was made decoy-aware against the Omyk_1.0 genome to reduce the occurrence of spurious mapping of reads. Gene level analyses were achieved using GTF transcript to gene annotations. Raw RNAseq reads and transcript counts are publicly available on the NCBI GEO repository under the following accession numbers: GSE156595 - IHNV challenge and GSE156930 - *Fp* challenge.

Differential transcript usage (DTU) analysis was performed using DRIMSeq (Nowicka and Robinson et al., 2016). Prior to DTU analysis, datasets from the two disease

challenges were separately filtered to retain only transcripts detected across all samples with a minimum expression of ten or greater (Love et al., 2018). Analysis modeled the independent effects of trout strain (Commercial vs. Select), disease status (Sham vs. Infected), and disease stage (Early vs. Late) in each disease challenge. Statistical significance was assessed by stageR (cite) using a two-stage statistical testing framework to detected DTU with an overall false discovery rate corrected threshold of $\alpha \leq 0.05$.

Transcript counts were summarized at the gene level, normalized by library size, and then transformed by variance stabilizing transformation (VST). Sample wise Euclidean distances were calculated from the VST gene level datasets for multivariate analysis of each disease challenge using permutational multivariate analysis of variance (PERMANOVA) using three-way full crossed models including trout strain, disease status, and disease stage. Principle components analysis was also used to visualize the multivariate relationship between samples. Differential gene expression (DGE) analysis was conducted separately by disease challenge at the gene level using DESeq2 (Love et al. 2014). For DGE analysis, the experimental factors trout strain (Commercial vs. Select), disease status (Sham vs. Infected), and disease stage (Early vs. Late) were combined to generate unique experimental groups (i.e. Commercial_Sham_Early, etc.) and pairwise contrasts were used to test meaningful group comparisons. Log fold change shrinkage was achieved using ashr (Stephens 2017). The significance threshold for differentially expressed genes (DEG) required a false discovery rate (FDR) corrected $\alpha \leq 0.05$ and shrunken log2 fold-change $\geq | 1.5 |$.

Gene ontology (GO) enrichment analysis was used to determine functional roles of transcripts involved in DTU or DGE. For DTU, transcripts identified as differential were used for enrichment analysis separately by experimental factor and disease challenge. As for the DEG, pairwise contrasts of interests were combined to yield three datasets for GO enrichment analysis: 1) select strain – sham vs. infected – early and late stages 2) select strain vs. commercial strain – infected – early and late stages 3) select strain – infected – early vs. late stage. Deduplicated lists of transcripts representing the differential genes were used for GO enrichment analysis. All GO enrichment analysis was conducted using a Fisher's Exact test (FDR corrected $\alpha \leq 0.05$) with DTU or DGE transcript lists serving as the test sets and the remaining annotated trout transcriptome as the reference set. Significantly enriched GO

terms were summarized at the levels of biological process (BP), molecular function (MF), and cellular component (CC).

Gut Microbiota Sequencing and Analysis

Microbial DNA was isolated from gut content samples using a DNeasy PowerSoil Pro 96 kit automated on the Qiacube HT (Qiagen) after homogenization on a TissueLyzer. Due to salt carryover among some samples, a Genomic DNA Clean and Concentrate 96 kit (Zymo Research; Irvine, CA) was used to clean all samples. Fluorometry (Quant-iT PicoGreen; ThermoFisher) was used to quantify final DNA, with 10 ng μL^{-1} used as input to generate custom two-step PCR dual-barcoded V3V4 16S rRNA gene sequencing libraries. Final libraries were quantified by fluorometry and pooled equimolarly. Size distribution was confirmed using a DNA 1000 kit (Agilent) before final quantification using Kapa Library Quantification Kit (Kapa Biosystems; Wilmington, MA). Libraries were sequenced on a MiSeq (Illumina) using a 600-cycle V3 kit (Illumina; San Diego, CA) at the Hagerman Fish Culture Experiment Station.

Raw 16S rRNA gene reads are publicly available on the NCBI repository (https://www.ncbi.nlm.nih.gov) under BioProject Accession PRJNA659058. As with the RNAseq data, microbiota data were analyzed separately by disease challenge. Raw sequencing reads were demultiplexed and primers were removed with dbcAmplicons (https://github.com/msettles/dbcAmplicons). DADA2 (Callahan et al. 2016) was used to trim (Forward – 275 bp, Reverse – 215 bp) and error filter (2 expected errors) sequences prior to denoising reads into chimera checked amplicon sequence variants (ASV). Taxonomy was applied to ASV using the Silva v138 database (Quast et al., 2012). Phylogenetic trees were constructed from microbiota ASV using a GTR model (Wright, 2016; Schliep, 2011) to enable calculation of phylogenetically informed distance metrics (UniFrac). The packages phyloseq (McMurdie and Holmes, 2013) and vegan (Oksanen et al. 2013) were used to filter and analyze microbiota data. All ASV assigned to the order Chloroplast or the family Mitochondria as well as singletons were removed.

Within sample diversity (alpha diversity) was assessed by observed ASV (obsASV) richness and Shannon Diversity. Alpha diversity of the water microbiota and gut microbiota were compared by Mann-Whitney U test. A three-way fully crossed ANOVA was used to test alpha diversity by trout strain, disease status, and disease stage. Between sample

diversity (beta diversity) in the form of weighted (wUniFrac) and unweighted (uwUniFrac) sample distances was tested by PERMANOVA using the same fully crossed three-way model described above. Differential abundance (DA) analysis was conducted at the bacterial genus level using DESeq2 with *poscount* size factor estimation under the same design scheme detailed above for RNAseq DGE analysis.

Results

Disease Challenge

In both challenges, mortalities exhibited classical signs of disease. The IHNV challenged mortalities displayed exophthalmia, hemorrhaging, scoliosis, and pale gills and skin. The *Fp* challenged mortalities showed pale gills, skin lesions, as well as muscle and fin erosion. No mortalities in either trout strain were observed in IHNV or *Fp* mock challenged tanks. The IHNV challenge produced moderate mortality, with similar mortality between the commercial (52.9 ± 7.4 %; mean CPM ± sd) and select (51.1 ± 3.9 %) strain (Figure 4.2 A). No significant difference in total mortality ($p = 0.732$), CPM ($p = 0.678$), or Kaplan-Meier survival estimates ($p = 0.48$) were detected by trout strain in the IHNV challenge. Conversely, mortality was higher in the *Fp* challenge in both strains, with significant differences between the commercial (94.8 ± 5.6 %) and select (70.4 ± 4.6 %) strain in total mortality ($p = 0.006$), CPM ($p = 0.004$), and Kaplan-Meier estimates ($p \leq 0.0001$) (Figure 4.2 B).

Immune Assays

IHN neutralization titers showed no significant difference ($p = 0.725$) by trout strain and infected fish showed much higher levels than controls (Table 4.1). The pooled sera from the mock challenged select strain group showed minor background IHN neutralizations titers but was lower than infected tanks, while the commercial mock challenged group showed no background (Table 4.1). In the IHNV challenge, serum lysozyme activity was significantly higher in the select strain at both early (4 dpc; $p = 0.048$) and late (20 dpc; $p = 0.0052$) sampling timepoints. However, at the conclusion of the study the commercial strain showed elevated ($p = 0.03$) alternative complement activity according to ACH50 assays (Figure 4.3). In the *Fp* challenge, the select strain showed higher lysozyme activity at early ($p = 0.035$) and late timepoints, as well as ACH50 activity at the late timepoint. Albeit, the high mortality in the commercial strain in the *Fp challenge* left only one tank replicate, therefore, statistical

comparisons at the late timepoint were not possible (Figure 4.3). At the early timepoint the IHNV challenge induced a higher level of lysozyme activity compared to that observed in the *Fp* challenge. At the late timepoint the pattern was reversed with higher lysozyme activity observed in the *Fp* challenged fish, though as mentioned above statistical tests were not possible due to the reduced sample size in the commercial strain at that timepoint. Alternative complement activity was upregulated in the *Fp* challenge compared to the IHNV trial (Figure 4.3).

Intestinal RNAseq

From the 32 pooled samples from the IHNV trial, 678.4 million raw paired end reads (10.6 M ± 1.17, mean ± sd) were generated and 502.3 million reads were mapped (74.03 ± 1.35 %) to the rainbow trout transcriptome index. Reads from the IHNV challenge were mapped to 44,849 genes in total. Due to high mortality only one tank of the commercial strain trout was available for sampling at the late timepoint, and therefore only 28 samples were sequenced. From these samples 617.4 million raw reads (11.03 M ± 3.04) were generated that yielded 454.1 million mapped reads (73.64 ± 1.56%). Reads from the *Fp* dataset were mapped to 44,778 genes in total.

After filtering, transcripts from 6,005 genes were tested for DTU in the IHNV trial. In total, 943 transcripts corresponding to 544 genes were found to be involved in DTU according to differences by trout strain (53.7%), stage of disease (39.3%), and disease status (7.0%) (Figure 4.4 A-B). Gene ontology enrichment analysis was used to interpret the functional role of transcripts involved in DTU by experimental factor, and after reducing the results to only the most specific enriched terms, 21 GO terms were enriched from the DTU based on trout strain, while 63 and 37 GO terms were enriched from the transcripts involved in DTU by IHNV stage and IHNV status, respectively (Figure 4.5 A, C and E; Table 4.2). Transcripts from 5,850 genes passed the filtering threshold in the *Fp* challenge dataset, with 1,034 transcripts representing 610 genes found to be differential transcribed by strain (56%), disease stage (34%), and disease status (9.3%) (Figure 4.4 C-D). After reducing GO terms to only the most informative terms, the transcripts involved in DTU in the *Fp* challenge showed 75, 58, and 9 significantly enriched GO terms by trout strain, stage of disease, and disease status, respectively (Figure 4.5 B, D, and F; Table 4.3).

Normalized and transformed (VST) expression profiles showed significant multivariate differences across the experimental factors in both the IHNV (Table 4.4) and *Fp* (Table 4.5) challenges and PCA biplots showed separation in sample pools according to experimental factors (Figure 4.7). In the IHNV challenge, main effects of strain ($p = 0.005$), disease status ($p = 0.001$), and stage of disease ($p = 0.001$) had significant influences on expression profile (Figure 4.7 A), while disease status and stage also showed significant interacting effects ($p = 0.004$) (Table 4.4). The *Fp* challenge showed similar trends, with strain ($p = 0.002$), disease status ($p = 0.001$), and stage of infection ($p = 0.001$) having significant influences on multivariate gene expression profiles (Figure 4.7 B). Although, not only did disease status and stage show significant interactions ($p = 0.003$) but strain effects were also found to change significantly across stages of the disease ($p = 0.025$). No other significant interactions were detected (Table 4.5).

Pairwise DGE analysis between experimental groups in the IHNV challenge showed the greatest difference in gene expression between sham and IHNV infected groups at the early timepoint, in both the commercial (790 DEG) and select trout (621 DEG) (Figure 4.8A). Comparisons between IHNV infected individuals at the early and late stage of infection also showed a high number of DEG in the commercial (493) and select strain (355), with many of those genes overlapping with early sham vs. infected DEG (233 shared DEG) (Figure 4.8 A). In total, 137 DEG were detected between the commercial and select strain trout infected with IHNV through the early (30.7%) and late (69.3%) stages of infection. At the late stage of infection, comparisons between the sham and infected fish showed few DEG, with only four and eight DEG in the commercial and select strain, respectively (Figure 4.8 A). Combined DEG datasets involving the select rainbow trout strain were utilized to evaluate function of DEG in the IHNV challenge. Gene ontology enrichment analysis of the 629 DEG between the sham and IHNV infected select strain fish (early and late) detected 55 significantly enriched GO terms after reducing to only the most specific GO terms (Table 4.6; Figure 4.8A). Differentially expressed genes between the early and late stages of IHNV infection in the select trout (355 DEG) showed enrichment for 34 GO terms (Table 4.6; Figure 4.8 B).

In the *Fp* challenge, by far the most DEG were detected between the early and late timepoints in *Fp* infected select trout (1,403 DEG), with 673 DEG that were not differentially

expressed in any other comparison (Figure 4.8 B). Comparisons between the sham and infected select trout at the early timepoint in the *Fp* challenge also showed considerable DGE (896 DEG). It should be noted that the commercial strain at the late stage of *Fp* infection had a reduced sample size due to high mortality, which may influence some comparisons to that group. Irrelevant, at the late stage, sham vs. infected comparison in the commercial strain detected 604 DEG (Figure 4.8 B). When comparing *Fp* infected fish by strain, 549 DEG were detected across the early (48.5%) and late (51.5%) timepoints. The least DEG (60 DEG) were detected between the sham and infected select trout at the late stage of infection (Figure 4.8 B). Enrichment analysis of GO terms showed that the 956 DEG between the sham and *Fp* infected fish from the select strain were enriched for 53 specific GO terms(Table 4.7; Figure 4.9 A).With the most DEG (1,403), comparisons between the early and late stage of infection in select strain fish showed 44 significantly enriched GO terms (Table 4.7; Figure 4.9 B), after reducing to only the most specific terms. Comparisons of the DEG between infected commercial and select trout throughout the *Fp* challenge detected 53 enriched GO terms (Table 4.7; Figure 4.9 C).

Gut Microbiota

From the 86 microbiota samples (6 water samples; 80 gut samples, n = 5 $tank^{-1}$) in the IHNV challenge, 2.6 million raw reads (30,809 ± 6,745 reads $sample^{-1}$; mean ± sd) were assigned to ASV and passed filtering constraints (no Mitochondria, Chloroplast, or singletons). From the *Fp* challenge samples, two samples (1 Commercial Sham Early and 1 Commercial Infected Early) were poorly sequenced with few reads passing filtering and were therefore removed from the dataset. This resulted in a total of 66 trout gut samples in the *Fp* microbiota dataset. When including the six water samples, 2.46 million reads (34,115 reads $sample^{-1}$ ± 19,752) were assigned to ASV and passed filtering parameters.

The inflowing water microbiota during the disease challenges had a richness of 202 ± 5.6 obsASV at the early stage sampling but was significantly reduced (paired t-test, p = 0.017) 133 ± 18.5 at the late stage sampling. In the IHNV challenge, there were no difference in the number of obsASV between the water and gut microbiota samples (Mann-Whitney U, p = 0.063), although the water microbiota did show significantly reduced Shannon diversity (p = 0.007), compared to the gut samples. In the *Fp* challenge, no differences between water and gut alpha diversity were detected according to either obsASV (p = 0.068) or Shannon

Diversity ($p = 0.927$). At the phylum level, the water microbiota was dominated by Proteobacteria (73.8% ± 0.13; mean ± sd) and to a lesser extent Bacteroidota (9.8% ± 0.87), while Actinobacteria, Bdellovibrionota, Patescibacteria and Cyanobacteria all accounted for roughly 3% of the water microbiota profile. At the genus level the most dominant genera was *Thiobacillus* (64.6% ± 1.9), with all ASV belonging to the species *sajanensis*. *Flavobacterium* (7.7% ± 6.9), *Mycobacterium* (3.7% ± 3.9), *OM27_clade* (2.8% ± 3.1), and *Crenothrix* (1.6% ± 1.2) rounded out the remaining top five most abundant bacterial genera detected in the water supply.

In the IHNV trial, alpha diversity richness (obsASV) was significantly influenced ($p < 0.05$) by disease stage, although significant disease status by stage interactions were also detected (Figure 4.10 A). Similarly, Shannon diversity showed significant effects of strain, yet strain by disease status interactions were also significant during the IHNV challenge (Table 4.4). In the *Fp* trial, disease status and stage had highly significant main effects ($p \leq 0.001$) on richness (obsASV) and diversity (Shannon), although main effects were found to have significant interactions in both alpha diversity metrics (Table 4.5; Figure 4.10 B). In both challenges, the infection tended to reduce both bacterial richness and diversity, though by the late-stage infected fish more closely resembled sham controls (Figure 4.10 A-B).

Multivariate analysis by means of PERMANOVA was used to determine the effect of experimental factors on the gut microbiota phylogenetic composition. In the virus challenge, infection status and disease stage showed significant interaction in abundance weighted microbial composition (wUniFrac), though abundance unweighted bacterial composition (uwUniFrac) showed significant main effects of IHNV infection, disease stage, and strain by stage and disease status by stage interaction effects (Table 4.4, Figure 4.10 C). Much greater shifts in gut microbiota were detected in the *Fp* challenge, with only trout strain and strain by disease by stage interactions not found to significantly influence the quantitative gut bacterial profile (wUniFrac, Table 4.5). When ignoring the abundance of gut bacteria and focusing on presence-absence (uwUniFrac) (Figure 4.10 D), trout strain, *Fp* infection, and disease stage all showed significant main effects; although, strain by disease and disease by stage interactions were also detected (Table 4.5).

The most abundant bacterial phyla in mock-challenged (Sham) gut samples were Firmicutes and Proteobacteria in both the IHNV (51.7% ± 32.7and 37.3% ± 36.0,

respectively) and *Fp* (64.6% ± 32.5 and 27.3% ± 34.9, respectively) challenge. In the IHNV challenge, the early infected fish gut microbiota was still dominated by Firmicutes (59.2% ± 28.8) and Proteobacteria (21.3% ± 21.0) and the same trend was observed in the late IHNV infected fish (59.7% ± 28.5 and 24.8% ± 29.7 respectively). Conversely, in the *Fp* challenge, the early infected gut microbiota was dominated by Bacteroidota (i.e. Bacteroidetes) (67.5% ± 37.4), most of which was *F. psychrophilum* (63.1% ± 35.2), as well as Proteobacteria (25.7 % ± 37.5). However, by the late stage of infection, at the phylum level the *Fp* infected gut microbiota more closely resembled that of the mock-controls (Firmicutes – 51.7% ± 27.9; Proteobacteria – 40.4% ± 29.4).

Differential abundance (DA) testing at the genus level, identified genera with DA between comparisons of experimental groups (Figure 4.11 A-B). In the IHNV challenge, 54 genera showed DA between select trout infected with IHNV and select trout mock infected (Sham) at early (27 DA) and late (27 DA) stages of the infection (Figure 4.11A and Figure 4.12 A). When comparing select IHNV infected trout to commercial IHNV infected select trout at both early (20 DA) and late (6 DA) stages, 26 genera showed DA (Figure 4.11A and Figure 4.12 B). Comparing the select trout in the early stage of infection to the select trout in the late stage of infection identified 27 DA genera (Figure 4.11A and Figure 4.12 C). In the *Fp* challenge, 58 genera were DA between select trout infected with *Fp* and select trout mock infected at early (56 DA) and late (2 DA) stages of the infection (Figure 4.11B and Figure 4.13 A). When comparing select *Fp* infected trout to commercial *Fp* infected select trout at both early (39 DA) and late (17 DA) stages, 56 genera showed DA (Figure 4.11A and Figure 4.13 B). Comparing the select trout in the early stage of infection to the select trout in the late stage of infection identified 88 DA genera (Figure 4.11A and Figure 4.13 C).

Discussion

Differences in Response to Viral and Bacterial Disease by Trout Strain

Here the superior disease resistance of the ARS-UI select strain of trout was shown in the bacterial *Fp* challenge, but not in the viral IHNV challenge (Figure 4.2). A slightly reduced IHNV viral challenge dose (0.5×10^5 PFU mL^{-1}) was chosen in hopes of enabling sampling of survivors at the late stage of infection; however, a higher challenge dose may have produced higher overall mortality and showed more separation in IHNV susceptibility between the two trout strains. In the *Fp* challenge, mortality rates were much higher than in

the IHNV trial, and clear separation in disease susceptibility between the two trout strains became apparent around 8 dpc when the commercial strain began experiencing significantly more daily mortalities than the selected strain (Figure 4.2).

Another line of selectively breed trout has been generated at the National Center for Cool and Cold Water Aquaculture (NCCCWA) through multiple generations of family-based selection specifically for innate resistance to *Flavobacterium psychrophilum* (ARS-Fp-R). During both experimental challenges and natural outbreaks of CWD the ARS-Fp-R line has been found to be significantly less susceptible to mortality than a control line (ARS-Fp-C), susceptible line (ARS-Fp-S), or hatchery line of rainbow trout, with the ARS-Fp-R line presenting only 2.8% mortality after being challenged intraperitoneally with 2.1 x 10^7 CFU of *F. psychrophilum* 259-93, compared to 57.6% mortality in a control line of trout (Wiens et al., 2018). Of interest, the ARS-UI select strain utilized in this study was strictly selected over multiple generations for growth performance on plant-based diets and no selection for innate immunity was involved. Despite this, the ARS-UI select trout were observed to display greater innate resistance to *Fp* challenge than a commercial strain. The ARS-UI select strain did show just over 70% mortality when challenged intramuscularly with 3.14 x 10^5 CFU with *F. psychrophilum* 259-93, which suggests the ARS-Fp-R strain's resistance to *Fp* may be superior to that of the ARS-UI strain, though side-by-side comparisons would be needed to confirm this hypothesis. However, the ARS-UI strain has the advantage of rapid growth on sustainable all plant diets. Regardless, the performance of both select strains highlights the benefits of selective breeding in aquaculture to generate desirable immune phenotypes and improve production efficiencies.

Serving as a key defense against bacterial septicemia, serum lysozyme is an antimicrobial enzyme found in circulation that cleaves peptidoglycan polymers that form bacterial cell walls and can render pathogens incompetent through lysis. Lysozyme is typically thought to be most effective against Gram negative bacterial pathogens because the peptidoglycan residues are most assessable to lysozyme hydrolysis in these microbes. As a Gram-negative pathogen *Fp* should be susceptible to lysozyme activity, although it has been shown that *Fp* is less susceptible than other bacterial pathogens such as *Aeromonas salmonidica* (Brown et al. 1997) and has been shown to resist lysozyme up to concentration of 2 mg mL^{-1} (Starliper et al., 2012). In the present study, lysozyme activity was significantly

higher in the ARS-UI select trout in both the viral and bacterial disease challenge. Interestingly, lysozyme activity was elevated at the early stage of the viral infection, although slightly higher lysozyme activity levels were detected at the late stage of the *Fp* challenge (Figure 4.3 A and C). While lysozyme is not likely to have any protective effects against viral infections, its activity was seemingly stimulated by the upstream immune signaling in response to detection of viral infection. The higher serum lysozyme activity of the select strain during the early stage of the *Fp* challenge could at least partially explain the strains reduced mortality in the *Fp* challenge.

The alternative complement system involves a powerful nonspecific humoral response that protects fish from a wide range of pathogens including viruses and bacteria with the ability to opsonize and lyse invading pathogens (Sunyer and Tort 1995). In fact, complement is thought to be required for salmonids to neutralize rhabdoviruses, such as IHNV, and while it is like that the classical complement pathway is involved, the exact mechanism of complement dependent antibody neutralization are not fully delineated in teleost (Ellis 2001). In the present study, the commercial strain showed significantly higher alternative complement activation, as measured by ACH50, than the select strain, although there were no differences in cumulative mortalities or complement dependent IHNV neutralization titers between the two trout strains. This suggests the elevated ACH50 level of the commercial strain was not enough to offer any advantage in viral protection. Unfortunately, ACH50 activity could only be assayed in this study at the late stages of disease, after the acute infections had already subsided, so it would have been interesting to compare ACH50 activity between the two strain at an early stage of the challenges. In the *Fp* challenge, the select strain appeared to have elevated serum alternative complement activity compared to the commercial strain trout, although statistical comparisons were not possible due to high mortality of the commercial strain only leaving one replicate tank for serum assays.

Determining the physiological mechanisms behind the ARS-UI select strain's innate resistance to disease was among the primary motivation behind characterizing the gut transcriptional and microbiota response to IHNV and *Fp* in this study. Previous studies have shown the select strain to be resistant to intestinal inflammation (Venold et al., 2012) and there is also evidence the gut microbiota of the select strain is different from that of control

strains of trout (Blaufuss et al., 2020; unpublished data). Therefore, it was hypothesized that differences in gut health, in terms of both host transcriptional response and gut microbial ecology, may impact the select strains innate disease resistance.

Differential transcript usage analysis, which has been rarely, if ever, conducted in aquaculture species, showed a significant number of genes that were differentially transcribed according to strain genetics. In both disease challenges, more DTG were detected according to trout strain than any other axes of variation in the study design, with 292 and 331 DTG across trout strain in the IHNV and *Fp* challenges, respectively (Figure 4.4). GO enrichment analysis using the list of isoforms involved in strain-wise DTU in the IHNV challenge showed an enrichment in genes involved in nutritional pathways unrelated to the disease challenge, such as alpha-linolenic and linoleic fatty acid metabolism, fatty-acyl-CoA binding, and linoleoyl-CoA desaturase activity. Although, pathways which may represent adaptive responses to viral pathogens such as nucleotide-excision repair, DNA damage recognition, macrophage migration inhibition factor binding, 15-hydroxyprostoglandin dehydrogenase activity were also highlighted by GO enrichment analysis of DTU isoforms. Other adaptive strain-wise DTU enriched GO terms included Jun amino-terminal kinase (JNK) signaling (Table 4.3; Figure 4.5 A), which controls apoptosis, inflammation, cytokine production, and cellular metabolism (Weston and Davis 2007), as well as DBIRD complex, which controls transcript elongation and alternative splicing for a wide range of exons, particularly in A-T rich regions of the genome (Close et al., 2012).

Similarly, in the *Fp* challenge, strain-wise DTU showed GO enrichment for functions seemingly unrelated to the disease challenge such as centrosome localization; however, DTU also showed enrichment in GO terms like antigen processing and presentation, IL-1β receptor binding, and negative regulation of cell population proliferation, which may provide adaptive differences in response to disease between the two strains. Interestingly, in both disease challenges isoforms involved in DTU between the two trout strains identified differences in very related terms such as response to oxidative stress, response to reactive oxygen species, oxidation-reduction process, and cellular oxidant detoxification (Figure 4.5 A-B) or linoleic and alpha-linolenic fatty acid metabolic processes (Table 4.2 and 4.3). This suggest there are differences in isoform usage in free radical detoxification and fatty acid metabolism pathways between our select line of trout and the commercial strain, though

further analysis would be needed to determine if this correlates to meaningful alterations at the phenotypic level.

Multivariate intestinal gene level analysis showed highly significant differences between trout strains in both disease challenge ($p \leq 0.005$) (Table 4.4 and Table 4.5), although strain separation was much more apparent in the *Fp* challenge (Figure 4.6 B). However, in terms of DGE, comparisons between the two strains produced among the least DEG in both disease challenges (Figure 4.7 A-B). In addition, GO enrichment analysis of DEG between the commercial and select strain yielded no enriched GO terms in the IHNV challenge. Although, in the *Fp* challenge, DEG between the two strains were enriched for GO terms involved in oxygen transport, hydrogen peroxide biosynthesis, carbohydrate binding, defense response to pathogens, cellular oxidant detoxification, acute phase response, and immune response-regulating cell surface receptor signaling pathway (Table 4.7; Figure 4.9 C). The reoccurrence of differences in cellular oxidant detoxification pathways at the gene level, as well as the previously discussed DTU, suggests oxidative stress metabolism at least in the intestine is significantly different in the ARS-UI select strain compared to that of the commercial group. Marancik et al. (2015) compared whole-body transcriptome-wide gene expression responses to *Fp* infections in the ARS-Fp-R, ARS-Fp-S (Susceptible), and ARS-Fp-C (Control) lines of trout and after controlling for other experimental factors, only identified 21 genes to be differentially expressed between the three trout strains. Alternatively, in the present study 137 and 549 DEG were detected by trout strain in the IHNV and *Fp* challenges, respectively.

Comparisons of the gut microbiota results in this study showed less separation between the two trout strains then did transcriptome data. The only effect of trout strain on gut bacterial alpha diversity, or within richness or diversity, was on Shannon diversity in the IHNV challenge. The commercial trout were found to have higher diversity, primarily in the mock-challenged fish, explaining the significant strain by disease status effects on IHNV gut microbiota diversity (Table 4.4). Furthermore, the only multivariate differences in gut microbiota profile according to host strain was in the *Fp* challenge according to uwUniFrac, but strain by disease status interactions were also apparent (Table 4.5; Figure 4.10 D). Although comparing the separation in trout strains in the *Fp* challenge based on either microbiota profile (Figure 4.10 D) or intestinal gene expression profile (Figure 4.6B), it is

likely that differences in host gene expression profiles explain more of the differences in disease resistance between the two trout strains.

Differential abundance analysis comparing the gut microbiota between the select and commercial strain, identified the fewest DA of any pairwise experimental group comparisons in the IHNV challenge (Figure 4.11 A), however, greater strain-wise DA was detected in the *Fp* challenge. In both challenges a similar number of bacterial genera were found to be significantly higher in abundance in both the commercial and the select strain (Figure 4.12 B; Figure 4.13 B). Interestingly, in the IHNV challenge microbes from the genus *Shewanella*, a genus commonly detected in the gut microbiome of teleost, were found to be significantly more abundant in the select strain trout at both early and late stages of the trail; however, in the *Fp* trial this same genus of bacteria were enriched in the commercial trout at the early stage of infection (Figure 4.12 B; Figure 4.13 B). A similar but inverse pattern was observed for *Brevibacterium* as well. Interestingly, most the DA showing enrichment in the select strain during the *Fp* challenge occurred during the late stage of infection. This is likely due to the select trout having nearly fully recovered and returned to the microbiota composition of mock-challenged individuals by the late samples, although the commercial trout had not recovered (Figure 4.10 D).

To summarize the differences in trout strain detected in the present study. Our ARS-UI select strain showed superior resistance to experimental *Fp* infection, and elevated serum lysozyme activity. DTU analysis highlighted potential differences in fatty acid metabolism in the select strain, and both DTU and DGE analysis detected differences in pathways related to oxidative stress in the two strains of trout. In addition, adaptive differences in the select strain of trout included differential isoform usage and gene expression related to an array of innate and adaptive immune functions. Analysis of gut microbiota showed less differences between trout strains than host gene expression, though some key differences were detected, and many differentially abundant bacterial genera were detected between the two strains.

Impacts of IHNV on Intestinal Transcription and Microbiota

Drolet et al. (1994) proposed that IHNV infections were initiated by two major routes; either through entry to the circulatory system via the gills or by an oral-intestinal entry that also leads to systemic viremia. Others have confirmed the importance of the oral-intestinal route of entry for IHNV, and the virus has been shown capable of transiently

infecting epithelial intestinal cells before spreading to the heart and central circulation through the highly vascularized connective tissue surrounding the gastrointestinal tract (Bootland and Leong 2011). This suggests IHNV infections could have both direct and indirect effects on intestinal transcriptomic responses. Directly by transiently infecting intestinal cells, or indirectly through alterations to global immune regulation, as would occur during a systemic viral infection.

Results from the present study showed rather classical transcriptional responses to viral infection during the IHNV challenge with evidence for both direct and indirect viral immune stimulation. Enrichment analysis of DTU showed significant effects of IHNV infections on genes involved in genome housekeeping, including multiple terms related to DNA damage repair, heterochromatic assembly, chromosomal condensation, and regulation of RNA polymerase, which may represent adaptive switches in isoform usage that enable the intestinal cells to cope with viral attempts to hijack cellular genomic machinery. In addition, IHNV caused DTU with GO enrichment for specific terms like intracellular viral transport, regulation of inflammatory response, and definitive hemopoiesis which are evidence of the adaptive role that DTU is playing in the intestine in viral response. Interestingly, GO terms for male gonad development, embryo development, and spermatid development were also enriched by the IHNV DTU dataset; however, immature all female fish were used in the study and only the intestine was sampled, so these terms may come from spurious or non-functional DTU. Comparing early to late stages of the IHNV infection also identified many potentially adaptive examples of DTU, with GO terms such as cellular response to interferon-gamma, viral RNA genome replication, interleukin-12 mediated signaling pathway, as well as many terms involved in energy homoeostasis that likely enabled fish to partition energy throughout the 20 day infection.

The overall intestinal gene expression profile was found to be significantly shifted by the IHNV infection ($p = 0.001$), though disease status by stage interactions were also detected, as the IHNV infected gene expression returned to similar profiles of the mock-challenged fish by the late stage of infection. In terms of DGE, the IHNV infection induced the highest number of DEG in both strains of trout as well as between early and late stages of the disease (Figure 4.7). The DEG between the sham and IHNV infected fish showed GO enrichment for classical responses to viral infection. Terms related paracrine immune

signaling such as IL-1β, INF-α and -γ cell signaling and negative regulation of JAK-STAT receptor signaling, as well as protein ubiquitination, defense response to virus, response to exogenous dsRNA, cellular response to virus, and circadian regulation of gene expression were all enriched in the IHNV infected DEG (Table 4.6; Figure 4.8). Most of these same terms were also enriched when comparing the early stage (acute infection) to the late stage (recovering survivors) of IHNV infection as well. This classical response to virus at the intestinal gene expression level further provides evidence that IHNV infection induces direct viral responses in the gut of rainbow trout. Recently, Dong et al., (2020) used RT-qPCR to quantify the expression level of seventeen immune related transcripts across eight different tissues of rainbow trout seven days following a bath immersion challenge with IHNV and showed that infection upregulated the expression of many classical viral response related genes (Mx1, INFαR, RIG-1, etc) in the intestine, when compared to control fish. In that study the expression of viral response genes in the intestine followed a similar pattern to that of other tissues; however, expression responses in the other tissues (buccal mucosa, pharyngeals, head kidney, and spleen) were more exaggerated. Furthermore, those authors quantified IHNV copy number in each tissue, and while highest in the head kidney and spleen, levels were appreciably high along the alimentary canal and intestine as well (Dong et al., 2020). Taken together with the results of the present study, it appears IHNV infection induce a global systemic antiviral response, even though the pathogen is known to have a tropism for hematopoietic tissues.

The virome of a host is known to be an interacting part of the microbiota and interactions between enteric viruses and bacteria can be complex, with multidirectional interactions with synergistic or competitive outcomes. In their review of interactions between mammalian enteric viruses and the transkingdom gut microbiota, Pfeiffer and Virgin (2016) showed that gut microbiota could modulate response to viral infection through direct or indirect interactions. For example, human poliovirus is stabilized and delivered to host cells through interactions with bacterial microbiota cell walls, while mouse mammary tumor virus disguises itself by biding LPS within the enteric environment which can lead to IL-10 induced tolerance of the viral antigen through TLR signaling (Pfeiffer and Virgin 2016). However, at the same time, gut bacteria can offer a protective role against viral invasion through regulation of innate immune responses, as mice with antibiotic depleted bacterial gut

microbiota are highly susceptible to mortality from respiratory influenza virus due to a lack of an adequate steady state of innate immune stimulation (Ichinohe et al., 2011). Furthermore, Yoshimizu and Ezura (1999) identified antiviral substances produced by *Aeromonas* and *Vibrio* isolated from the intestinal microbiota of three fish species, and when those antiviral compound producing bacteria where fed to rainbow trout the fish showed elevated resistance to IHNV challenge. Those authors then collected intestinal homogenates from the fish and showed *in vitro* inhibition of IHNV, although it is likely the intestinal homogenates also contained soluble immune effectors derived from the host. Regardless, this suggests that gut microbiota certainly play a role in host response to viral pathogenesis.

As only an observational study the microbiota, the results reported here do not definitively provide evidence for the role of the bacterial microbiota in the response to viral infection; however, a number of metrics related to the gut bacterial communities were found to be affected by IHNV infection. The IHNV infection caused a significant shift in phylogenetic presence-absence profile of gut bacterial communities (uwUniFrac, $p = 0.001$; Table 4.4), though by the late recovery stage of infection communities more closely matched mock-infected controls (Figure 4.10 C; Table 4.4). Interestingly, the IHNV infection was found to increase the abundance of 50 bacterial genera, while decreasing the abundance of *Domibacillus*, an unannotated genus in the family Rhizobiaceae, and *Clostridium_sensu_stricto_12* (Figure 4.12 A). As a typical plant associated microbe, the Rhizobiaceae may have been of dietary origin and the DA could be due the infected fish not consuming similar levels of feed as the mock-challenge fish, as all fish were offered feed throughout the study. The genera *Proteus* was found to be more abundant in IHNV infected fish during both the early and late stage of infection, these free-living opportunistic saprophytic microbes are found in the soil and water where they are known to decompose animal matter. A similar pattern was observed for *Arthrobacter, Actinomyces, Hyphomicrobium, Massilia, Crenobacter, Peptococcus,* and *Lachnospiraceae*. *Arthrobacter, Actinomyces, Peptococcus,* and *Lachnospiraceae* are all common gut microbes and it is interesting that their abundance would be increased by IHNV infection, while *Hyphomicrobium, Massilia, Crenobacter,* and *Lawsonella* are not common gut microbiota though most do have nitrogen fixation or denitrification capabilities. Dong et al. (2020) recently reported the first evidence of effects of IHNV on the gut microbiota of rainbow

trout, and those authors found an increase in bacterial richness and diversity along the alimentary canal during infection and also detected an enrichment of more operational taxonomic units (OTU) in the infected fish then in the control groups. In their report, those authors suggested that the increase in gut bacteria during IHNV infection, suggested the disease induced gut microbiota dysbiosis with the communities being overwhelmed with opportunistic pathogens. The data presented here may slightly be in agreement with the acute IHNV infection showing an increase in opportunistic bacteria, although the majority of the more highly abundant bacteria genera during acute infection are not particularly known as pathogens so it is unclear whether this represents true dysbiosis. Although, there is certaily evidence that the ability of the trout to regulate their microbiota was significantly impacted by IHNV infection.

Impacts of F. psyschrophilum on Intestinal Transcription and Microbiota

Just as in the IHNV challenge, purposeful transcriptional responses to *Fp* were detected in the current study. For example, DTU detected during the *Fp* infection showed enrichment for GO terms related to cell chemotaxis and NFAT signaling cascade. Cell chemotaxis can be explained by activation and migration of the immune cells residing in the gastrointestinal associated lymphatic tissue, while NFAT signaling is a highly important, redundant system that serves crucial roles in T-cell development, activation, and differentiation (Macian 2005). In addition, terms related to cellular response to UV-B were enriched in DTU lists from *Fp* infected fish compared to sham controls, as well as early infection stages to late stage (Table 4.3). While not intuitive, this GO term involves genes related to apoptosis and lymphoid regulation and suggests these DTU have some functional capacity as well. Interestingly, response to zinc ion was also an enriched DTU function from the *Fp* infection status comparison and disease stage comparison. Zinc plays an integral role in matrix metalloproteinases, which are utilized by the host to remodel cellular and tissue structure possibly due to cell damage caused by infection but are also a primary virulence factors of *Fp;* however, more research will be needed to determine the utility of modifying zinc metabolism during *Fp* infection. In addition to the importance of DTU as part of the molecular response to *Fp*, as shown here, Paneru et al. (2016) showed that long non-coding RNA (lncRNA) are also an often-overlooked means of transcriptionally coping with bacterial infection. Those authors showed that in addition to DTU and DGE, expression of lncRNA is

yet another means of regulating anti-bacterial immunity, with lncRNA expression showing tight correlation, both positive and negative, with the expression of multiple key immune related exons (Paneru et al., 2016).

The intestinal gene expression profile of rainbow trout was found to be significantly shifted ($p = 0.001$) by *Fp* infection and was found to have interacting effects with disease stage ($p = 0.003$), with very clear separation in the profiles of infected an sham control trout at early stages of infection. Yet at late stages the profiles returned to that of the sham controls (Figure 4.6 B). In addition, comparisons between the sham and *Fp* infected fish as well as early vs. late stages of *Fp* infection showed the greatest number of DEG (Figure 4.7 B). The DEG between the *Fp* infected fish and sham controls, as well as early and late stages, showed GO enrichment for functions related to cell chemotaxis, complement activation and signaling, JAK-STAT cytokine signaling receptor activity, INF-α and -γ response, defense response to Gram-negative bacterium, fever generation, positive regulation of leukocyte migration, leukotriene metabolism, granulocyte chemotaxis, iron binding, regulation of apoptosis, and endocrine processes. Typically thought of as part of the classical viral response, the repeated evidence of activation of the INF-α and -γ activation during the *Fp* was slightly surprising. While conducting whole-body differential gene expression analysis between *Fp* infected ARS-Fp trout to uninfected controls, Marancik et al. (2015) found no evidence of differentially expressed INF genes, although they did find significant differences in expression of genes that are directly induced by INF signaling.

The GO terms related to complement activation in the intestine during *Fp* infection in the present study are in agreement with serum complement levels as ACH50 levels were by far highest in the *Fp* challenge (Figure 4.3). Furthermore, genes related to iron binding were induced by the *Fp* infection, and interestingly iron limited medium has been repeatedly shown to increase the production of outer membrane proteins (LaFrentz et al., 2009) that produce greater immunogenicity of *Fp* (Long et al., 2013). This suggests the host may increase iron binding capacity to starve *Fp* of an essential nutrient, which may not only slow the proliferation of the bacteria, but possibly also make it more susceptible host immune regulation due the increased expression of extracellular antigens which can be targeted by adaptive immune system to recognize and attack the pathogen.

Here, the effects of *Fp* infection on the gut microbiota of rainbow trout was found to be more profound than that of the viral INHV infection. The *Fp* infection significantly ($p \leq 0.001$) reduced both bacterial richness and diversity in the gut microbiota, though both were found to significantly interact with other factors as well (Table 4.5). The reduction in richness and diversity is explained by the dominance of *F. psychrophilum,* which was found to account for greater than 60% of the bacterial gut microbiota in infected fish at the early stage of infection. Because *Fp* is not thought to enter the fish through the oral-intestinal route and the intestine is not thought of as a primary target tissue for *Fp,* it is certainly worth taking note of the dominance of *Fp* in the gut microbiota at the early stage.

Disease status and stage of disease both had highly significant main effects and interaction effects on the overall microbial community in the *Fp* challenge, using both weighted and unweighted metrics (Table 4.5). Differential abundance analysis showed that *F. psychrophilum, Pelomonas, Pseudorhodobacter, Hafnia-Obesumbacterium,* and *Morganella* were all more abundant in infected fish, seemingly replacing a long list of common beneficial gut microbes (i.e. *Bacillus, Lactobacillus, Streptococcus, Pediococcus, Lactococcus, Enterococcus, Lachnospiraceae,* and others) that showed higher abundance in uninfected controls (Figure 4.13 A). Interestingly, DA analysis between early and late stage of the *Fp* challenge, show that many of those beneficial microbes that were lost at the early acute phase of the infection, began regaining abundance by the late recovery phase of the infection (Figure 4.13).

Together, results from this study suggest that during acute infection, *Fp* has strong negative interactions with common commensal gut bacteria and becomes dominant in the gut of fish. However, it appears that in fish that survive the infection, commensal gut bacteria quickly recolonize the enteric environment. To this authors knowledge, no other study has evaluated the dynamics of the gut microbiota during a *Fp* infection in salmonids using high-throughput molecular techniques. Recently, Brown et al. (2019) compared the gut microbiota of the ARS-Fp-R and ARS-Fp-S select lines of rainbow trout to identify whether differences in gut microbiota explained some differences in the disease resistance between the two strains; although, that study did not observe the bacterial ecology during or after *Fp* infection. In their results, only minor difference in microbiota metrics were observed with no

significant difference in overall microbial communities between the two strains with disparity in *Fp* resistance.

Concluding Remarks

The study design undertaken here aimed to explore the effects of a common viral and bacterial pathogen on the intestinal transcription and microbiota, while including variation in host genetics to compare the performance of our selected ARS-UI strain to a commercial control line. The superior innate immune performance of the select strain was seen in immunological assays and *Fp* disease susceptibility challenges, but no differences in viral susceptibility were observed by host strain. For the first time, it is reported here that rainbow trout utilize DTU to alter the isoforms used to express many key genes with potentially adaptive roles during viral and bacterial disease response. In addition to DTU, intestinal DGE analysis showed somewhat classical responses to the viral and bacterial challenge, with significant differences in adaptive functions between the two host strains detected as well. In the IHNV challenge, functions related to genomic repair, transcriptional regulation, cell differentiation, viral RNA replication, as well as, chemokine, cytokine, and interferon signaling were all enriched, as were many other GO terms, in the sham vs. infected comparisons. Similar enrichments were seen in the *Fp* challenge with functions related to chemokine, cytokine, and interferon signaling enriched, complement activation, and iron metabolism, cytoskeleton reorganization and maintenance, metalloendopeptidase activity, and fever generation, as well as others, were detected in the *Fp* infected fish. This report is the first study to characterize the gut microbiota of trout during *Fp* challenge using resolute high-throughput molecular methods and is the only the second to do so during an IHNV infection, although this study is the first to track the dynamics across early and late stages of disease progression. Microbiota results highlighted many key effects of host strain, infection status, and disease stage on bacterial ecology of rainbow trout. Differential abundance analysis at the bacterial genus level showed somewhat similar patterns to DGE group comparisons, with hundreds of potential biomarker genera identified across the experimental factors in both pathogen challenges. Of note, the IHNV infection appeared to increase the abundance of a set of non-enteric opportunistic bacteria, while the *Fp* challenge showed a depletion of healthy microbes that were replaced by *F. psychrophilum* which dominated the gut bacterial communities during infection. The novel integrated multi-systems approach

taken in this study along with the inclusion of multiple axes of variation provide unique insights into the regulation of pathogens and commensal microbiota in rainbow trout. Results reported here have widespread implications on selective breeding and fish health, while also bolstering our basic understanding of host-microbiota-pathogen interactions in metazoans.

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Table 4.1. Results of a plaque neutralization assay conducted on sera samples taken from two strains of rainbow trout at the end (20 days post challenge) of an experimental challenge with Infectious Hematopoietic Necrosis Virus (IHNV).

Fish Strain	Tank	IHNV Neutralization Titer
Commercial	Infected 1	160
	Infected 2	40
	Infected 3	80
	Sham 1	0
Select	Infected 1	80
	Infected 2	80
	Infected 3	80
	Sham 1	20
Assay Controls	Positive	320
	Negative	0

Table 4.2. Summary of GO enrichment analysis performed on transcripts identified as participating in DTU during an IHNV challenge in rainbow trout. Enrichment analysis was performed separately for each experimental factor: disease (IHNV vs. Sham), stage (Early vs. Late infection), and trout strain (Commercial vs. Select). All enriched GO terms were over enriched and are listed by biological process (BP), cellular component (CC), and molecular function (MF).

Factor	GO ID	GO Category	GO Name	FDR
Disease	GO:0008584	BP	male gonad development	0.002
Disease	GO:1903311	BP	regulation of mRNA metabolic process	0.008
Disease	GO:0000717	BP	nucleotide-excision repair, DNA duplex unwinding	0.013
Disease	GO:0000715	BP	nucleotide-excision repair, DNA damage recognition	0.013
Disease	GO:0070911	BP	global genome nucleotide-excision repair	0.015
Disease	GO:0075733	BP	intracellular transport of virus	0.015
Disease	GO:0006296	BP	nucleotide-excision repair, DNA incision, 5'-to lesion	0.015
Disease	GO:0031509	BP	subtelomeric heterochromatin assembly	0.015
Disease	GO:0031508	BP	pericentric heterochromatin assembly	0.015
Disease	GO:1902340	BP	negative regulation of chromosome condensation	0.015
Disease	GO:0006297	BP	nucleotide-excision repair, DNA gap filling	0.016
Disease	GO:0042276	BP	error-prone translesion synthesis	0.016
Disease	GO:0019509	BP	L-methionine salvage from methylthioadenosine	0.016
Disease	GO:0031167	BP	rRNA methylation	0.016
Disease	GO:0061418	BP	regulation of transcription from RNA polymerase II promoter in response to hypoxia	0.016
Disease	GO:0042769	BP	DNA damage response, detection of DNA damage	0.016
Disease	GO:0007566	BP	embryo implantation	0.017
Disease	GO:0071596	BP	ubiquitin-dependent protein catabolic process via the N-end rule pathway	0.018

Disease	GO:0009950	BP	dorsal/ventral axis specification	0.023
Disease	GO:0006166	BP	purine ribonucleoside salvage	0.025
Disease	GO:0035264	BP	multicellular organism growth	0.033
Disease	GO:0001649	BP	osteoblast differentiation	0.037
Disease	GO:0060964	BP	regulation of gene silencing by miRNA	0.038
Disease	GO:0030307	BP	positive regulation of cell growth	0.040
Disease	GO:0007286	BP	spermatid development	0.045
Disease	GO:0060216	BP	definitive hemopoiesis	0.045
Disease	GO:0050727	BP	regulation of inflammatory response	0.046
Disease	GO:0000245	BP	spliceosomal complex assembly	0.046
Disease	GO:0001740	CC	Barr body	0.016
Disease	GO:0017061	MF	S-methyl-5-thioadenosine phosphorylase activity	0.007
Disease	GO:0008131	MF	primary amine oxidase activity	0.008
Disease	GO:0000179	MF	rRNA (adenine-N6,N6-)-dimethyltransferase activity	0.012
Disease	GO:0048038	MF	quinone binding	0.016
Disease	GO:0003723	MF	RNA binding	0.017
Disease	GO:0098770	MF	FBXO family protein binding	0.018
Disease	GO:0000979	MF	RNA polymerase II core promoter sequence-specific DNA binding	0.020
Disease	GO:0031492	MF	nucleosomal DNA binding	0.038
Stage	GO:0071346	BP	cellular response to interferon-gamma	0.000
Stage	GO:0046855	BP	inositol phosphate dephosphorylation	0.001
Stage	GO:0050847	BP	progesterone receptor signaling pathway	0.004
Stage	GO:0006886	BP	intracellular protein transport	0.004
Stage	GO:2000323	BP	negative regulation of glucocorticoid receptor signaling pathway	0.004
Stage	GO:0090501	BP	RNA phosphodiester bond hydrolysis	0.009
Stage	GO:0032469	BP	endoplasmic reticulum calcium ion homeostasis	0.010
Stage	GO:0021693	BP	cerebellar Purkinje cell layer structural organization	0.010
Stage	GO:0009060	BP	aerobic respiration	0.012
Stage	GO:0042254	BP	ribosome biogenesis	0.022
Stage	GO:0010739	BP	positive regulation of protein kinase A signaling	0.026
Stage	GO:0050878	BP	regulation of body fluid levels	0.027
Stage	GO:0060661	BP	submandibular salivary gland formation	0.029
Stage	GO:0048664	BP	neuron fate determination	0.029
Stage	GO:0048549	BP	positive regulation of pinocytosis	0.029
Stage	GO:0008611	BP	ether lipid biosynthetic process	0.029
Stage	GO:0051835	BP	positive regulation of synapse structural plasticity	0.029
Stage	GO:0090135	BP	actin filament branching	0.029
Stage	GO:0031274	BP	positive regulation of pseudopodium assembly	0.029
Stage	GO:0060501	BP	positive regulation of epithelial cell proliferation involved in lung morphogenesis	0.029
Stage	GO:0039694	BP	viral RNA genome replication	0.035
Stage	GO:0043552	BP	positive regulation of phosphatidylinositol 3-kinase activity	0.035
Stage	GO:0035722	BP	interleukin-12-mediated signaling pathway	0.035

Stage	GO:0099159	BP	regulation of modification of postsynaptic structure	0.042
Stage	GO:0006448	BP	regulation of translational elongation	0.042
Stage	GO:0006021	BP	inositol biosynthetic process	0.042
Stage	GO:0003161	BP	cardiac conduction system development	0.042
Stage	GO:0038189	BP	neuropilin signaling pathway	0.042
Stage	GO:0030307	BP	positive regulation of cell growth	0.044
Stage	GO:0006553	BP	lysine metabolic process	0.045
Stage	GO:0005719	CC	nuclear euchromatin	$\leq 1E^{-3}$
Stage	GO:0015935	CC	small ribosomal subunit	$\leq 1E^{-3}$
Stage	GO:0044613	CC	nuclear pore central transport channel	0.001
Stage	GO:0022626	CC	cytosolic ribosome	0.021
Stage	GO:0071261	CC	Ssh1 translocon complex	0.026
Stage	GO:0030895	CC	apolipoprotein B mRNA editing enzyme complex	0.029
Stage	GO:0000322	CC	storage vacuole	0.029
Stage	GO:0042721	CC	TIM22 mitochondrial import inner membrane insertion complex	0.029
Stage	GO:0051233	CC	spindle midzone	0.029
Stage	GO:0043197	CC	dendritic spine	0.031
Stage	GO:0043025	CC	neuronal cell body	0.034
Stage	GO:0031105	CC	septin complex	0.042
Stage	GO:0003735	MF	structural constituent of ribosome	0.004
Stage	GO:0004362	MF	glutathione-disulfide reductase activity	0.004
Stage	GO:0031403	MF	lithium ion binding	0.004
Stage	GO:0004540	MF	ribonuclease activity	0.004
Stage	GO:0070180	MF	large ribosomal subunit rRNA binding	0.010
Stage	GO:0008609	MF	alkylglycerone-phosphate synthase activity	0.017
Stage	GO:0008934	MF	inositol monophosphate 1-phosphatase activity	0.017
Stage	GO:0052832	MF	inositol monophosphate 3-phosphatase activity	0.017
Stage	GO:0052833	MF	inositol monophosphate 4-phosphatase activity	0.017
Stage	GO:0047280	MF	nicotinamide phosphoribosyltransferase activity	0.026
Stage	GO:0034191	MF	apolipoprotein A-I receptor binding	0.029
Stage	GO:0008475	MF	procollagen-lysine 5-dioxygenase activity	0.029
Stage	GO:0032427	MF	GBD domain binding	0.029
Stage	GO:0031435	MF	mitogen-activated protein kinase kinase kinase binding	0.029
Stage	GO:0019842	MF	vitamin binding	0.034
Stage	GO:0003746	MF	translation elongation factor activity	0.035
Stage	GO:0102148	MF	N-acetyl-beta-D-galactosaminidase activity	0.042
Stage	GO:0004563	MF	beta-N-acetylhexosaminidase activity	0.042
Stage	GO:0050661	MF	NADP binding	0.042
Stage	GO:0004449	MF	isocitrate dehydrogenase (NAD+) activity	0.042
Stage	GO:0050660	MF	flavin adenine dinucleotide binding	0.046
Strain	GO:0055114	BP	oxidation-reduction process	$\leq 1E^{-3}$
Strain	GO:0098869	BP	cellular oxidant detoxification	0.001
Strain	GO:0043651	BP	linoleic acid metabolic process	0.011
Strain	GO:0036109	BP	alpha-linolenic acid metabolic process	0.015
Strain	GO:0000715	BP	nucleotide-excision repair, DNA damage recognition	0.017

Strain	GO:0007031	BP	peroxisome organization	0.017
Strain	GO:0007254	BP	JNK cascade	0.017
Strain	GO:0060119	BP	inner ear receptor cell development	0.018
Strain	GO:0000463	BP	maturation of LSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	0.027
Strain	GO:0000302	BP	response to reactive oxygen species	0.044
Strain	GO:0048471	CC	perinuclear region of cytoplasm	0.004
Strain	GO:0044609	CC	DBIRD complex	0.017
Strain	GO:0044194	CC	cytolytic granule	0.017
Strain	GO:0004386	MF	helicase activity	$\leq 1E^{-3}$
Strain	GO:0035718	MF	macrophage migration inhibitory factor binding	0.003
Strain	GO:0016213	MF	linoleoyl-CoA desaturase activity	0.011
Strain	GO:0016404	MF	15-hydroxyprostaglandin dehydrogenase (NAD+) activity	0.017
Strain	GO:0004362	MF	glutathione-disulfide reductase activity	0.017
Strain	GO:0008413	MF	8-oxo-7,8-dihydroguanosine triphosphate pyrophosphatase activity	0.017
Strain	GO:0000062	MF	fatty-acyl-CoA binding	0.020
Strain	GO:0050178	MF	phenylpyruvate tautomerase activity	0.035

Table 4.3. Summary of GO enrichment analysis performed on transcripts identified as participating in DTU during a *Flavobacterium psychrophilum* challenge in rainbow trout. Enrichment analysis was performed separately for each experimental factor: disease (*Fp* vs. Sham), stage (Early vs. Late infection), and trout strain (Commercial vs. Select). Over enriched GO terms are listed in red, while stable or under enriched terms are green. GO terms are listed by biological process (BP), cellular component (CC), and molecular function (MF).

Factor	GO ID	GO Category	GO Name	FDR
Disease	GO:0060326	BP	cell chemotaxis	0.003
Disease	GO:0071493	BP	cellular response to UV-B	0.003
Disease	GO:0071236	BP	cellular response to antibiotic	0.003
Disease	GO:0010043	BP	response to zinc ion	0.007
Disease	GO:0033173	BP	calcineurin-NFAT signaling cascade	0.045
Disease	GO:0005576	CC	extracellular region	0.008
Disease	GO:1904724	CC	tertiary granule lumen	0.011
Disease	GO:0008009	MF	chemokine activity	0.001
Disease	GO:0016504	MF	peptidase activator activity	0.037
Stage	GO:0045104	BP	intermediate filament cytoskeleton organization	0.004
Stage	GO:0006040	BP	amino sugar metabolic process	0.006
Stage	GO:0061734	BP	parkin-mediated stimulation of mitophagy in response to mitochondrial depolarization	0.006
Stage	GO:0071493	BP	cellular response to UV-B	0.006
Stage	GO:0006427	BP	histidyl-tRNA aminoacylation	0.006
Stage	GO:1905461	BP	positive regulation of vascular associated smooth muscle cell apoptotic process	0.006
Stage	GO:0071236	BP	cellular response to antibiotic	0.006
Stage	GO:0019896	BP	axonal transport of mitochondrion	0.006
Stage	GO:0001825	BP	blastocyst formation	0.011
Stage	GO:0051958	BP	methotrexate transport	0.011

Stage	GO:0010043	BP	response to zinc ion	0.011
Stage	GO:0048662	BP	negative regulation of smooth muscle cell proliferation	0.018
Stage	GO:0015884	BP	folic acid transport	0.018
Stage	GO:0010040	BP	response to iron(II) ion	0.018
Stage	GO:0030097	BP	hemopoiesis	0.019
Stage	GO:0006430	BP	lysyl-tRNA aminoacylation	0.026
Stage	GO:0009085	BP	lysine biosynthetic process	0.026
Stage	GO:0043328	BP	protein transport to vacuole involved in ubiquitin-dependent protein catabolic process via the multivesicular body sorting pathway	0.026
Stage	GO:0034497	BP	protein localization to phagophore assembly site	0.026
Stage	GO:0048203	BP	vesicle targeting, trans-Golgi to endosome	0.033
Stage	GO:0120162	BP	positive regulation of cold-induced thermogenesis	0.033
Stage	GO:1900060	BP	negative regulation of ceramide biosynthetic process	0.033
Stage	GO:0042759	BP	long-chain fatty acid biosynthetic process	0.033
Stage	GO:1904707	BP	positive regulation of vascular associated smooth muscle cell proliferation	0.033
Stage	GO:0033559	BP	unsaturated fatty acid metabolic process	0.033
Stage	GO:0006664	BP	glycolipid metabolic process	0.037
Stage	GO:0055114	BP	oxidation-reduction process	0.037
Stage	GO:0006879	BP	cellular iron ion homeostasis	0.037
Stage	GO:1900025	BP	negative regulation of substrate adhesion-dependent cell spreading	0.041
Stage	GO:0098974	BP	postsynaptic actin cytoskeleton organization	0.048
Stage	GO:0090156	BP	cellular sphingolipid homeostasis	0.048
Stage	GO:0031305	CC	integral component of mitochondrial inner membrane	0.006
Stage	GO:0098588	CC	bounding membrane of organelle	0.011
Stage	GO:0043564	CC	Ku70:Ku80 complex	0.033
Stage	GO:0035339	CC	SPOTS complex	0.033
Stage	GO:0000151	CC	ubiquitin ligase complex	0.041
Stage	GO:0005840	CC	ribosome	0.043
Stage	GO:0097433	CC	dense body	0.048
Stage	GO:0031625	MF	ubiquitin protein ligase binding	0.002
Stage	GO:0016887	MF	ATPase activity	0.003
Stage	GO:0004821	MF	histidine-tRNA ligase activity	0.006
Stage	GO:0005229	MF	intracellular calcium activated chloride channel activity	0.011
Stage	GO:0015350	MF	methotrexate transmembrane transporter activity	0.011
Stage	GO:0008518	MF	folate:anion antiporter activity	0.011
Stage	GO:0031418	MF	L-ascorbic acid binding	0.018
Stage	GO:0004824	MF	lysine-tRNA ligase activity	0.026
Stage	GO:0004353	MF	glutamate dehydrogenase [NAD(P)+] activity	0.026
Stage	GO:0008475	MF	procollagen-lysine 5-dioxygenase activity	0.033
Stage	GO:0035650	MF	AP-1 adaptor complex binding	0.033
Stage	GO:0004360	MF	glutamine-fructose-6-phosphate transaminase (isomerizing) activity	0.041
Stage	GO:0003994	MF	aconitate hydratase activity	0.041

Stage	GO:0050661	MF	NADP binding	0.048
Stage	GO:0098973	MF	structural constituent of postsynaptic actin cytoskeleton	0.048
Stage	GO:0016504	MF	peptidase activator activity	0.048
Stage	GO:0102148	MF	N-acetyl-beta-D-galactosaminidase activity	0.048
Stage	GO:0004563	MF	beta-N-acetylhexosaminidase activity	0.048
Stage	GO:0007166	BP	cell surface receptor signaling pathway	0.013
Stage	GO:0004888	MF	transmembrane signaling receptor activity	0.006
Strain	GO:0019243	BP	methylglyoxal catabolic process to D-lactate via S-lactoyl-glutathione	$\leq 1E^{-3}$
Strain	GO:0019882	BP	antigen processing and presentation	$\leq 1E^{-3}$
Strain	GO:0098869	BP	cellular oxidant detoxification	$\leq 1E^{-3}$
Strain	GO:0043651	BP	linoleic acid metabolic process	0.005
Strain	GO:0015986	BP	ATP synthesis coupled proton transport	0.005
Strain	GO:0036109	BP	alpha-linolenic acid metabolic process	0.006
Strain	GO:0006183	BP	GTP biosynthetic process	0.006
Strain	GO:0006228	BP	UTP biosynthetic process	0.006
Strain	GO:0006506	BP	GPI anchor biosynthetic process	0.006
Strain	GO:0006635	BP	fatty acid beta-oxidation	0.007
Strain	GO:0006915	BP	apoptotic process	0.007
Strain	GO:0001916	BP	positive regulation of T cell mediated cytotoxicity	0.007
Strain	GO:2000568	BP	positive regulation of memory T cell activation	0.007
Strain	GO:0008285	BP	negative regulation of cell population proliferation	0.012
Strain	GO:0002237	BP	response to molecule of bacterial origin	0.012
Strain	GO:0002115	BP	store-operated calcium entry	0.015
Strain	GO:0045541	BP	negative regulation of cholesterol biosynthetic process	0.015
Strain	GO:0006625	BP	protein targeting to peroxisome	0.015
Strain	GO:0000463	BP	maturation of LSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	0.015
Strain	GO:0032729	BP	positive regulation of interferon-gamma production	0.015
Strain	GO:0045717	BP	negative regulation of fatty acid biosynthetic process	0.015
Strain	GO:0002191	BP	cap-dependent translational initiation	0.015
Strain	GO:0090140	BP	regulation of mitochondrial fission	0.015
Strain	GO:2000583	BP	regulation of platelet-derived growth factor receptor-alpha signaling pathway	0.015
Strain	GO:0006979	BP	response to oxidative stress	0.016
Strain	GO:2000114	BP	regulation of establishment of cell polarity	0.025
Strain	GO:1900260	BP	negative regulation of RNA-directed 5'-3' RNA polymerase activity	0.025
Strain	GO:0006241	BP	CTP biosynthetic process	0.025
Strain	GO:0006471	BP	protein ADP-ribosylation	0.027
Strain	GO:0006637	BP	acyl-CoA metabolic process	0.034
Strain	GO:0051642	BP	centrosome localization	0.036
Strain	GO:0090230	BP	regulation of centromere complex assembly	0.036
Strain	GO:0002159	BP	desmosome assembly	0.048
Strain	GO:0009635	BP	response to herbicide	0.048
Strain	GO:1905072	BP	cardiac jelly development	0.048
Strain	GO:0070972	BP	protein localization to endoplasmic reticulum	0.050

Strain	GO:0042613	CC	MHC class II protein complex	$\leq 1E^{-3}$
Strain	GO:0005840	CC	ribosome	0.001
Strain	GO:0005777	CC	peroxisome	0.006
Strain	GO:1990429	CC	peroxisomal importomer complex	0.007
Strain	GO:0044609	CC	DBIRD complex	0.007
Strain	GO:0045261	CC	proton-transporting ATP synthase complex, catalytic core F(1)	0.023
Strain	GO:0005789	CC	endoplasmic reticulum membrane	0.031
Strain	GO:0042627	CC	chylomicron	0.036
Strain	GO:0031093	CC	platelet alpha granule lumen	0.036
Strain	GO:0005753	CC	mitochondrial proton-transporting ATP synthase complex	0.041
Strain	GO:0120115	CC	Lsm2-8 complex	0.048
Strain	GO:0016281	CC	eukaryotic translation initiation factor 4F complex	0.048
Strain	GO:0004416	MF	hydroxyacylglutathione hydrolase activity	$\leq 1E^{-3}$
Strain	GO:0017176	MF	phosphatidylinositol N-acetylglucosaminyltransferase activity	$\leq 1E^{-3}$
Strain	GO:0003997	MF	acyl-CoA oxidase activity	$\leq 1E^{-3}$
Strain	GO:0003735	MF	structural constituent of ribosome	0.001
Strain	GO:0071949	MF	FAD binding	0.002
Strain	GO:0016213	MF	linoleoyl-CoA desaturase activity	0.005
Strain	GO:0016404	MF	15-hydroxyprostaglandin dehydrogenase (NAD+) activity	0.007
Strain	GO:0023030	MF	MHC class Ib protein binding, via antigen binding groove	0.007
Strain	GO:0004362	MF	glutathione-disulfide reductase activity	0.007
Strain	GO:0004550	MF	nucleoside diphosphate kinase activity	0.013
Strain	GO:0098808	MF	mRNA cap binding	0.015
Strain	GO:0005229	MF	intracellular calcium activated chloride channel activity	0.015
Strain	GO:0050178	MF	phenylpyruvate tautomerase activity	0.015
Strain	GO:0047750	MF	cholestenol delta-isomerase activity	0.015
Strain	GO:0004601	MF	peroxidase activity	0.024
Strain	GO:0042289	MF	MHC class II protein binding	0.025
Strain	GO:0005150	MF	interleukin-1, type I receptor binding	0.025
Strain	GO:0000009	MF	alpha-1,6-mannosyltransferase activity	0.025
Strain	GO:1990380	MF	Lys48-specific deubiquitinase activity	0.026
Strain	GO:0050661	MF	NADP binding	0.027
Strain	GO:0035718	MF	macrophage migration inhibitory factor binding	0.036
Strain	GO:0030527	MF	structural constituent of chromatin	0.036
Strain	GO:0033149	MF	FFAT motif binding	0.036
Strain	GO:0046933	MF	proton-transporting ATP synthase activity, rotational mechanism	0.037
Strain	GO:0051920	MF	peroxiredoxin activity	0.037
Strain	GO:0015279	MF	store-operated calcium channel activity	0.048
Strain	GO:0007186	BP	G protein-coupled receptor signaling pathway	0.025

Table 4.4. Statistical summary of intestinal RNAseq and gut microbiota results from an IHNV challenge in rainbow trout. PERMANOVA were conducted separately by disease challenge using a fully crossed design (Strain * Disease Status * Disease Stage) and 999 permutations.

Factor	RNAseq	Microbiota			
	Euclidean	ObsASV	Shannon H	wUniFrac	uwUniFrac
Strain	**0.005**	0.214	**0.003**	0.088	0.205
Disease	**0.001**	0.166	0.132	0.290	**0.001**
Stage	**0.001**	**≤ 1E^{-3}**	0.364	0.072	**0.020**
Strain:Disease	0.432	0.155	**0.012**	0.335	0.265
Strain:Stage	0.571	0.840	0.920	**0.037**	**0.010**
Disease:Stage	**0.004**	**0.015**	0.195	0.439	**0.001**
Strain:Disease:Stage	0.538	0.749	0.811	0.474	0.295

Table 4.5. Statistical summary of intestinal RNAseq and gut microbiota results from an *Flavobacterium psychrophilum* challenge in rainbow trout. PERMANOVA were conducted separately by disease challenge using a fully crossed design (Strain * Disease Status * Disease Stage) and 999 permutations.

Factor	RNAseq	Microbiota			
	Euclidean	ObsASV	Shannon H	wUniFrac	uwUniFrac
Strain	**0.002**	0.878	0.168	0.604	**0.034**
Disease	**0.001**	**≤ 1e^{-3}**	**≤ 1e^{-3}**	**0.001**	**0.001**
Stage	**0.001**	**≤ 1e^{-3}**	**≤ 1e^{-3}**	**0.001**	**0.001**
Strain:Disease	0.240	0.478	**0.027**	**0.012**	**0.025**
Strain:Stage	**0.025**	**0.038**	0.162	**0.01**	0.148
Disease:Stage	**0.003**	**≤ 1e^{-3}**	**≤ 1e^{-3}**	**0.001**	**0.003**
Strain:Disease:Stage	0.187	0.137	0.748	0.192	0.075

Table 4.6. Summary of enriched gene ontology terms from the differentially expressed intestinal gene lists from comparisons of experimental groups (Factor) of rainbow trout during an experimental IHNV disease challenge. Only DEG from comparisons including the select rainbow trout strain were included in GO analysis. Enrichment analysis was conducted using a Fisher's Exact Test (FDR ≤ 0.05) with DEG serving as the test set and the remaining annotated transcriptome serving as the reference set.

Factor	GO ID	GO Category	GO Name	FDR
Select Sham vs IHNV	GO:0016567	BP	protein ubiquitination	≤ 1E^{-3}
Select Sham vs IHNV	GO:0060326	BP	cell chemotaxis	≤ 1E^{-3}

Select Sham vs IHNV	GO:0035457	BP	cellular response to interferon-alpha	$\leq 1E^{-3}$
Select Sham vs IHNV	GO:0051607	BP	defense response to virus	$\leq 1E^{-3}$
Select Sham vs IHNV	GO:0071357	BP	cellular response to type I interferon	$\leq 1E^{-3}$
Select Sham vs IHNV	GO:0043330	BP	response to exogenous dsRNA	$\leq 1E^{-3}$
Select Sham vs IHNV	GO:0038003	BP	opioid receptor signaling pathway	$\leq 1E^{-3}$
Select Sham vs IHNV	GO:0002474	BP	antigen processing and presentation of peptide antigen via MHC class I	$\leq 1E^{-3}$
Select Sham vs IHNV	GO:0098586	BP	cellular response to virus	$\leq 1E^{-3}$
Select Sham vs IHNV	GO:1903093	BP	regulation of protein K48-linked deubiquitination	0.007
Select Sham vs IHNV	GO:0019835	BP	cytolysis	0.009
Select Sham vs IHNV	GO:0060334	BP	regulation of interferon-gamma-mediated signaling pathway	0.016
Select Sham vs IHNV	GO:0034121	BP	regulation of toll-like receptor signaling pathway	0.023
Select Sham vs IHNV	GO:0046426	BP	negative regulation of receptor signaling pathway via JAK-STAT	0.027
Select Sham vs IHNV	GO:0009435	BP	NAD biosynthetic process	0.032
Select Sham vs IHNV	GO:2000660	BP	negative regulation of interleukin-1-mediated signaling pathway	0.032
Select Sham vs IHNV	GO:0032922	BP	circadian regulation of gene expression	0.046
Select Sham vs IHNV	GO:0048731	BP	system development	0.002
Select Sham vs IHNV	GO:0043087	BP	regulation of GTPase activity	0.002
Select Sham vs IHNV	GO:0065008	BP	regulation of biological quality	0.005
Select Sham vs IHNV	GO:0044085	BP	cellular component biogenesis	0.007
Select Sham vs IHNV	GO:0006810	BP	transport	0.010
Select Sham vs IHNV	GO:0051276	BP	chromosome organization	0.011
Select Sham vs IHNV	GO:0007010	BP	cytoskeleton organization	0.013
Select Sham vs IHNV	GO:0051649	BP	establishment of localization in cell	0.022
Select Sham vs IHNV	GO:0007017	BP	microtubule-based process	0.022
Select Sham vs IHNV	GO:0030154	BP	cell differentiation	0.031

Select Sham vs IHNV	GO:0006357	BP	regulation of transcription by RNA polymerase II	0.038
Select Sham vs IHNV	GO:0009069	BP	serine family amino acid metabolic process	0.043
Select Sham vs IHNV	GO:0009653	BP	anatomical structure morphogenesis	0.043
Select Sham vs IHNV	GO:0043085	BP	positive regulation of catalytic activity	0.044
Select Sham vs IHNV	GO:0005615	CC	extracellular space	$\leq 1E^{-3}$
Select Sham vs IHNV	GO:0005579	CC	membrane attack complex	0.023
Select Sham vs IHNV	GO:0042613	CC	MHC class II protein complex	0.032
Select Sham vs IHNV	GO:0031981	CC	nuclear lumen	0.002
Select Sham vs IHNV	GO:0005856	CC	cytoskeleton	0.004
Select Sham vs IHNV	GO:0005694	CC	chromosome	0.022
Select Sham vs IHNV	GO:0003950	MF	NAD+ ADP-ribosyltransferase activity	$\leq 1E^{-3}$
Select Sham vs IHNV	GO:0008270	MF	zinc ion binding	$\leq 1E^{-3}$
Select Sham vs IHNV	GO:0004842	MF	ubiquitin-protein transferase activity	$\leq 1E^{-3}$
Select Sham vs IHNV	GO:0008009	MF	chemokine activity	$\leq 1E^{-3}$
Select Sham vs IHNV	GO:0005525	MF	GTP binding	$\leq 1E^{-3}$
Select Sham vs IHNV	GO:0047280	MF	nicotinamide phosphoribosyltransferase activity	$\leq 1E^{-3}$
Select Sham vs IHNV	GO:0097677	MF	STAT family protein binding	$\leq 1E^{-3}$
Select Sham vs IHNV	GO:0004985	MF	opioid receptor activity	$\leq 1E^{-3}$
Select Sham vs IHNV	GO:0004514	MF	nicotinate-nucleotide diphosphorylase (carboxylating) activity	0.001
Select Sham vs IHNV	GO:0070976	MF	TIR domain binding	0.001
Select Sham vs IHNV	GO:0003726	MF	double-stranded RNA adenosine deaminase activity	0.005
Select Sham vs IHNV	GO:0016874	MF	ligase activity	0.019
Select Sham vs IHNV	GO:0042289	MF	MHC class II protein binding	0.032
Select Sham vs IHNV	GO:0061578	MF	Lys63-specific deubiquitinase activity	0.049
Select Sham vs IHNV	GO:0035718	MF	macrophage migration inhibitory factor binding	0.049

Select Sham vs IHNV	GO:0051020	MF	GTPase binding	0.008
Select Sham vs IHNV	GO:0000981	MF	DNA-binding transcription factor activity, RNA polymerase II-specific	0.016
Select Sham vs IHNV	GO:0008092	MF	cytoskeletal protein binding	0.022
Select IHNV Early vs Late	GO:0060326	BP	cell chemotaxis	$\leq 1E^{-3}$
Select IHNV Early vs Late	GO:0016567	BP	protein ubiquitination	$\leq 1E^{-3}$
Select IHNV Early vs Late	GO:0035457	BP	cellular response to interferon-alpha	$\leq 1E^{-3}$
Select IHNV Early vs Late	GO:0071346	BP	cellular response to interferon-gamma	0.001
Select IHNV Early vs Late	GO:0038003	BP	opioid receptor signaling pathway	0.001
Select IHNV Early vs Late	GO:0043330	BP	response to exogenous dsRNA	0.003
Select IHNV Early vs Late	GO:1903093	BP	regulation of protein K48-linked deubiquitination	0.004
Select IHNV Early vs Late	GO:0009435	BP	NAD biosynthetic process	0.008
Select IHNV Early vs Late	GO:0034121	BP	regulation of toll-like receptor signaling pathway	0.009
Select IHNV Early vs Late	GO:0032922	BP	circadian regulation of gene expression	0.012
Select IHNV Early vs Late	GO:0001819	BP	positive regulation of cytokine production	0.015
Select IHNV Early vs Late	GO:0071357	BP	cellular response to type I interferon	0.018
Select IHNV Early vs Late	GO:2000660	BP	negative regulation of interleukin-1-mediated signaling pathway	0.018
Select IHNV Early vs Late	GO:0006996	BP	organelle organization	$\leq 1E^{-3}$
Select IHNV Early vs Late	GO:1901605	BP	alpha-amino acid metabolic process	0.029
Select IHNV Early vs Late	GO:0044085	BP	cellular component biogenesis	0.042
Select IHNV Early vs Late	GO:0051649	BP	establishment of localization in cell	0.047
Select IHNV Early vs Late	GO:0005576	CC	extracellular region	$\leq 1E^{-3}$
Select IHNV Early vs Late	GO:0043232	CC	intracellular non-membrane-bounded organelle	0.020
Select IHNV Early vs Late	GO:0043227	CC	membrane-bounded organelle	0.047
Select IHNV Early vs Late	GO:0003950	MF	NAD+ ADP-ribosyltransferase activity	$\leq 1E^{-3}$
Select IHNV Early vs Late	GO:0008009	MF	chemokine activity	$\leq 1E^{-3}$

Select IHNV Early vs Late	GO:0004842	MF	ubiquitin-protein transferase activity	$\leq 1E^{-3}$
Select IHNV Early vs Late	GO:0047280	MF	nicotinamide phosphoribosyltransferase activity	$\leq 1E^{-3}$
Select IHNV Early vs Late	GO:0097677	MF	STAT family protein binding	$\leq 1E^{-3}$
Select IHNV Early vs Late	GO:0004514	MF	nicotinate-nucleotide diphosphorylase (carboxylating) activity	$\leq 1E^{-3}$
Select IHNV Early vs Late	GO:0070976	MF	TIR domain binding	0.001
Select IHNV Early vs Late	GO:0004985	MF	opioid receptor activity	0.001
Select IHNV Early vs Late	GO:0008270	MF	zinc ion binding	0.002
Select IHNV Early vs Late	GO:0016887	MF	ATPase activity	0.005
Select IHNV Early vs Late	GO:0005525	MF	GTP binding	0.009
Select IHNV Early vs Late	GO:0042626	MF	ATPase-coupled transmembrane transporter activity	0.018
Select IHNV Early vs Late	GO:0061578	MF	Lys63-specific deubiquitinase activity	0.027
Select IHNV Early vs Late	GO:0019206	MF	nucleoside kinase activity	0.032

Table 4.7. Summary of enriched gene ontology terms from the differentially expressed intestinal gene lists from comparisons of experimental groups (Factor) of rainbow trout during an experimental *Flavobacterium psychrophilum* disease challenge. Only DEG from comparisons including the select rainbow trout strain were included in GO analysis. Enrichment analysis was conducted using a Fisher's Exact Test (FDR ≤ 0.05) with DEG serving as the test set and the remaining annotated transcriptome serving as the reference set.

Factor	GO ID	GO Category	GO Name	FDR
Select Fp Early vs Late	GO:0060326	BP	cell chemotaxis	$\leq 1E^{-3}$
Select Fp Early vs Late	GO:0018057	BP	peptidyl-lysine oxidation	$\leq 1E^{-3}$
Select Fp Early vs Late	GO:2000272	BP	negative regulation of signaling receptor activity	0.001
Select Fp Early vs Late	GO:0007259	BP	receptor signaling pathway via JAK-STAT	0.003
Select Fp Early vs Late	GO:0006457	BP	protein folding	0.003
Select Fp Early vs Late	GO:0042427	BP	serotonin biosynthetic process	0.004
Select Fp Early vs Late	GO:0071346	BP	cellular response to interferon-gamma	0.005
Select Fp Early vs Late	GO:0006626	BP	protein targeting to mitochondrion	0.007

Select Fp Early vs Late	GO:0030168	BP	platelet activation	0.008
Select Fp Early vs Late	GO:0006956	BP	complement activation	0.012
Select Fp Early vs Late	GO:0000050	BP	urea cycle	0.015
Select Fp Early vs Late	GO:0034340	BP	response to type I interferon	0.015
Select Fp Early vs Late	GO:0042742	BP	defense response to bacterium	0.018
Select Fp Early vs Late	GO:0060956	BP	endocardial cell differentiation	0.023
Select Fp Early vs Late	GO:0035457	BP	cellular response to interferon-alpha	0.033
Select Fp Early vs Late	GO:0001660	BP	fever generation	0.034
Select Fp Early vs Late	GO:0000256	BP	allantoin catabolic process	0.034
Select Fp Early vs Late	GO:0071615	BP	oxidative deethylation	0.034
Select Fp Early vs Late	GO:0042981	BP	regulation of apoptotic process	0.043
Select Fp Early vs Late	GO:0048585	BP	negative regulation of response to stimulus	0.043
Select Fp Early vs Late	GO:0008217	BP	regulation of blood pressure	0.049
Select Fp Early vs Late	GO:0006396	BP	RNA processing	0.012
Select Fp Early vs Late	GO:0005720	CC	nuclear heterochromatin	0.004
Select Fp Early vs Late	GO:0016021	CC	integral component of membrane	0.006
Select Fp Early vs Late	GO:0031526	CC	brush border membrane	0.009
Select Fp Early vs Late	GO:0005739	CC	mitochondrion	0.015
Select Fp Early vs Late	GO:0031012	CC	extracellular matrix	0.019
Select Fp Early vs Late	GO:0034364	CC	high-density lipoprotein particle	0.033
Select Fp Early vs Late	GO:0008009	MF	chemokine activity	$\leq 1E^{-3}$
Select Fp Early vs Late	GO:0005525	MF	GTP binding	$\leq 1E^{-3}$
Select Fp Early vs Late	GO:0004720	MF	protein-lysine 6-oxidase activity	$\leq 1E^{-3}$
Select Fp Early vs Late	GO:0050750	MF	low-density lipoprotein particle receptor binding	$\leq 1E^{-3-4}$
Select Fp Early vs Late	GO:0004222	MF	metalloendopeptidase activity	$\leq 1E^{-3}$

Select Fp Early vs Late	GO:0051082	MF	unfolded protein binding	0.001
Select Fp Early vs Late	GO:0005507	MF	copper ion binding	0.002
Select Fp Early vs Late	GO:0005539	MF	glycosaminoglycan binding	0.002
Select Fp Early vs Late	GO:0004510	MF	tryptophan 5-monooxygenase activity	0.004
Select Fp Early vs Late	GO:0004053	MF	arginase activity	0.015
Select Fp Early vs Late	GO:0033293	MF	monocarboxylic acid binding	0.018
Select Fp Early vs Late	GO:0005152	MF	interleukin-1 receptor antagonist activity	0.034
Select Fp Early vs Late	GO:0001872	MF	(1->3)-beta-D-glucan binding	0.034
Select Fp Early vs Late	GO:0020037	MF	heme binding	0.044
Select Fp Early vs Late	GO:0003723	MF	RNA binding	$\leq 1E^{-3}$
Select Sham vs Fp	GO:0019835	BP	cytolysis	$\leq 1E^{-3}$
Select Sham vs Fp	GO:0007259	BP	receptor signaling pathway via JAK-STAT	$\leq 1E^{-3}$
Select Sham vs Fp	GO:0006956	BP	complement activation	0.001
Select Sham vs Fp	GO:0034340	BP	response to type I interferon	0.001
Select Sham vs Fp	GO:0000050	BP	urea cycle	0.004
Select Sham vs Fp	GO:0050829	BP	defense response to Gram-negative bacterium	0.004
Select Sham vs Fp	GO:0002430	BP	complement receptor mediated signaling pathway	0.004
Select Sham vs Fp	GO:0035457	BP	cellular response to interferon-alpha	0.009
Select Sham vs Fp	GO:0051607	BP	defense response to virus	0.011
Select Sham vs Fp	GO:0001660	BP	fever generation	0.013
Select Sham vs Fp	GO:0010466	BP	negative regulation of peptidase activity	0.015
Select Sham vs Fp	GO:0001960	BP	negative regulation of cytokine-mediated signaling pathway	0.015
Select Sham vs Fp	GO:0086100	BP	endothelin receptor signaling pathway	0.021
Select Sham vs Fp	GO:0032928	BP	regulation of superoxide anion generation	0.021
Select Sham vs Fp	GO:0002687	BP	positive regulation of leukocyte migration	0.023

Select Sham vs Fp	GO:0006691	BP	leukotriene metabolic process	0.035
Select Sham vs Fp	GO:0060334	BP	regulation of interferon-gamma-mediated signaling pathway	0.039
Select Sham vs Fp	GO:0071621	BP	granulocyte chemotaxis	0.039
Select Sham vs Fp	GO:0008217	BP	regulation of blood pressure	0.041
Select Sham vs Fp	GO:0006527	BP	arginine catabolic process	0.046
Select Sham vs Fp	GO:0050886	BP	endocrine process	0.046
Select Sham vs Fp	GO:0006396	BP	RNA processing	0.001
Select Sham vs Fp	GO:0051649	BP	establishment of localization in cell	0.005
Select Sham vs Fp	GO:0015031	BP	protein transport	0.009
Select Sham vs Fp	GO:0006996	BP	organelle organization	0.009
Select Sham vs Fp	GO:0022613	BP	ribonucleoprotein complex biogenesis	0.021
Select Sham vs Fp	GO:0006417	BP	regulation of translation	0.022
Select Sham vs Fp	GO:0031526	CC	brush border membrane	0.002
Select Sham vs Fp	GO:0005579	CC	membrane attack complex	0.004
Select Sham vs Fp	GO:0070062	CC	extracellular exosome	0.009
Select Sham vs Fp	GO:0034364	CC	high-density lipoprotein particle	0.009
Select Sham vs Fp	GO:0098592	CC	cytoplasmic side of apical plasma membrane	0.013
Select Sham vs Fp	GO:1902494	CC	catalytic complex	0.001
Select Sham vs Fp	GO:0005654	CC	nucleoplasm	0.002
Select Sham vs Fp	GO:0005840	CC	ribosome	0.002
Select Sham vs Fp	GO:0098588	CC	bounding membrane of organelle	0.005
Select Sham vs Fp	GO:1990904	CC	ribonucleoprotein complex	0.028
Select Sham vs Fp	GO:0008009	MF	chemokine activity	$\leq 1E^{-3}$
Select Sham vs Fp	GO:0005525	MF	GTP binding	$\leq 1E^{-3}$
Select Sham vs Fp	GO:0004222	MF	metalloendopeptidase activity	0.002

Select Sham vs Fp	GO:0001848	MF	complement binding	0.002
Select Sham vs Fp	GO:0004875	MF	complement receptor activity	0.004
Select Sham vs Fp	GO:0004053	MF	arginase activity	0.004
Select Sham vs Fp	GO:0020037	MF	heme binding	0.008
Select Sham vs Fp	GO:0005152	MF	interleukin-1 receptor antagonist activity	0.013
Select Sham vs Fp	GO:0001872	MF	(1->3)-beta-D-glucan binding	0.013
Select Sham vs Fp	GO:0033293	MF	monocarboxylic acid binding	0.017
Select Sham vs Fp	GO:0004962	MF	endothelin receptor activity	0.021
Select Sham vs Fp	GO:0005539	MF	glycosaminoglycan binding	0.024
Select Sham vs Fp	GO:0001530	MF	lipopolysaccharide binding	0.039
Select Sham vs Fp	GO:0005506	MF	iron ion binding	0.043
Select Sham vs Fp	GO:0004866	MF	endopeptidase inhibitor activity	0.044
Select Sham vs Fp	GO:0003723	MF	RNA binding	$\leq 1E^{-3}$
Select vs. Commercial	GO:0015671	BP	oxygen transport	$\leq 1E^{-3}$
Select vs. Commercial	GO:0050665	BP	hydrogen peroxide biosynthetic process	$\leq 1E^{-3}$
Select vs. Commercial	GO:0098542	BP	defense response to other organism	0.003
Select vs. Commercial	GO:0098869	BP	cellular oxidant detoxification	0.007
Select vs. Commercial	GO:0006953	BP	acute-phase response	0.007
Select vs. Commercial	GO:0071615	BP	oxidative deethylation	0.007
Select vs. Commercial	GO:0002768	BP	immune response-regulating cell surface receptor signaling pathway	0.045
Select vs. Commercial	GO:0006355	BP	regulation of transcription, DNA-templated	0.003
Select vs. Commercial	GO:0005833	CC	hemoglobin complex	0.000
Select vs. Commercial	GO:0034364	CC	high-density lipoprotein particle	0.003
Select vs. Commercial	GO:0005790	CC	smooth endoplasmic reticulum	0.030
Select vs. Commercial	GO:0019815	CC	B cell receptor complex	0.030

Select vs. Commercial	GO:0031528	CC	microvillus membrane	0.030
Select vs. Commercial	GO:0005634	CC	nucleus	0.002
Select vs. Commercial	GO:0005344	MF	oxygen carrier activity	$\leq 1E^{-3}$
Select vs. Commercial	GO:0019825	MF	oxygen binding	$\leq 1E^{-3}$
Select vs. Commercial	GO:0020037	MF	heme binding	$\leq 1E^{-3}$
Select vs. Commercial	GO:0030246	MF	carbohydrate binding	$\leq 1E^{-3}$
Select vs. Commercial	GO:0016174	MF	NAD(P)H oxidase H2O2-forming activity	$\leq 1E^{-3}$
Select vs. Commercial	GO:0005525	MF	GTP binding	0.003
Select vs. Commercial	GO:0004601	MF	peroxidase activity	0.003
Select vs. Commercial	GO:0003796	MF	lysozyme activity	0.006
Select vs. Commercial	GO:0008009	MF	chemokine activity	0.007
Select vs. Commercial	GO:0005506	MF	iron ion binding	0.022
Select vs. Commercial	GO:0003677	MF	DNA binding	0.022

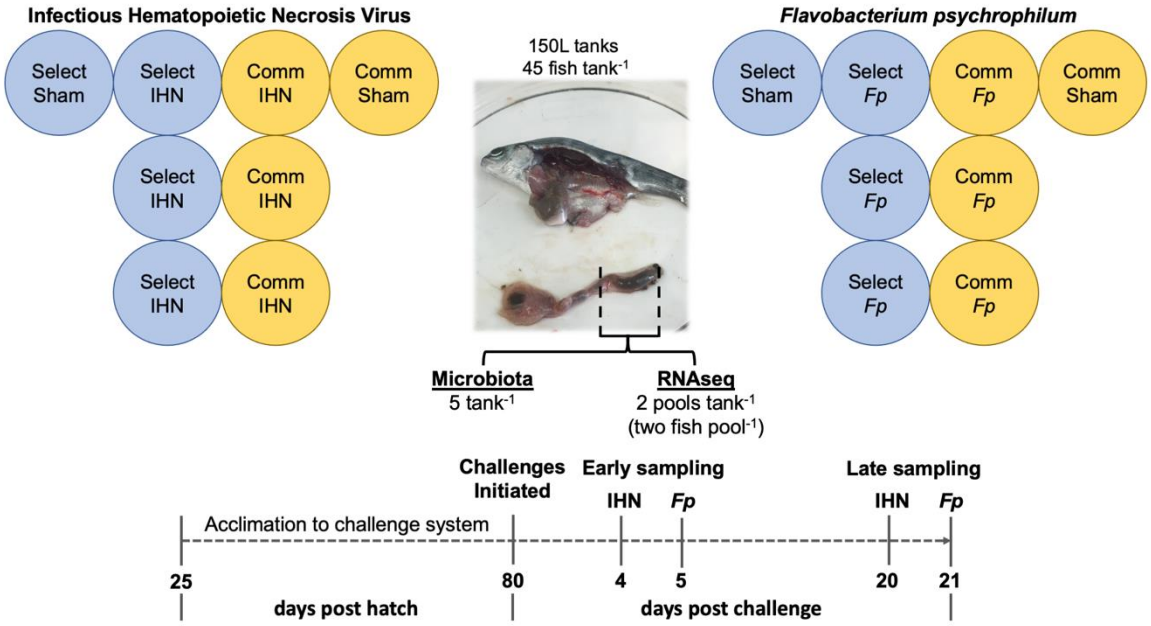

Figure 4.1. Study design schematic. Fish from a commercial (Comm) and selected (Select) strain of rainbow trout were reared alongside one another under equal conditions. Fish were challenged with Infectious Hematopoietic Necrosis Virus (IHN) or *Flavobacterium psychrophilum* (*Fp*) in triplicate tanks, with a single control tank of fish from each group mock-challenged (Sham). Intestinal transcriptome (RNAseq) and microbiota were characterized in samples collected at early and late stages of both disease.

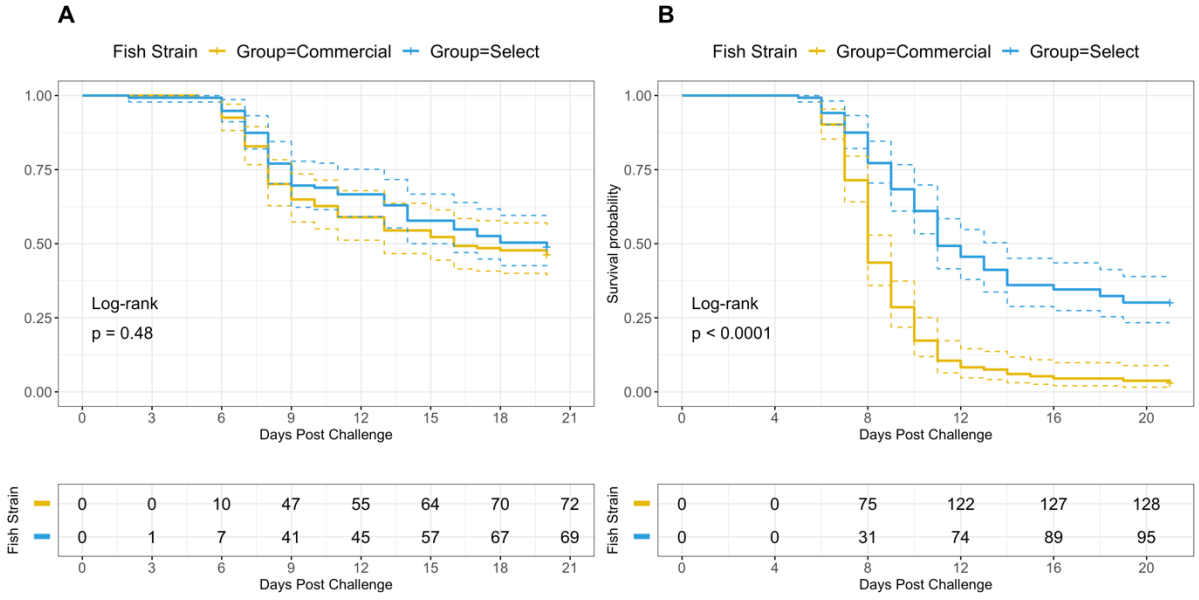

Figure 4.2. Kaplan-Meier survival estimator curves for two strains of rainbow trout (Yellow – Commercial; Blue – Select) challenged with Infectious Hematopoietic Necrosis Virus (A) or *Flavobacterium psychrophilum* (B). Line plots show survival probability by strain with dashed lines representing 95% CI. Cumulative mortalities are listed below plots. Listed p-values are derived from log rank tests of Kaplan-Meier product limit estimates.

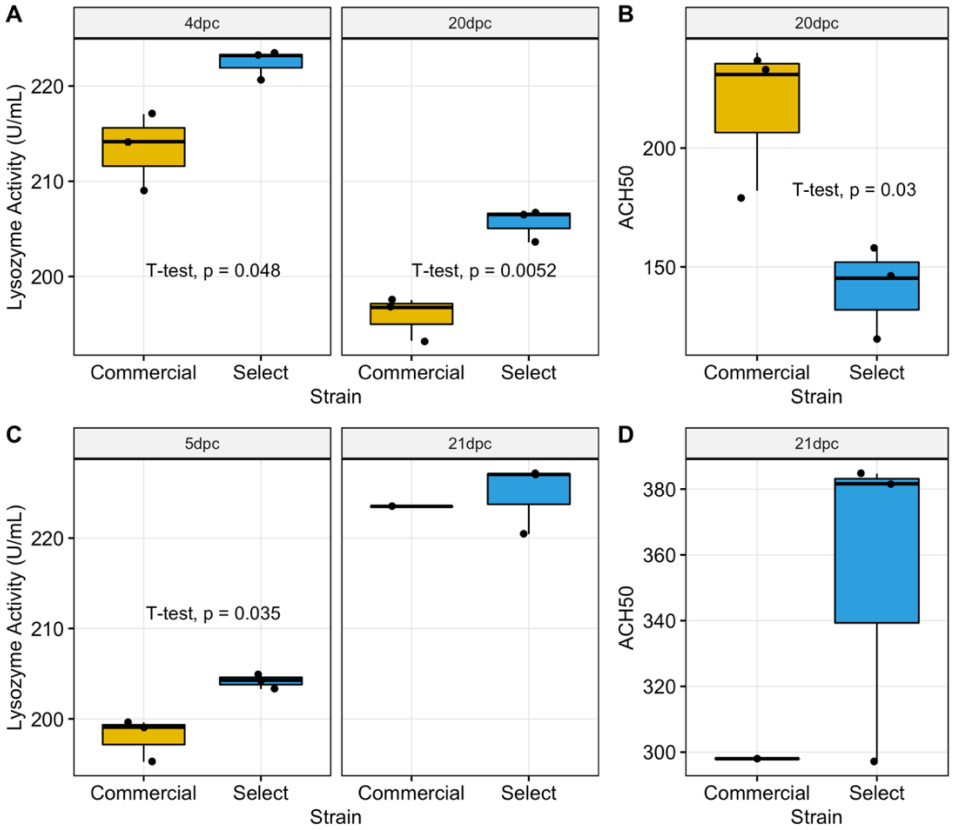

Figure 4.3. Results on circulating lysozyme (A and C) and alternative complement (ACH50; B and D) in two strains of rainbow trout infected with IHNV (A-B) or *Flavobacterium psychrophilum* (C-D). Separate paired t-tests were used to compare values between trout strains at each timepoint for each disease. Blood samples were collected at early (IHN – 4 days post challenge; *Fp* – 5 dpc) and late (IHN – 20 dpc; *Fp* – 21 dpc) timepoints in the progression of disease and pooled by tank (n = 3). ACH50 values represent the serum dilution required to yield 50% hemolysis of competent rabbit red blood cells. ACH50 assay was only conducted on late serum samples. Due to high mortality only one pooled tank sample was available for the commercial strain at late stage of the *Fp* challenge.

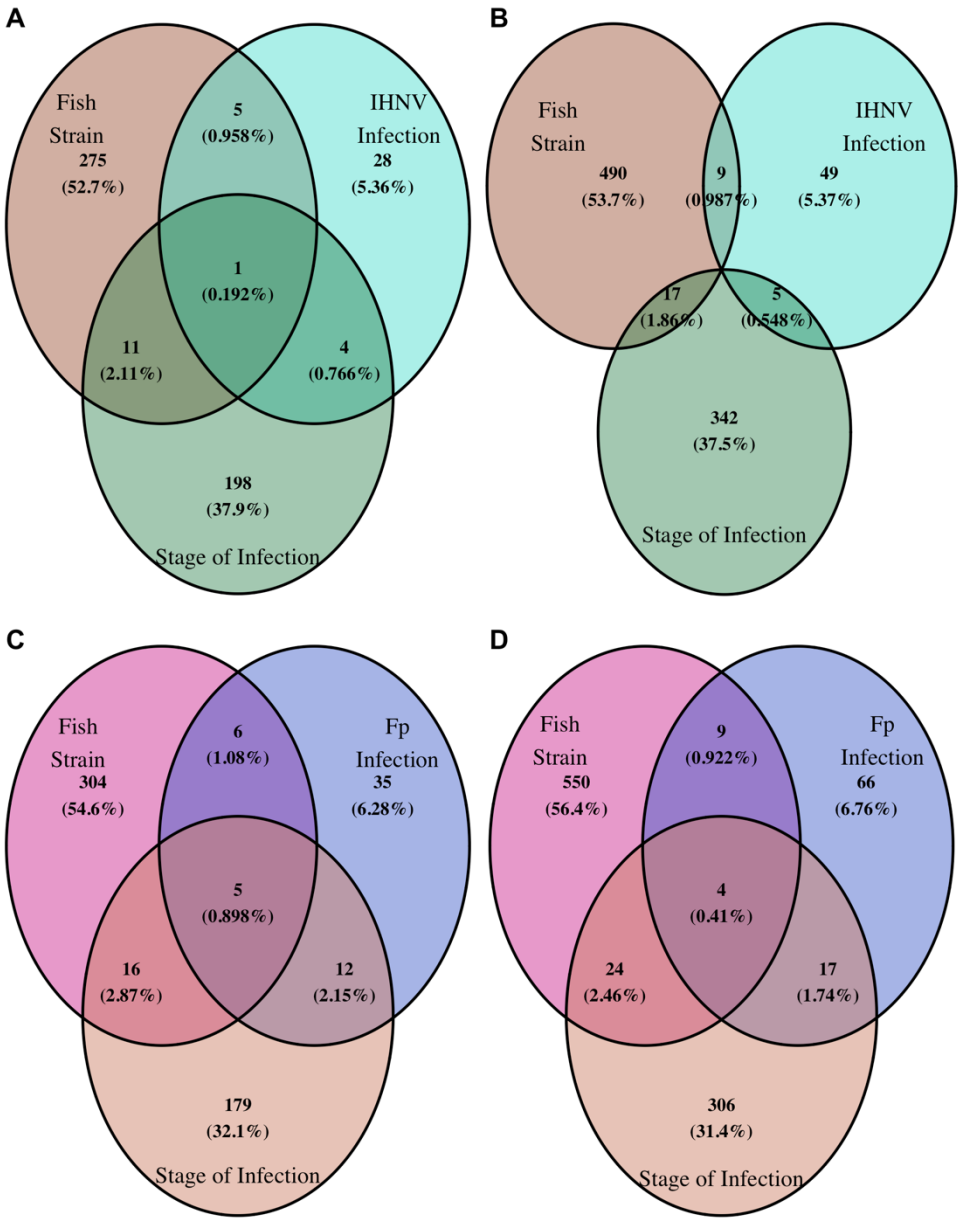

Figure 4.4. Venn diagrams summarizing the distribution of genes (A and C) and transcripts (B and D) involved in differential transcript usage (DTU) from intestinal tissue of rainbow trout infected with IHNV (A-B) and *Flavobacterium psychrophilum* (C-D). DTU analysis was conducted modeling the effects of trout strain (Commercial vs. Select), disease status (Sham vs. Infected), and stage of infection (Early vs. Late).

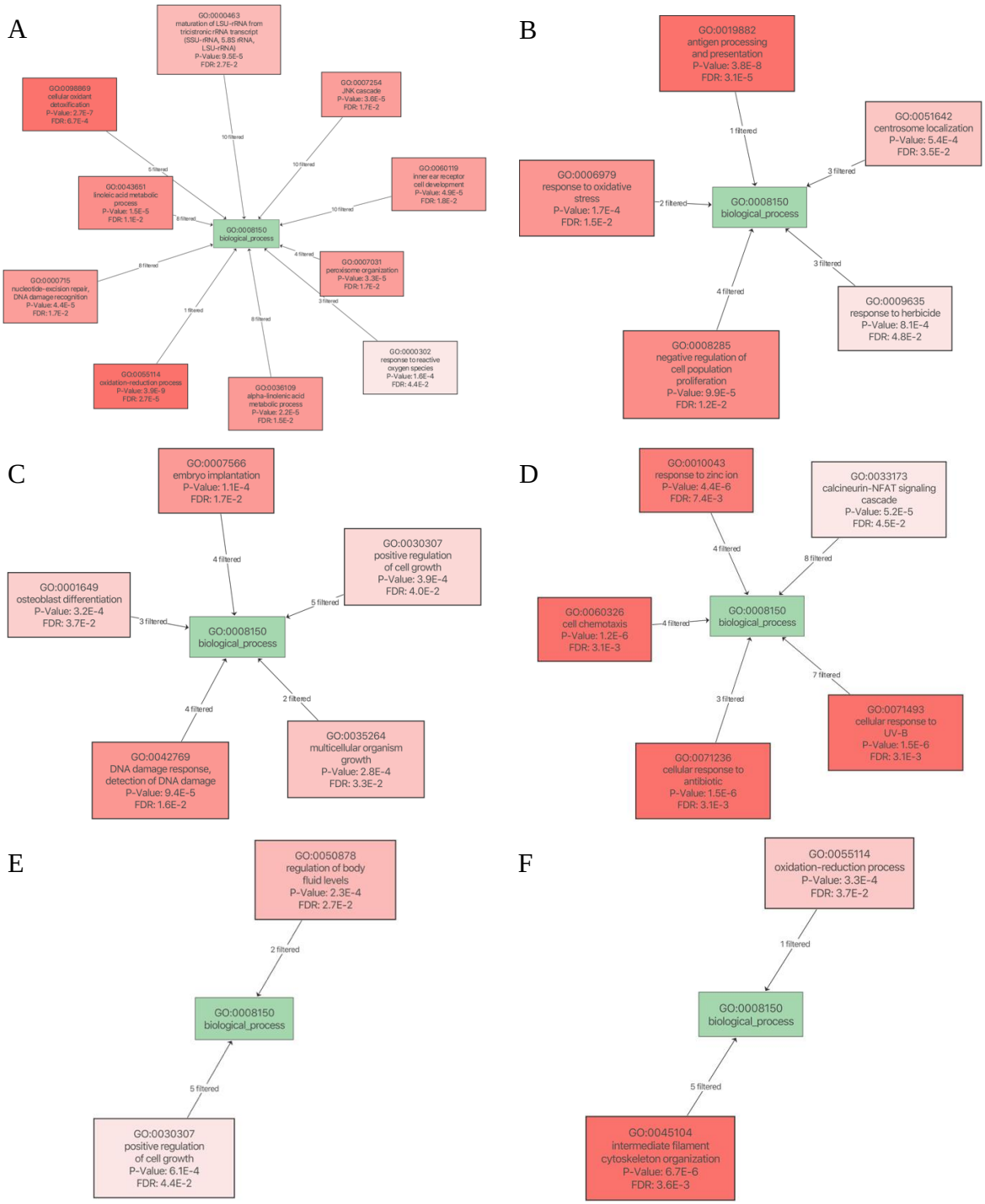

Figure 4.5. Enriched biological process gene ontologies from differentially transcribed genes according to rainbow trout strain (Commercial vs. Select) (A-B), disease status (Sham vs. Infected) (C-D), and disease stage (Early vs. Late) (E and F) during an IHNV (left side; A,C, and E) and *Flavobacterium psychrophilum* (right side; B, D, and F) disease challenge.

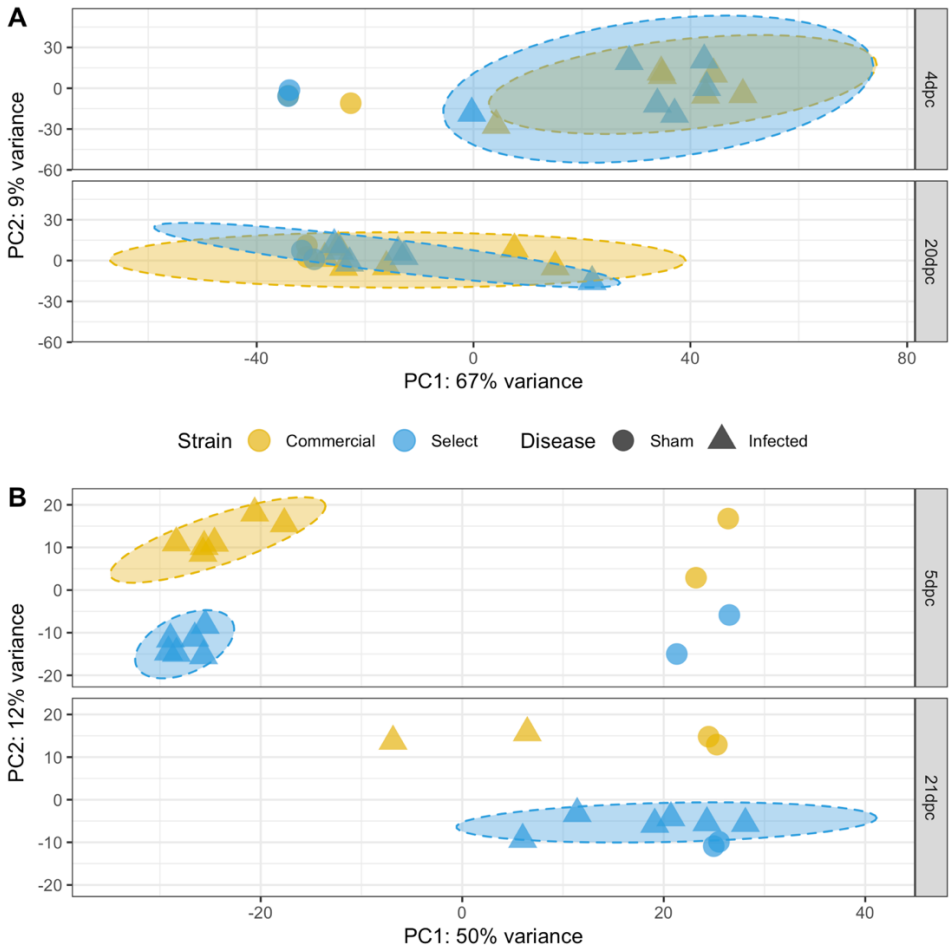

Figure 4.6. Principle component biplots of intestinal gene expression profiles from two strains of rainbow trout infected with IHNV (A) or *Flavobacterium psychrophilum* (B). A variance stabilizing transformation was applied to raw gene level RNAseq counts prior to principle components analysis. Samples were collected from mock challenged individuals (Sham; n = 2 pools) and infected individuals (n = 6 pools) from both strains at early (IHN – 4 days post challenge; *Fp* – 5 dpc) and late (IHN – 20 dpc; *Fp* – 21 dpc) timepoints in the progression of disease.

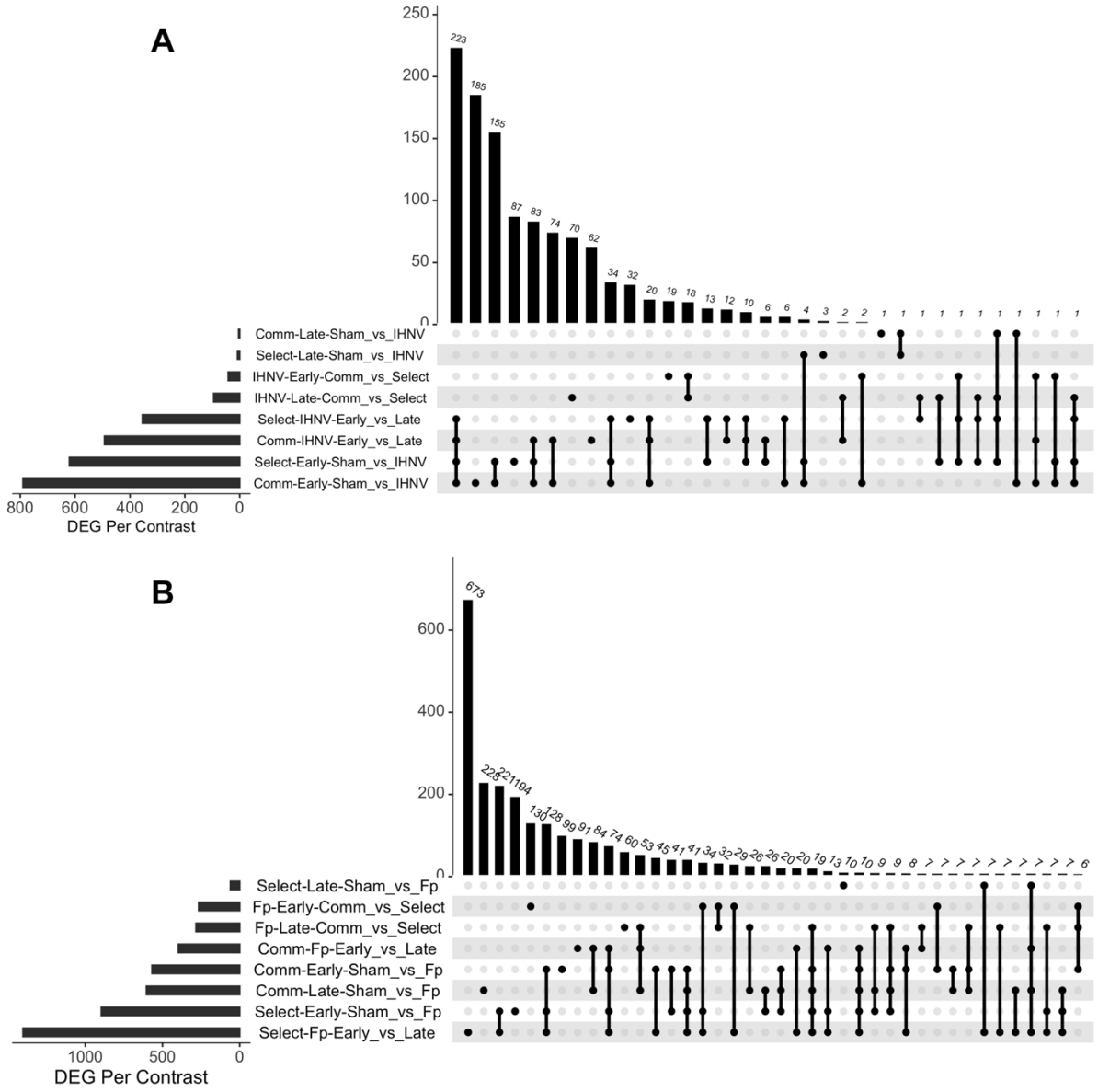

Figure 4.7. Upset plots depicting the overlap in differentially expressed genes (DEG) in the intestine of rainbow trout across experimental group comparisons during infectious hematopoietic necrosis virus (A) and *Flavobacterium psychrophilum* (B) disease challenges. Horizontal bars show the number of DEG by experimental group comparison and vertical bars show the number of overlapping and unique DEG according to comparison groupings listed in the dot plot, as an alternative approach to Venn diagrams.

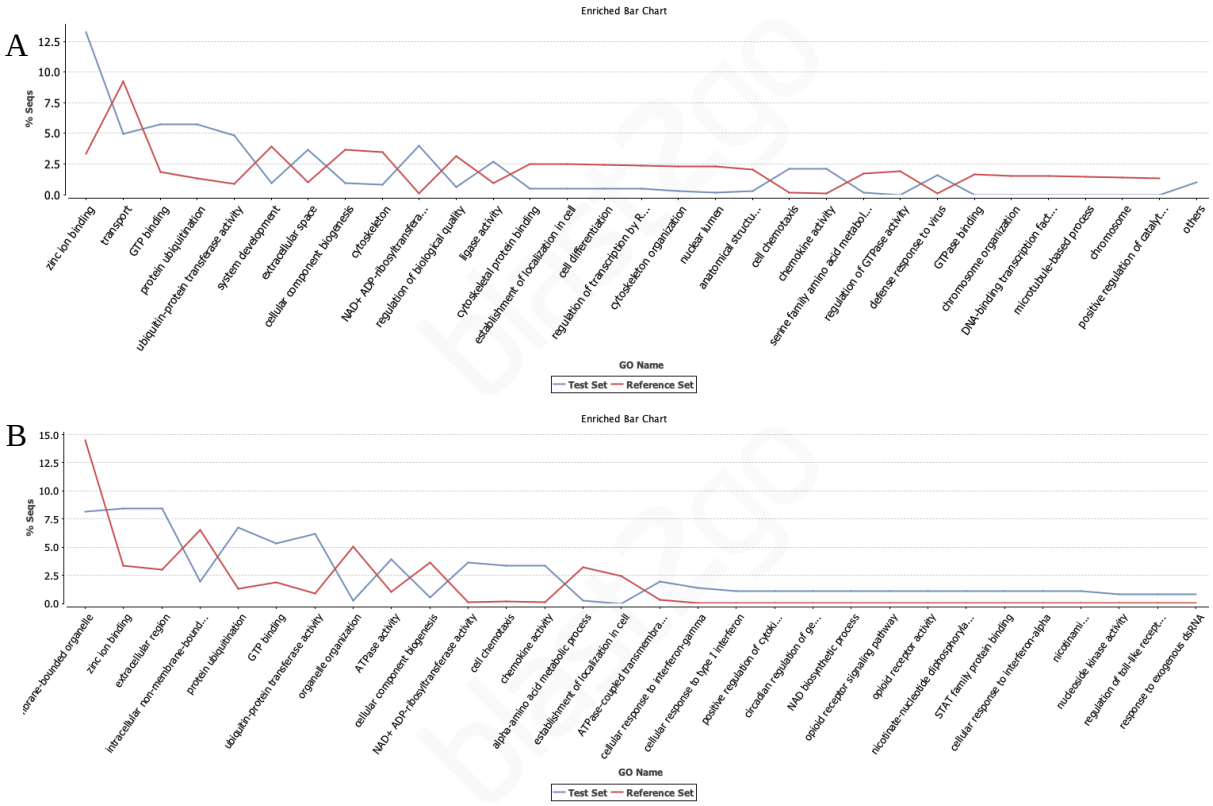

Figure 4.8. Top 30 most enriched GO terms based on differentially expressed genes in a selected strain of rainbow trout infected with Infectious Hematopoietic Necrosis Virus (IHNV). GO enrichment analysis was conducted separately for DEG based on comparisons of sham controls to IHNV infected fish (A), early versus late stages of infection (B), and IHNV infected select strain trout versus infected commercial trout (no significant GO enrichment). The full annotated rainbow trout transcriptome served as the Reference Set with a list of transcripts representing the DEG serving as the Test Sets. Only significant (FDR ≤ 0.05) over- or under-enriched GO terms are listed including biological process, molecular function, and cellular components categories. Terms with greater percentage in Reference Set are stable across the experimental factor, while the opposite indicates over-enriched functions.

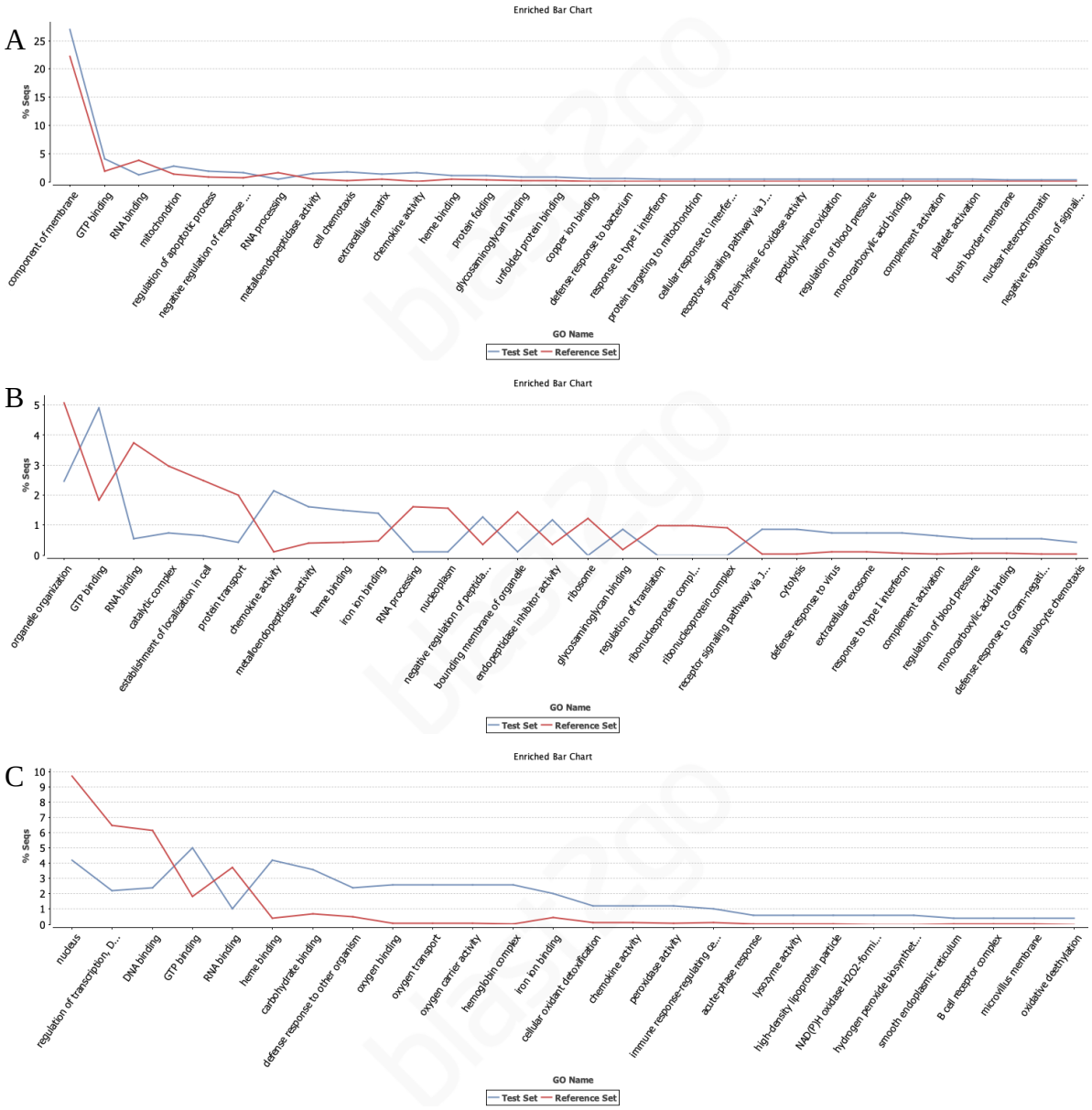

Figure 4.9. Top 30 most enriched GO terms based on differentially expressed genes in a selected strain of rainbow trout infected with *Flavobacterium psychrophilum* (Fp). GO enrichment analysis was conducted separately for DEG based on comparisons of sham controls to IHNV infected fish (A), early versus late stages of infection (B), and *Fp* infected select strain trout versus infected commercial trout (C). The full annotated rainbow trout transcriptome served as the Reference Set with a list of transcripts representing the DEG serving as the Test Sets. Only significant (FDR ≤ 0.05) over- or under-enriched GO terms are listed including biological process, molecular function, and cellular components categories. Terms with greater percentage in Reference Set are stable across the experimental factor, while the opposite indicates over-enriched functions.

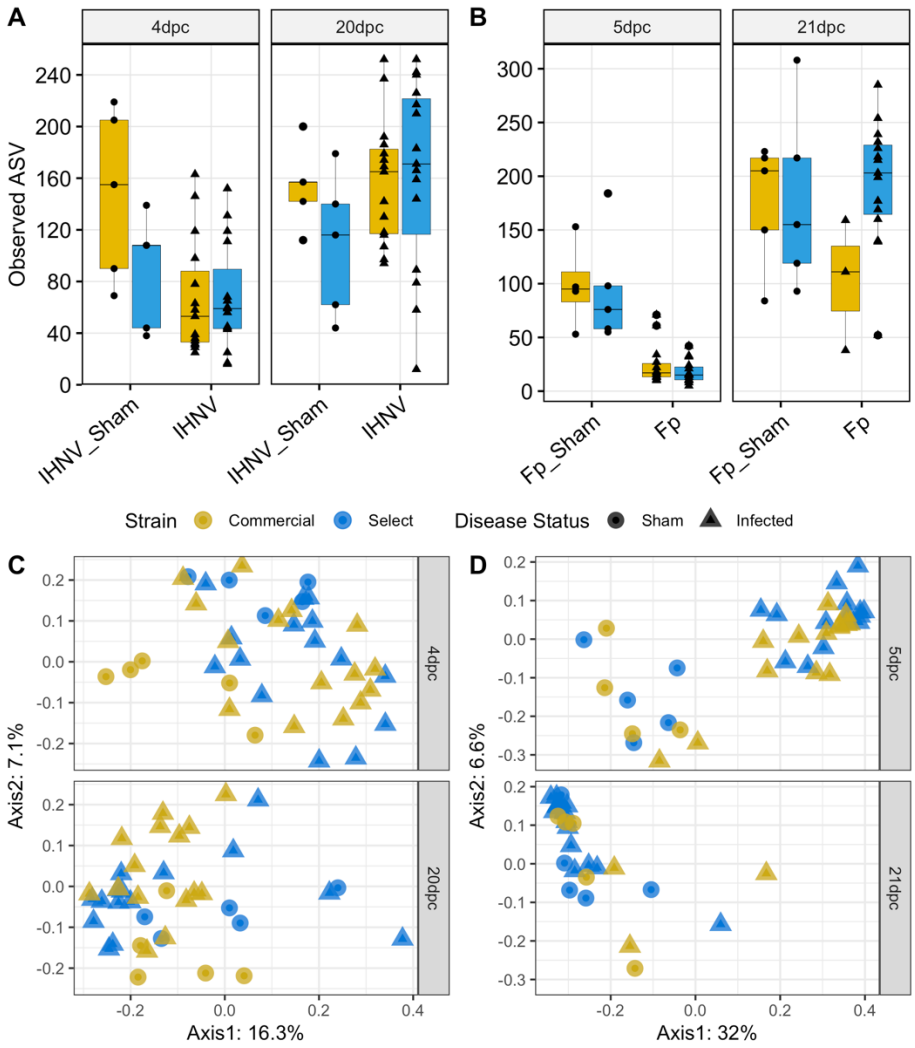

Figure 4.10. Summary of results on gut microbiota during disease challenge with IHNV (A and C) or *Flavobacterium psychrophilum* (B or D) in two strains of rainbow trout. Within sample richness is displayed as observed ASV (A-B) and between sample diversity is displayed as uwUniFrac principle coordinated analysis plots (C-D). Samples were collected from two strains of trout (Commercial vs. Select) at early (4 and 5 days post challenge) and late (20 and 21 dpc) timepoints. Sham samples (n = 5) were collected from mock challenged individuals, will infected sample (n=15) were collected from fish infected with IHNV (A and C) or *Fp* (B and D).

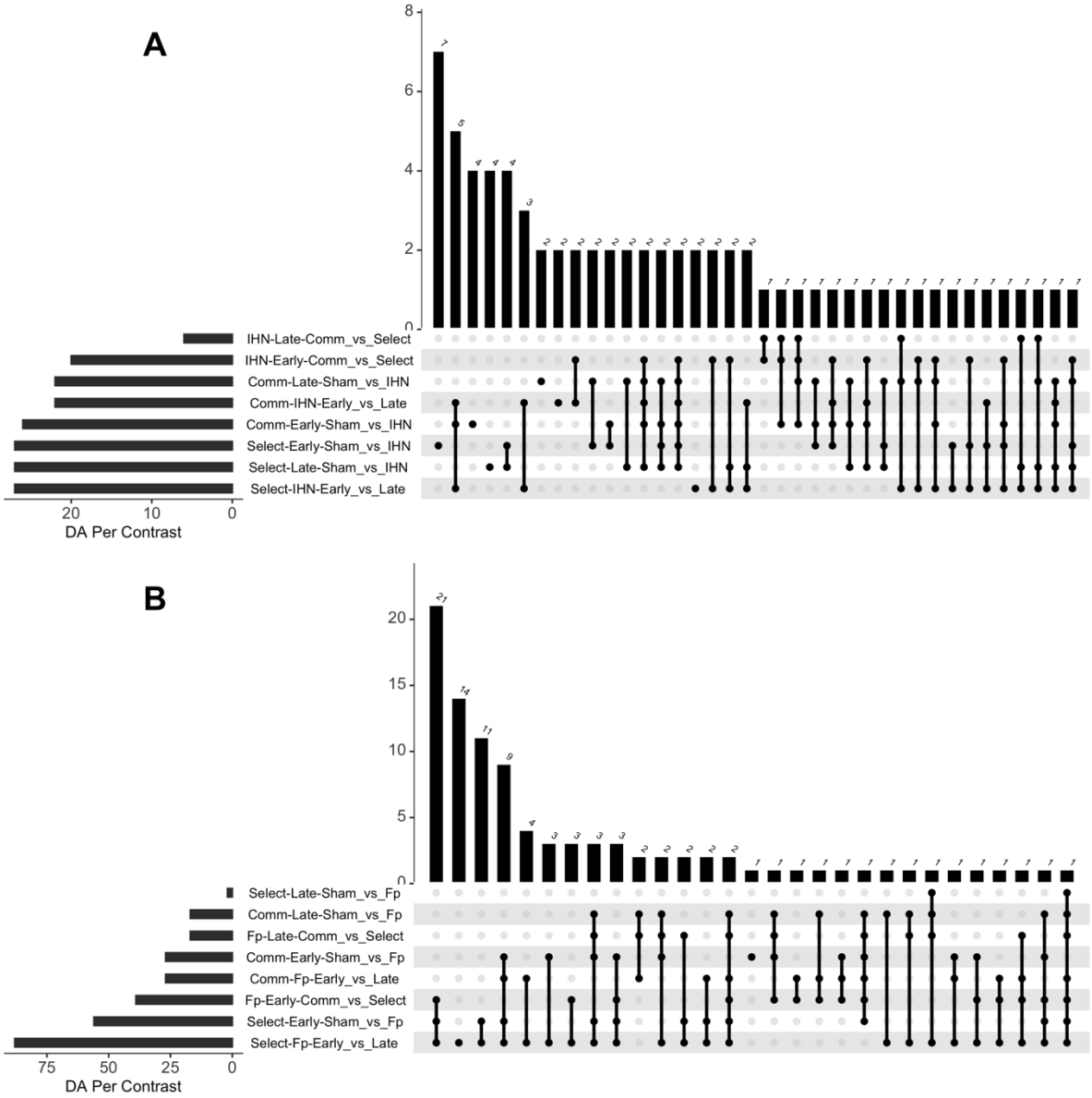

Figure 4.11. Upset plots depicting the overlap in differential abundance (DA) of bacterial genera in the intestine of rainbow trout across experimental group comparisons during infectious hematopoietic necrosis virus (A) and *Flavobacterium psychrophilum* (B) disease challenges. Horizontal bars show the number of DA by experimental group comparison and vertical bars show the number of overlapping and unique DA according to comparison groupings listed in the dot plot, as an alternative approach to Venn diagrams.

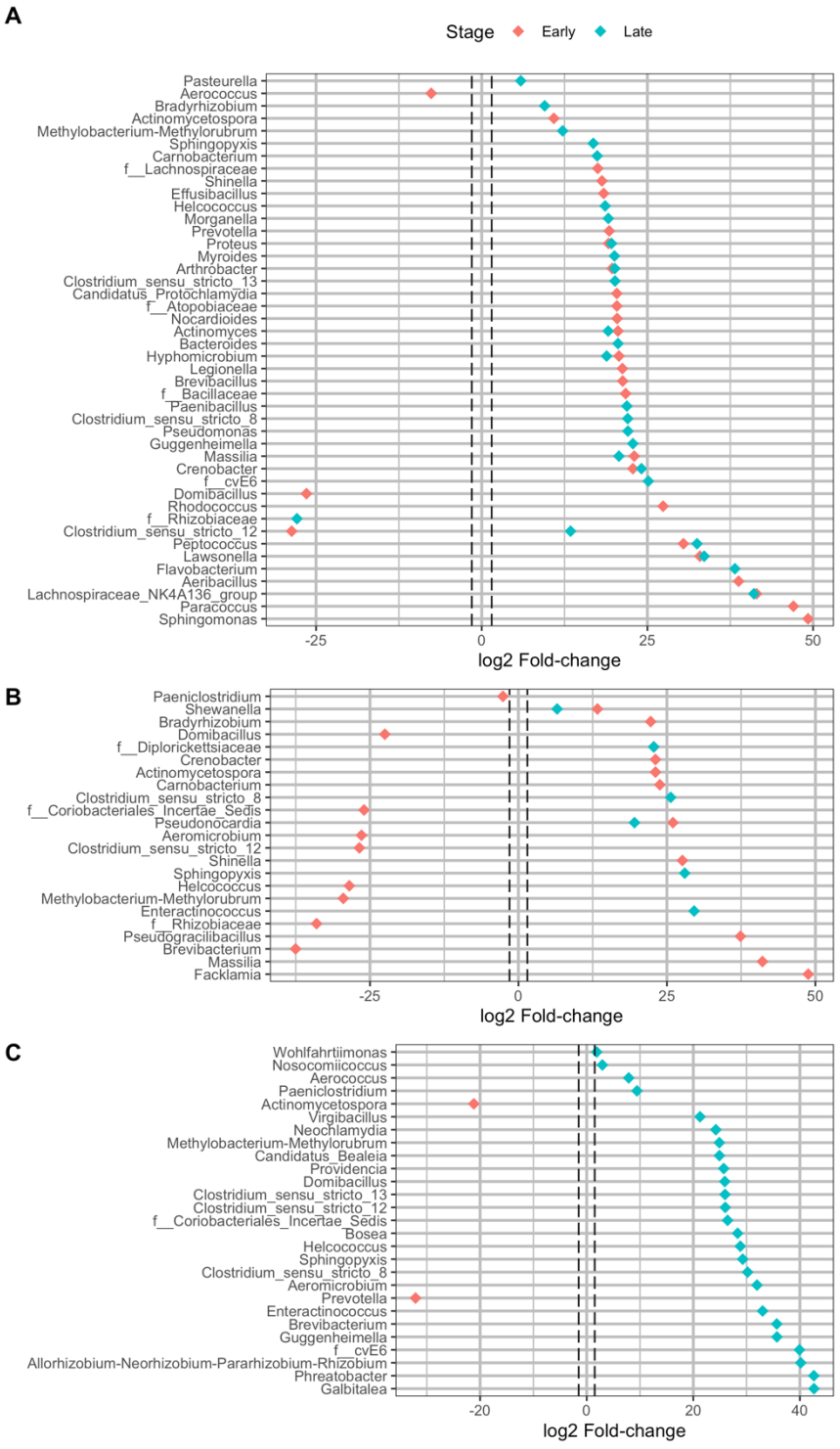

Figure 4.12. Genus level differential abundance analysis of the gut microbiota of rainbow trout infected with IHNV. Plots show: the influence of IHNV infection (positive lfc) compared to sham controls (negative lfc) in select trout (A), comparisons between a commercial (negative lfc) and select strain (positive lfc) of trout infected with IHNV at early and late stages of infection (B), and changes in the gut microbiota of select strain trout infected with IHNV at early (negative lfc) versus late (positive lfc) stages of infection (C). Microbial abundance was agglomerated at the genus level prior to DA analysis with DESeq2 (FDR corrected $p \leq 0.05$ and log2 fold-change > | 1.5 |.

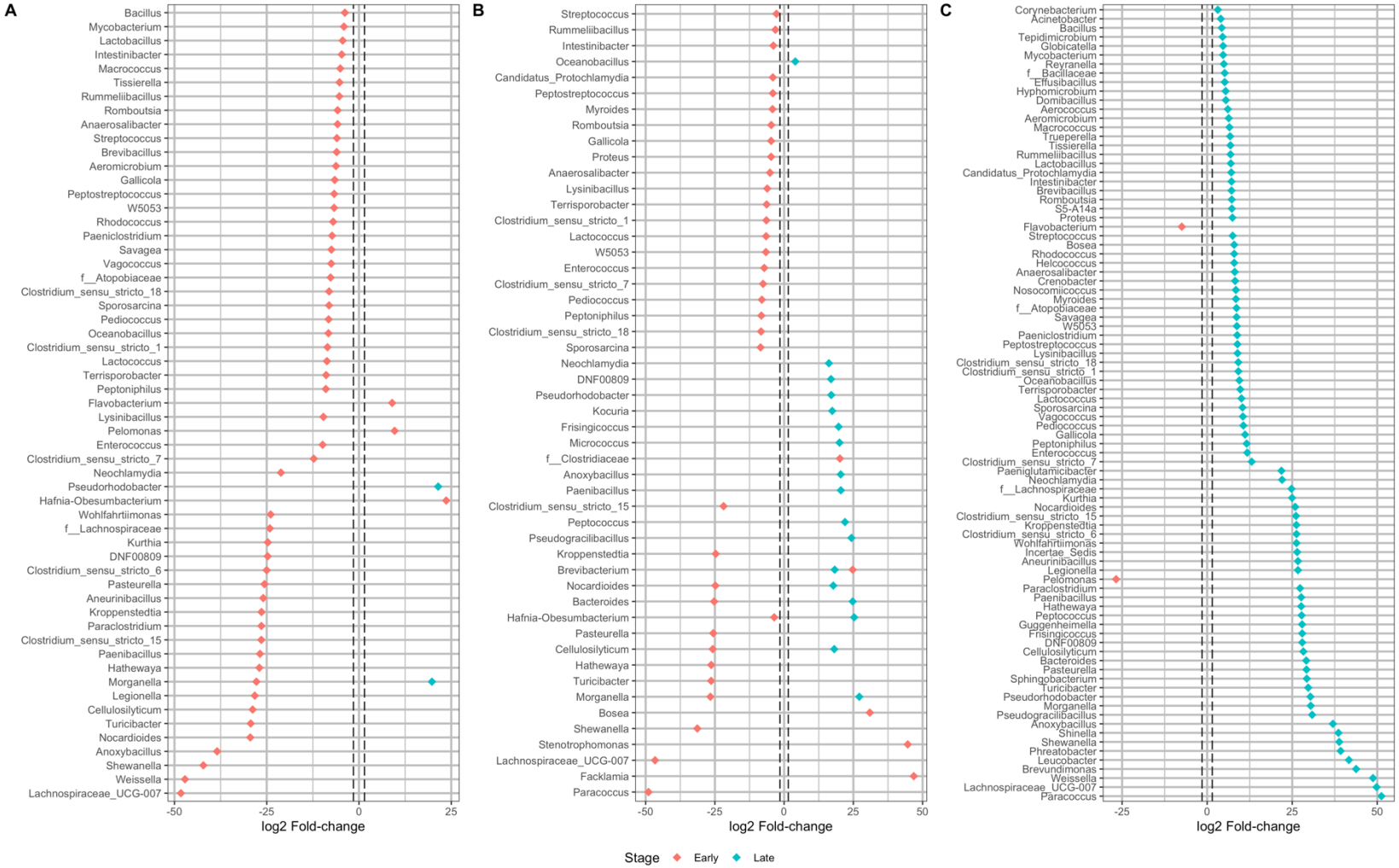

Figure 4.13. Genus level differential abundance analysis of the gut microbiota in two strain of rainbow trout infected with *Flavobacterium psychrophilum*. Plots show: the influence of *Fp* infection (positive lfc) compared to sham controls (negative lfc) in select trout (A), comparisons between a commercial (negative lfc) and select strain (positive lfc) of trout infected with *Fp* at early and late stages of infection (B), and changes in the gut microbiota of select strain trout infected with *Fp* at early (negative lfc) versus late (positive lfc) stages of infection (C). Microbial abundance was agglomerated at the genus level prior to DA analysis with DESeq2 (FDR corrected $p \leq 0.05$ and log2 fold-change > | 1.5 |.

Chapter 5: Conclusion

Synthesis of Research Findings and Collective Implications

To date, our understanding of host-microbiota-environment interactions in finfish is relatively poor in comparison to that of other important agricultural and biomedical model species. Therefore, the aim of the dissertation presented here was to advance our understanding of host microbiota interaction in commercially important salmonids, across multiple sources of variation that are commonly experienced in aquaculture production. The individual research chapters reported here offer novel insights on their own, though when taken in concert, trends across these studies may yield even more pragmatic implications. Both applied and basic, the implications for these studies will impact multiple areas of research related to aquaculture production of finfish.

Aquaculture with Microbial Ecology in Mind

With the many considerations that aquaculture practitioners must keep in mind throughout a production cycle, it is not surprising that the beneficial roles that commensal host associated microbes can play on the physiological function of finish is often overlooked. In fact, many traditional practices in aquaculture that are aimed at protecting against pathogenic microbes can have rather drastic unintended consequences on the mutualistic and commensal finfish microbiota. For example, while treatment of eggs with iodine, formalin, or hydrogen peroxide has greatly reduced the negative impacts of virulent bacterial and fungal egg pathogens, this process also severely perturbs the natural transfer and successional development of mucosal microbiota (DeSchryver et al., 2014). While the richness and diversity of gut microbiota detected in rainbow trout fry at early life stages (20 and 65 days post hatch [dph]) were stable in the research presented in Chapter 3 (Figure 3.2 A), there was an influx of traditional gut microbiota detected at 65 dph compared to the earlier timepoint (Figure 3.2 H). Because the trout eggs in that study were sterilized by iodine and formalin baths at various stages of development, following standard industry practices, it may suggest traditional microbiota that colonize salmonids are removed by disinfection processes early in life, though these commensal organisms can be slowly recruited from the environment to eventually recolonize host tissues. In agreement with this paradigm of microbiota recovery, in Chapter 4, viral and bacterial infections showed evidence that disease led to dysregulation

of gut microbiota communities; however, gut microbiota communities appeared to return to profiles similar to that of uninfected controls by the late recovery stages of the infections (Table 4.4; Table 4.5; Figure 4.10 C-D). This certainly is not to say that severe perturbations to microbial ecology simply delay or temporarily offset the natural microbiota colonization processes, as there is plenty of evidence that such perturbations to homeostatic microbiota colonization have lifelong negative impacts as well (Zaneveld et al., 2017). Regardless, aquaculture practitioners are likely to see benefits from remaining cognizant of the effects that their actions have not only on their finfish stocks, but also their associated beneficial and mutualistic mucosal microbiota. While a complete understanding is certainly still lacking, we are approaching a level of understanding related to fish health management and teleost microbiota that will soon serve to offer aquaculture producers more concrete recommendations for controlling pathogens without having such negative consequences on non-target microbes.

As reviewed in Chapter 1, direct-fed microbial probiotics and prebiotics have received a great deal of attention lately, though in many cases the products tested are not based on biologically motivated hypotheses. Instead, pre- and probiotic products used in aquaculture research are often simply products that were originally developed for terrestrial livestock (Van Doan et al., 2020). Utilizing high resolution untargeted culture independent microbiota data, similar to that generated here, allows us to prescreen the types of bacterial probiotics that are likely to be successful in colonizing mucosal tissues of the target species. Such an approach can detect natural finfish host-associated probiotic candidates, which have been shown to be more efficacious in improving fish performance (Van Doan et al., 2020). Furthermore, probiotics in aquaculture have been predominantly focused on direct-fed microbials aimed at the gut mucosa; however, the important functional roles that the microbiota of other mucosal tissues, such as the skin and gill, play on host physiology, as was reported in Chapter 3 (Figure 2.6), suggests that probiotics targeting other mucosal tissues may be advantageous as well. Finally, comparing the gut microbiota of a genetic strain with proven superior physiological performance, alongside a control line, enables detection of candidate microbes that potentially are associated with gains in host performance (Figure 3.2 G; Figure 4.12 B; Figure 4.13B). Although, it should also be noted that in all three research chapters presented here, there was much greater intra- and interindividual

variation in microbiota data with less reproducible trends, as compared to host transcriptional responses. This suggests that while potentially serving advantageous roles, microbiota manipulations will likely not achieve the same gains as could be achieved through improvements to host germplasm lines, at least based on our current knowledge.

Implications on Fish Health Management

In addition to direct-fed microbial probiotics, the broader category of functional feed ingredients has also attracted much research attention in aquaculture, which include non-nutritive compounds like pre- and probiotics, as well as nutrients that have functional benefits when fed above basal nutritional requirements (Waagbo and Remo 2020). In the first research chapter presented here (Chapter 2), functional feed ingredients (mannan-oligosaccharides and coconut oil) were found to only minorly impact the microbiota and have no impact on sea lice resistance in near market sized Atlantic salmon (Figure 2.2; Figure 2.3). Conversely, while studies are certainly mixed, some research has shown functional feeds to have positive impacts on both gut microbiota (Hoseinifar et al., 2019) and sea lice resistance (Barrett et al., 2020). While no dietary additive treatments were included in the research presented here in Chapter 3, the commercial trout fry starter diet utilized in that study is known to contain pre- and probiotic supplements, which were confirmed by the 16S rRNA gene sequencing results (Figure 3.2 E and F). In addition, many of the probiotic bacteria that were found to be abundant in the diet were also detected at relatively high abundance in the gut of the rainbow trout fry receiving that diet (Figure 3.2 F), potentially showing a benefit of the functional ingredients on gut microbiota composition. This suggests that the impacts of functional feeds on fish microbiota may be age dependent, as only very minor effects were detected in large market sized Atlantic salmon, but significant evidence of probiotic transfer was detected in rainbow trout first feeding fry.

Despite the lack of effects of functional feeds on microbiota and lice resistance in Chapter 2, the dietary treatments did show significant impacts on expression of some immune related host genes (i.e. CD4, FOXP3, IgT, MHC2, IL10, IL17a, and MBLc - Figure 2.8; multivariate profile - Figure 2.9) across various mucosal tissues. Similarly, Tacchi et al. (2011) found a diet containing nucleotides, two prebiotics, and two vitamin functional feed additives to reduce protein turnover rates, plasma protein expression, and innate immune

response in the liver and muscle of 65 g Atlantic salmon. Those authors suggested these effects would lead to reduced energetic costs and improved growth rates; however, such effects may also have negative consequences under various different conditions. While functional feed ingredients do show evidence for improved finfish physiology, the contradictory and often minor effects of functional feeds suggest further research is still required to optimize their utilization in aquafeeds.

Offering superior gains in immune modulation to nutritional additives, vaccine development holds great potential to overcome many of the issues related to disease in aquaculture. There has been notable success in vaccine development in aquaculture over the last forty years, with some twenty-four commercially licensed vaccines now available for various diseases (Adams 2019). Though, there are certainly significant hurtles related to aquaculture vaccine development that have delayed the commercialization of more vaccine treatments in aquaculture. The challenges in vaccine development in aquaculture include cost of development, lack of effective adjuvants, lack of fundamental understanding of immune responses related to adaptive immunity, as well as the cost of intraperitoneal (IP) injection delivery methods (Adams 2019). While not directly applicable to vaccine development, the results on mucosal immune responses of salmonids in the works presented here offers potential insights to strengthen our attempts to properly vaccinate finfish. Recently, the mucosa of aquatic species has become a target for vaccine development due to our improved understanding of the important role that mucosa associated lymphoid tissues (MALT) play in adaptive immunity at both the local and systemic level, as well as the potential ease of delivery for mucosal vaccines compared to vaccines which require IP injection (Wilson et al., 2020). In Chapter 2, the expression of a range of genes related to innate and adaptive immune signaling, which have relevance in vaccine development, were characterized and compared across the three primary mucosal tissues (gut, gill, and skin) (Figure 2.8). Such data can be utilized to determine which mucosal tissues may offer the most robust response to vaccination. For example, MHC2, a biomarker of antigen presenting cells that are required for initiation of adaptive immune response, was found to be highest in the gut compared to the gill, skin, and even peripheral blood leukocytes. Also, the expression of B-cell immunoglobulin (Ig) receptors, which serve a critical role in effective adaptive immune response, were shown here to be most highly expressed in systemic circulation; however,

CD4 and FOXP3 T regulatory cell markers, which also play critical roles in vaccine response showed mixed expression levels across the tissues (Figure 2.8). Furthermore, the data from Chapter 4 related to the response of the gut associated lymphoid tissue (GALT) of rainbow trout in response to viral and bacterial infection challenge, highlights the classical viral and bacterial pathogen responses that occur in the intestine, even when the pathogens are not known to specifically target that mucosal tissue (Table 4.6; Table 4.7). Such results may suggest that even pathogens that cause disease by infecting central immune tissues can be properly vaccinated against through mucosal delivery methods. This is validated by results from a vaccination study in channel catfish *Ictalurus punctatus* by Zhang et al. (2017) that showed that a protective systemic response to vaccination against *Flavobacterium columnare* could be achieved by mucosal delivery, and gill mucosal associated lymphoid tissue (GiALT) transcriptional profiles of vaccinated individuals showed an alteration in genes related to sensitization and proliferation of secretory cells. Together, such data will serve to increase our basic understanding of adaptive immune response, both centrally and in peripheral MALT, while also potentially enabling more effective mucosal vaccine delivery strategies, both of which will bolster out ability to generate effective commercial vaccines.

Findings Related to Genetic Selection

Selective breeding holds possibly the greatest potential to improve production efficiencies of aquaculture. The ARS-UI select trout, developed through a selective breeding program involving a collaboration between the USDA-ARS and University of Idaho, is a great example of the gains that can be made through selective breeding of finfish. While the selection program has focused strictly on selecting families based on growth performance on sustainable all plant-protein diets, select strain progeny also exhibit other superior phenotypes. The strains superior growth on traditional fishmeal-based diets, as well as, alternative sustainable plant-based diets, has been repeatedly shown (Overturf et al., 2013; Blaufuss et al., 2020; Lee et al., 2020) and over eight generations growth rates have nearly doubled in comparison to the founding strains (Abernathy et al., 2017). The results presented in Chapter 2 add to this evidence by showing that the select strain also grows more rapidly at early stages of ontogenetic development, in comparison to a commercial line of rainbow trout (Figure 3.1). In addition to growth performance, the select strain shows resistance to the

development of soybean meal induced intestinal enteritis, with previous studies showing differences in targeted intestinal gene expression, intestinal histology, and gut microbiota compared to a control strain (Venold et al. 2012, Blaufuss et al., 2020). While dietary effects were not explored in the studies on the select strain that are reported here, the results certainly support the paradigm that the select strain displays unique and adaptive transcriptional responses and associated differences in bacterial microbiota (Chapters 3 and 4). In addition, as the first published comparison of the select strains resistance to viral and bacterial disease challenges, the results of Chapter 4 provide clear evidence that selection has also improved innate immune responses in the ARS-UI fish. In comparison to a commercial strain, the select trout exhibited repeatable elevations in serum lysozyme activity and significantly less susceptibility to mortality when challenged with *Flavobacterium psychrophilum* (Figure 4.2 F; $p \leq 0.0001$). Such results suggest more efforts in aquaculture should be focused on achieving gains through selective breeding.

Currently only about 10% of aquaculture production utilizes stocks improved through genetic selection, although encouragingly when properly executed the gains reported from selection of aquatic species often substantially outweigh that seen for terrestrial livestock (Yanez et al., 2015). As aquaculture researchers work to advance selective breeding programs, genomic information can provide valuable insights on selection criteria. Genomic signatures of selection, or regions of the genome that contain adaptive functions related to observable variation in the phenotype under selection, are most commonly identified through methods such as quantitative trait loci (QTL) mapping or genome wide associate studies (GWAS) that rely on dense genome-wide single polymorphism (SNP) data. Although, transcriptomics can also be a very powerful tool for identifying adaptive signatures of selection at the level of transcription (Abdelrahman et al., 2017). Comparing signatures of genetic selection identified across the three experiments conducted in Chapters 3 and 4 makes findings more robust, as differential traits that are repeatedly detected are likely not false-positives and are of functional relevance. Of note, analysis of intestinal transcription in the select strain compared to a commercial control line across developmental ontogeny and viral or bacterial disease challenge highlighted repeating themes. Genes related to immune cell surface receptor signaling (*i.e. CD4 and CD5 expression, immunoglobulin leucine-rich repeats, B-cell receptor expression, cytoxic and memory T-cell function, antigen processing*

and presentation, DBIRD complex, NOD-like receptor expression, etc.), cytokine signaling (*i.e. IFNγ transcription, acute-phase response, macrophage migration signaling, etc.),* mineral (*i.e. iron and zinc binding*), peptide (*i.e. carboxypeptidase, neutral amino acid transporter, proteosome, etc*.), lipid (i.*e. linoleic fatty acid metabolism, fatty acyl-CoA synthesis, HDL particles, etc*.) and energy (i.e. *ATP binding, glycerol-3-phosphate dehydrogenase,* etc.) metabolism, as well as intracellular genomic machinery (i.e. *endonucleases, polymerase, ribosomal RNA production, nucleotide excision repair, etc.*) were all found to be repeatedly detected biomarkers for selection in the ARS-UI strain (Table 3.3; Table 4.2; Table 4.3; Table 4.7). In a separate study previously conducted by Abernathy and others (2017), RNA sequencing of liver and muscle samples was used to detect biomarkers for selection of the ARS-UI strain and despite the differences in bioinformatic methods and tissues analyzed those results show much overlap with the genes and biological functions identified here. Together these works provide valuable insights on the physiological mechanisms behind the performance of the ARS-UI select strain and lends knowledge to future attempts at genetic selection in aquaculture.

Evidence of Adaptive Differential Transcript Usage

From genome to phenome there are multiple compounding molecular mechanisms that an organism utilizes to physiologically cope with developmental and environmental pressures. Allelic diversity (Campbell et al., 2014), genome chromatin structure and epigenetic regulation (Marandel et al., 2016; Panserat et al., 2017), regulatory RNA expression (Paneru et al., 2018), gene expression (Abernathy et al., 2017), protein expression and modification (Martin et al., 2003) and metabolomic shifts (Palma 2019) have all been shown to be molecular mechanism by which salmonids control their phenotypic responses. Advances in untargeted transcriptome sequencing and bioinformatic methods have now enabled analysis of genome wide isoform usage that suggests differential transcript usage (DTU) is yet another adaptive molecular mechanism utilized by organisms. For examples, while evaluating transcript usage in zebrafish embryos pre- and post-activation of zygotic transcription, Aanes et al. (2013) detected hundreds of genes with DTU, with many involved in cell-cell communications critical for embryonic ontogeny.

Transcriptome wide analysis of isoform usage has not been conducted in fish species other than zebrafish prior to that which was reported here (Qian et al., 2014), at least to the authors knowledge. Here, evidence was provided that DTU is an adaptive molecular mechanism that is utilized by rainbow trout to cope with ontogenetic development (Table 3.4) as well as viral (Table 4.2) and bacterial infections (Table 4.3). In addition, it was repeatedly shown that genetic selection led to detectable differences in isoform usage (Table 3.2; Table 4.2; Table 4.3). In many cases, the significant DTU events were in genes known to play important roles in organismal response to the experimental factors being tested, suggesting these isoform switches are not occurring by happenstance. Recently, Vaneechoutte and others (2017) combined 26 publicly available transcriptomic datasets on the model plant species *Arabidopsis thaliana* and detected reproducible patterns of DTU for some 8,148 genes; however, of those only about 1 in 5 caused direct changes to protein sequences. Therefore, more detailed analyses of the DTU candidates presented in the research chapters here will be needed to determine which of these isoform switching events have consequence on protein sequence and phenotypic function.

Shortcomings and Future Considerations

Targeted (RT-qPCR) and untargeted (RNA sequencing) gene expression profiling, as was done here, is a powerful molecular technique that provides a snapshot of the transcriptional activity of tissues or cell populations in response to experimental factors. However, while interpreting such data one must be cognizant that transcriptional responses do not always correlate to equivalent consequences on phenotype. For some genes, transcript levels serve as a valid proxy for protein expression, although for many genes the succession from transcript to phenotype is rather convoluted and involves multiple regulatory steps, which are overlooked by focusing analayses at the level of transcription. Processes related to post-transcriptional modification, translation initiation, post-translational peptide modification and trafficking, protein-protein interaction, as well as, protein ubiquitination can all muddle the quantitative relationship between transcription and phenotype, with studies from model organisms suggesting that only about 40% of the variation in protein expression can be explained by mRNA levels (Martin et al., 2016). While characterization at the protein level would be advantageous, current hurtles related to cost and data

interpretation in high-throughput un-targeted proteomics makes transcriptomics still one of the most attractive methods for studying organismal responses (Martin et al., 2016). However, as costs decrease and unsupervised data interpretation methods improve, proteomic data is likely to increase the accuracy and resolution of future studies.

Recent efforts to advance genomic resources in aquaculture have made great strides and annotated genomic and transcriptomic references are now available for many of the most valuable species in aquaculture. However, in comparison to other research models (i.e. man, rat, mouse, chicken, pig, cattle, zebrafish, *Arabidopsis*, *Saccharomyces, E. coli,* etc.) gene annotations and functional characterizations are still rather sparse in aquaculture finfish. While the gene ontology analysis utilized in the research presented here offers useful information on gene functions by utilizing BLASTx searches against a large database of well annotated proteins from model and non-model organisms, similar analysis in more well studied species requires much less effort and yields more resolute insights. In addition, highly annotated genomes enable more fruitful pathway analyses that help synthesize transcriptome-wide expression findings into meaningful biological interpretations. While similar pathway analyses can be conducted in non-model organisms by reannotating data with hits to the most similar genes in model organisms, much resolution is lost in such an approach due to the appreciable phylogenetic distance between teleost and man or rodent models. Nevertheless, as high-quality nucleotide sequencing data continues to become easier to generate and analyze it is expected that genomic resources related to aquaculture species will continue to become more sophisticated, in turn yielding even more effective interpretations of molecular data.

Characterizing microbiota composition using 16S rRNA gene sequencing has its weaknesses as well. Focusing on one region of the 16S rRNA, as was done in the work presented here, can have limited power to discriminate between some bacteria with high sequence homology. It should be noted that much work was taken to ensure the V3V4 16S rRNA gene sequencing primers used in the present work (Table 2.1) were the most effective for characterizing salmonid microbiota. However, using targeted amplicon sequencing to characterize bacterial communities lacks any definitive information on the functional capacity of the microbiome. Although, functional prediction techniques, such as the PICRUSt2 analysis performed in Chapter 2, have greatly improved over previous

implementations and, as was shown here can provide rich inferences on bacterial function in the absence of metagenomic data. Furthermore, full length 16S rRNA gene sequencing and shotgun metagenomics are becoming more practical with third generation long read sequencing, such as that of PacBio® (Pacific Biosciences, Menlo Park, CA, US) and Oxford NanoPore® (Oxford NanoPore Technologies, Oxford, UK). One problem that all molecular approaches to microbiota characterization face is the difficulty in discriminating nucleic acids from the targeted viable microorganisms from off-target quantification of transient DNA contamination from the environment or non-viable bacteria. For this reason, some researchers isolate microbial RNA from the sampled environment and conduct 16S rRNA gene sequencing following cDNA conversion, to ensure that only nucleic acids from microbes that are actively transcribing will be quantified (Legrand et al., 2018). Viability-PCR can also be utilized to overcome this issue, by first treating raw sample material with propidium monoazide prior to DNA extraction to render nucleic acids not protected by competent cell walls incapable of PCR amplification (Cenciarini-Borde and La Scola 2009). Additionally, advances in flow cytometer have made it a rather valuable tool for quantifying viable microbiota communities (Duygan et al., 2020), though this method has its own limitations as well.

Typically, genomics assisted genetic selection requires a relatively rich panel SNPs, which are commonly first identified using whole genome sequencing or reduced representation methods such as RADseq. However, it is also possible to identify SNPs using transcriptomic data (Zhao et al., 2019), which can focus SNP discovery to within functional protein coding regions of the genome when using mRNA selected transcriptomic data, like that generated here. Therefore, the wealth of RNA sequencing data generated in the work presented here could serve as a very useful resource for discovery of SNPs in rainbow trout, and more specifically the ARS-UI select strain. Salem et al (2012) showed that even low depth (3-6 million reads population^{-1}) RNA sequencing data could be utilized to identify SNPs, as they detected 54 SNPs with 22 of those showing significant association with differences between slow and fast growing populations of rainbow trout. Interestingly, those authors also showed that 48 of those 54 SNPs were also polymorphic in outgroups of trout broodstock, including the ARS-UI strain (Salem et al., 2012). In the future it would be useful

to further analyize the datasets presented here to potentially confirm and add to the list of previously detected SNPs.

In the disease challenge trials in Chapter 4, mild mortalities were observed in viral IHNV challenges, while excess mortality in the bacterial *Flavobacterium psychrophilum* challenge slightly biased comparisons between the commercial and select strain at the late stage of infection by leaving too few surviving fish to sample. This highlights the importance of considering pathogen dose while conducting experimental disease challenges. In future studies, research similar to that conducted here should be conducted across a range of doses and include later sampling timepoints to determine whether signatures of the effects of disease on the microbiota and host immune system linger in surviving individuals even after disease is overcome. Such findings would have valuable applied implications on aquaculture production, where cohorts of fish are often exposed to disease outbreaks. As such, a better understanding of any persistent impacts of acute infection on surviving fish would be useful.

While the results presented here are some of the most resolute data on host-microbiota interactions in an aquaculture species, difficulties in comparing multi-omics datasets with meaningful biological interpretations limits the utility of the findings. The Procrustes analysis used throughout the research chapters presented here is an ideal test, similar to but more powerful than Mantel's Test, for correlating multivariate datasets across response variables, such as mapping bacterial function to bacterial phylogeny (Figure 2.7) or comparing host gene expression responses to microbiota community dynamics (Figure 2.10; Figure 3.5 B). However, this technique only compares multivariate responses as a whole and cannot identify interactions between specific genes and bacteria. A recent study on the interactions between the gut microbiome and host colonic gene expression in human patients with cystic fibrosis used Spearman rank correlations to conduct thousands of FDR corrected pairwise correlations between expression of differentially regulated genes and genus level microbiota abundance (Dayama et al., 2020). Those authors then built a covariance network of significantly correlated gene-microbe interactions to identify bacterial genera that showed positive and negative associations with biomarker gene expression (Dayama et al., 2020). Host-microbe bipartite covariance networks, similar to the microbe-microbe sparse inverse covariance networks constructed in Chapter 2 (Figure 2.5), have been utilized to identify interactions between host genes and specific microbiota as well, though there are many

considerations to conducting such analysis effectively (Jiang et al., 2019). The research presented here could benefit from similar analyses; however, one must thoughtfully consider potential for biases that can be induced by applying inappropriate filtering and transformations to the respective datasets prior to correlation and their respective effects on interpretations of results. While such correlations do not substantiate any causative link between host genes and microbiota, they would provide targeted hypotheses for more detailed future research. As mentioned above, the decreasing cost and analytical burden of generating and interpreting multi-omics datasets are sure to provide an increase in studies relating host and microbiota data in more insightful ways.

The best methods for proving causative relationships between host physiology and mucosal microbiota involve axenic, gnotobiotic, or antibiotic depleted models in a fashion similar to reverse genetics, which has been so useful in describing gene functions. Unfortunately, other than seminal works from Rawls and others (Rawls et al., 2004; Rawls et al., 2006; Kanther et al., 2010; Semova et al., 2012) conducted in zebrafish, few studies have taken similar approaches to delineating the role of microbiota in aquaculture fish species. To date many aquaculture practitioners and researchers overlook or ignore the physiological relevance of mucosal microbiota communities; however, when designed properly data from such experiments on the functional roles of microbiota often provide insights that are difficult to overlook (Semova et al., 2012). Therefore, there is still a need for more microbiota studies that go beyond simply observing responses to natural sources of variance, as was done here, and begin utilizing thoughtful targeted manipulations of finfish microbiota to gain a more resolute understanding of mucosal microbiota function.

Concluding Remarks

The results presented here offer novel insights into the effects that factors such as dietary ingredients, mucosal tissue, host genetics, ontogenetic development, and viral or bacterial infection have on salmonid gene expression and mucosal bacterial microbiota, as well as their interactions. Each of the three studies reported here offer valuable insights that will have short- and long-term impacts on aquaculture production; however, when taken together trends across such studies offer even more concrete insight that will have broad applied and basic implications related to microbial control in aquaculture, development of

effective probiotic and functional feed supplementation, fish health management and vaccine development, selective breeding and genomic selection, as well as, molecular organismal transcriptional responses (i.e. adaptive DTU detection), and basic research related to host-microbiota-environment interactions. These findings have useful insights for aquaculture researchers and academics, commercial aquafeed and fish health companies, aquaculture farmers and operations managers, as well as more broad basic implications for comparative organismal biology researchers. While these data provide valuable insights on host-microbiota interactions and their physiological relevance to salmonid aquaculture, continued advances in our research capacities is expected to help fill the critical knowledge gaps that currently exist in future studies, with recommendations offered here on important research questions which remain unanswered.

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