Impact of Brewing Industry Byproducts Used as Feed Additives for Aquaculture-Raised Fish: Studies of the Host-Microbe Relationship

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Abstract

Aquaculture, the cultivation of aquatic organisms in a controlled environment, offers both economic and nutritional benefits to human society. As there is an increased demand to feed a growing human population, many wild-caught fisheries have struggled due to the overexploitation of resources. Currently, production relies heavily on wild-caught fish to produce fishmeal to feed farm-raised fish. The demand for alternative materials in fish feeds has grown rapidly as fishmeal resources have become limited.

Antibiotic resistance emergence in aquaculture systems is another area of concern. Reducing antibiotic use via alternate prophylactic measures to increase host health is an essential area of research; modulation of the host intestinal bacterial community via prebiotics is one possibility. Prebiotics refer to non-digestible food ingredients that are thought to stimulate the growth of beneficial bacteria, consequently benefiting host health by indirectly reducing the possibility of bacterial pathogen proliferation. This occurs through various measures such as competition for space and resources. The intestinal bacterial community has a significant impact on a variety of host factors that include host development, physiology, immunity, and nutrient acquisition. In turn, there are multiple factors impacting the bacterial community, including the presence of pathogens and/or antibiotics, environmental conditions, host genetics, and the diet consumed.

To promote environmental sustainability and improve production and animal health in aquaculture, a collaboration was created with Anheuser-Busch of the brewing industry and

Maltento, a functional ingredient company. With breweries around the globe, Anheuser-Busch produces consistent, food grade byproducts that are safe for human consumption. Two of the most prevalent brewery byproducts are brewer's spent yeast (BSY) and brewer's spent grain (BSG). BSY contains a variety of beneficial nutrients such as proteins, essential amino acids, and carbohydrates. BSG is high in fiber but low in protein; however, black soldier fly larvae can be cultured on BSG to convert the low-value product into insect biomass to be used in fish feed, as insects themselves are full of beneficial lipids and proteins. The objective of the work presented in this thesis was to evaluate the efficacy of using low-value brewery waste products, converted into high-value feed additives, for aquaculture practices. Specifically, the effects of dietary feed additives on the production, health, and intestinal bacterial community of aquaculture-raised rainbow trout were examined. Inadvertently, benefits of the feed additives on fish subjected to chronic and acute thermal stress were also assessed.

Overall, the results of the study found that the feed additives did not significantly change the production efficiency of the rainbow trout, though some increase in growth was observed. When subjected to chronic thermal stress conditions, fish fed the experimental diets outperformed those fed the control diet regarding growth parameters. The intestinal bacterial community of the fish was significantly altered from the beginning of the trial compared to the end of the trial, though differences were not attributed to the feed additives. Instead, the resulting intestinal dysbiosis is believed to have stemmed from the physiological response of the fish to thermal stress conditions. When the fish underwent an acute thermal stress event, causing mortality, fish fed three of the five experimental diets were found to have higher survival rates compared to the control. Ultimately, results of this project suggest that the BSY and BSG-fed insect feed additives may have increased the health and robustness of the fish during a period of

thermal stress. However, further research under controlled conditions is needed to evaluate if the observed host health benefits can directly be attributed to the feed additives.

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General Audience Abstract

Aquaculture refers to the method of rearing aquatic organism such as fish and shellfish under controlled conditions. Within the food industry, aquaculture is one of the fastest growing sectors, and provides important economic and nutritional benefits to humans. Additionally, aquaculture is an important alternative to fisheries that rely on catching fish from the natural environment. Wild-caught fisheries have struggled due to the overfishing, and unfortunately, many aquaculture practices still rely on wild-caught fisheries to produce fishmeal used in feed for carnivorous fish. Research into alternate protein sources to use in fish feed has been on the rise. Additionally, as the emergence of multi-drug resistant bacteria continues to increase, reducing antibiotic use has become a priority across all fields whether it be healthcare or the food industry.

Within aquaculture, using alternative prophylactic measures such as prebiotics to increase animal health and disease resistance could lead to the overall reduction of antibiotic use. Prebiotics are non-digestible food ingredients believed to help the beneficial bacteria within the intestinal track to grow. In turn, the increased numbers of beneficial bacteria reduce the possibility of pathogenic bacteria invading and establishing a presence in the intestinal track. The intestinal microbiome refers to the various organisms, such as bacteria, viruses, and fungi, that live commensally within the host digestive tract. The bacterial community within the intestinal microbiome has many important roles, including effects on host development, physiology, immunity, and nutrient acquisition. Many factors also impact the bacterial community, including

the presence of pathogens and/or antibiotics, environmental conditions, host genetics, and the diet consumed.

To promote environmental sustainability and improve production and animal health in aquaculture, a collaboration was created with Anheuser-Busch of the brewing industry and Maltento, a functional ingredient company. With breweries around the globe, Anheuser-Busch produces consistent, food grade byproducts that are safe for human consumption. Two of the most prevalent brewery byproducts are brewer's spent yeast (BSY) and brewer's spent grain (BSG). These low-value waste products can consequently be converted into high-value feed additives for use in aquaculture. The objective of the work presented in this thesis was to evaluate the effects of BSY and BSG-fed insect dietary feed additives on the production, health, and intestinal bacterial community of aquaculture-raised rainbow trout. Unintentionally, benefits of the feed additives on fish subjected to chronic and acute high-temperature thermal stress were also explored.

Overall, the results of the study found that while the feed additives did not significantly increase the growth of the rainbow trout, benefits were still observed. When subjected to chronically high-water temperatures, fish fed the experimental diets outperformed those fed the control diet regarding growth parameters. The intestinal bacterial community of the fish was significantly altered from the beginning of the trial compared to the end of the trial, though differences are not believed to be caused by the feed additives. Instead, the resulting shift in the bacterial community is believed to have stemmed from the stress-response of the fish triggered by high water temperature. When the fish underwent an acute thermal stress event, which caused mortality, fish fed three of the five experimental diets were found to have higher survival rates compared to the control. Ultimately, results of this project suggest that the feed additives may

have increased the health and robustness of the fish while undergoing thermal stress. However, further research under controlled conditions is needed to evaluate if the observed host health benefits can be attributed directly to the feed additives.

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Chapter One

Literature Review

Aquaculture

Aquaculture, the cultivation of aquatic organisms in a controlled environment, is an important alternative to traditional wild-caught fisheries. As there is an increased demand to feed a growing human population, many wild-caught fisheries have struggled due to the overuse of natural resources. Overfishing leads to a reduction in biodiversity and can cause detrimental effects to ecosystems. Due to the fear of short-term economic costs, many wild-caught fisheries are reluctant to cooperate in rebuilding efforts (Worm et al., 2009). As the rate of overexploitation continues to increase, a decline in total biomass and average body size of certain fish species has become apparent. Additionally, species collapse and extinction rates remain high (Worm et al., 2009). Aquaculture has been shown to be more profitable than wild-caught fisheries and may lessen the overfishing of certain species (Diana, 2009). The "Blue Transformation" is a term used to denote to the push towards improved sustainability, food security, and production maximization in aquatic food systems (FAO, 2022).

Currently, aquaculture production relies on wild-caught fish to produce farm-raised fish, a value that can be quantified via the 'Fish-In Fish-Out' (FIFO) ratio (Kaushik and Troell, 2010). However, the estimated FIFO ratio published for certain production practices, such as salmon, may not be entirely accurate (Jackson, 2010). The FIFO ratio is often portrayed as a much higher value than reality as the FIFO ratio does not consider that unused fishmeal or fish oil gets used for production of other aquatic organisms. Instead, the current calculation considers excess as waste. It is necessary to consider the production of all organisms in combination, since in reality the leftover fishmeal or fish oil is utilized (Jackson, 2010). Regardless, aquaculture dominated worldwide consumption of fish oil and fishmeal, consuming 90% and 71% of available

resources, respectively, in 2008 (Bostock et al., 2010). The demand for alternative materials in fish feeds has grown rapidly as resources have become limited (Naya-Català et al., 2021).

Aquaculture is the fastest growing sector in the food industry, reaching annual growth rates as high as 9.5 percent, with an overall average increase of 6.7 percent between the years 1990-2020. In the past thirty years, the annual output of global aquaculture production has increased by 609 percent, and fish consumption has doubled to over 20 kg per capita. In 2020, a record high of 87.5 million tons of aquatic animals were produced primarily for human consumption (FAO, 2022). The reliance on aquaculture continues to grow rapidly, therefore, it is important to conduct research on methods that can improve efficiency, sustainability, and animal welfare in the industry. Use of alternative diets including feed additives and their influence on the fish microbiome is one active area of research.

Fish Microbiome

The microbiome is a complex, dynamic community composed of fungi, archaea, protists, viruses, and the most abundant of all, bacteria (Berg et al., 2020). Due to the extreme influence the microbiome has on physiological processes, the microbiome has even been considered as an organ (Baquero and Nombela, 2012; Anwar et al., 2019). Some essential physiological functions provided by the microbiome include protection from pathogens (Kim et al., 2017), immune system development and regulation (Cerf-Bensussan and Gaboriau-Routhiau, 2010), and nutrient acquisition and metabolism (Foster et al., 2017). Additionally, there are a myriad of factors that can influence the microbiome (Figure 1.1). Regarding fish production, some factors include salinity, temperature, geographical location, host life stage, pathogens and antibiotics, diet, and farm management (Egerton et al., 2018).

The fish microbiome has been studied far less than that of mammals, and not much is known in comparison (Llewellyn et al., 2014). Despite the limited research, the microbiome of fish has been found to be more diverse than expected and previously believed (Llewellyn et al., 2016). Compared to all other body parts of the fish, the bacterial community of the gastrointestinal (GI) tract is the most complex in terms of both diversity and density (Midhun and Arun, 2023). The bacterial community can be separated into two groups: autochthonous and allochthonous (Hao and Lee, 2004). The autochthonous bacteria, also known as indigenous, are those that colonize the mucosal surface and are therefore more stable, prevalent, and arguably important. The allochthonous, or transient bacteria, live freely in the GI tract and are considered to have a more temporary and changing association with the host (Midhun and Arun, 2023).

Different sampling methods can be used to test for the different groups of bacteria, as it has been shown that the mucosa and feces have different bacterial community compositions (Zoetendal et al., 2002; Ringel et al., 2015). Further, the makeup and metagenomic functions of the mucosa-associated bacterial community cannot be accurately obtained from solely feces, which is one of the most common sampling methods for microbiome research (Zmora et al., 2018). However, it should be noted that the bacterial community of the feces and mucosal layer cannot be completely separated and will contain overlap in the samples (Tang et al., 2020).

Core Microbiome

It is often true that different rearing environments can change the composition of the microbiome. Nevertheless, the concept of a "core" microbiome is also important. Hosts that are cultivated and maintained under the same conditions will share similar microbial taxonomic compositions (Wong et al., 2013). Some research hypothesizes that the microbiome of fish simply mimics the surrounding aquatic environment (Nayak, 2010). However, on the contrary, it

has been discovered that fish contain specialized bacterial communities just like mammals that are significantly different from the surrounding water column (Smith et al., 2015; Li et al., 2017; Lyons et al., 2017; Midhun and Arun, 2023). For example, four dominant phyla (Tenericutes, Firmicutes, Proteobacteria, and Spirochaetota) were discovered in rainbow trout across all samples despite being obtained from different rearing environments on different diets (Lyons et al., 2017). At the family level, common inhabitants of the rainbow trout intestinal tract across studies include *Mycoplasmataceae* (specifically *Mycoplasma* spp.)*, Enterobacteriaceae, Brevinemataceae,* and *Fusobacteriaceae* (Hines et al., 2023a). The GI bacterial community is important to study since it is essential for various physiological functions and has countless impacts on the host.

Molecular-based Methods to Analyze the Bacterial Community

Traditional methods used for studying bacterial diversity rely on the isolation and cultivation of bacteria in a laboratory setting. Though, these traditional methods result in bottlenecks in the estimation of bacterial diversity due to the vast majority of bacteria being unculturable using traditional methods (Cai et al., 2013). Fortunately, molecular methods such as initially Sanger sequencing, and more recently next generation sequencing (NGS), have been developed and can detect the presence of bacteria without the use of traditional culturing methods (Figure 1.2; van Dijk et al., 2014; Graspeuntner et al., 2018).

The small ribosomal subunit in prokaryotes, which contains 16S rRNA, is highly conserved and universal in all bacteria, making the 16S rRNA gene a target for DNA sequencing (Clarridge, 2004). The 16S rRNA gene is composed of both conserved and variable regions. Specifically, there are nine hypervariable regions denoted V1-9, making the choice of which primer set to use a crucial decision. Different biases are introduced according to the regions

sequenced, leading to different estimations of bacterial diversity. Generally, the V3V4 region is preferred due to the identification of more taxa and less bias, among other reasons (Cai et al., 2013; Graspeuntner et al., 2018).

The development of NGS platforms has been revolutionary, allowing for millions of DNA fragments to be sequenced in parallel. The resulting sequences can then be analyzed using bioinformatics methods (Figure 1.2) and reference genomes found in databases (Li et al., 2009; Behjati and Tarpey, 2013). One of the most popular NGS platforms is known as Illumina sequencing. Illumina sequencing was commercialized in 2005 and remains the leading platform to this day, partly due to the method having the highest throughput with the lowest cost (van Dijk et al., 2014; Illumina, 2017). Illumina sequencing employs the sequence by synthesis (SBS) method. SBS uses cleavable fluorescent nucleotide analogs as reversible terminators on DNA that is immobilized via binding to a fixed adapter sequence (Ju et al., 2006; van Dijk et al., 2014; Illumina, 2017). Additionally, the sequence is directly detected without the need for electrophoresis. Each nucleotide base has a unique fluorophore capped to the 3'-OH group which is automatically detected by a laser and sequencer (Ju et al., 2006). Overall, NGS allows for a much more comprehensive method of studying the microbiome.

Intrinsic Factors Affecting the Fish Microbiome

Host Age

Host age is an important contributing factor to the development of the microbiome. The bacterial community of the GI tract is highly dynamic and varies according to life stage (Egerton et al., 2018). Contrary to mammals, where microbial diversity increases with age (Yatsunenko et al., 2012), research has shown that richness, diversity, and stability of the GI bacterial

community may decline as fish age (Llewellyn et al., 2016; Zhao et al., 2020). Bacteria which are better adapted to the surrounding environment will outcompete those which are not. For example, in freshwater salmon, the genus *Cetobacterium* was found to be the most abundant at 22% in young fish but dropped to less than 1% in the adult stage. On the other hand, abundant genera found in young salmon continued to increase in abundance as they transitioned into an adult, which contributed to the overall decline in diversity as prominent bacteria became even more dominant (Zhao et al., 2020). One hypothesis is that differences in feeding behaviors between juvenile and adult fish directly correlate to the age-related bacterial community shifts that are observed (Orlov et al., 2006).

Host Genetics and Phylogeny

Host genetics and phylogeny have a significant impact on the microbiome. Research on the microbiome of vertebrates has found strong similarities between closely related lineages as opposed to distantly related lineages (Ochman et al., 2010; Moeller et al., 2013). Although some studies on mammals have concluded that diet is more indicative than host phylogeny (Ley et al., 2008), other studies on fish have found the opposite. This is not to say that diet has little effect, but instead to demonstrate that host phylogeny and genetics play a considerable role in microbiome development. For example, a study on five cyprinid fish species cohabitated in the same environment found that the intestinal bacterial communities clustered first by species, then by feeding habits (Li et al., 2017). These findings were concordant with another report on wild populations of three-spined stickleback, where both food and environment were controlled. The analysis concluded that similarities among the microbial communities were the most correlated with the host genotype for species that were closely related (Smith et al., 2015). Gender of the host has also been found to have no significant effect in some studies (Smith et al., 2015;

Stephens et al., 2016; Tarnecki et al., 2017). It is hypothesized that the host's genetics may be associated with the regulation of the microbiome and can play a part in promoting the colonization of organisms that are directly beneficial to the host (Benson et al., 2010). Variations in immune response due to genetics may be another factor in the filtering of bacterial communities that are able to colonize the intestinal tract (Smith et al., 2015). In conclusion, it is clear that a correlation exists between host genetics and phylogeny and the microbiome.

Host Physiology

Rainbow trout (*Oncorhynchus mykiss*), a freshwater fish native to the Pacific coast of North America, is one of the most popular farmed-fish species around the globe (D'Agaro et al., 2022; FAO, 2023). Production of rainbow trout has risen drastically since the 1950s, with the species being easy to spawn, fast growing, and highly tolerant to a range of environmental conditions (Verdile et al., 2020; FAO, 2023). Rainbow trout are a carnivorous species, with nutrient requirements including amino acids, fatty acids, proteins, lipids, and various vitamins and minerals (Kamalam J et al., 2020). Differences in nutrient requirement among fish species lead to shifts in the bacterial community of the GI tract. Carnivorous species require essential long-chain omega-3 fatty acids (Miller et al., 2008), whereas herbivorous fish appear to rely on short-chain fatty acids produced by bacterial fermentation (Hao et al., 2017). Additionally, carnivorous fish have a short intestinal tract relative to omnivorous and herbivorous fish, with herbivorous fish having the longest and most convoluted (Kramer and Bryant, 1995). The intestines of carnivorous species are adapted for the metabolism of a nutrient dense, protein rich diet. Therefore, carbohydrate metabolism is low compared to other fish species that are omnivorous or herbivorous (Buddington et al., 1997; Verdile et al., 2020). However, when

necessary, research has shown that glucose can be stored and utilized by rainbow trout, which is unusual for carnivorous fish species (Polakof et al., 2010).

Extrinsic Factors Affecting the Fish Microbiome

Environment

Regarding the production of aquaculture-raised rainbow trout, various studies have been performed on aspects such as stocking density and water quality. Stocking density is an important factor to consider, as it can negatively impact the growth and welfare of rainbow trout (Ellis et al., 2002). Injury occurrence is often used as a welfare indicator, and fin damage is commonly associated with high stocking densities (Latremouille, 2003; Hoyle et al., 2007). Social interactions and swimming behavior are also affected by stocking density (Boujard et al., 2002; Bégout Anras and Lagardère, 2004; Turnbull et al., 2005). Decreased growth rates have been observed when the stocking density is high in farmed rainbow trout, with food accessibility being one of the main factors. Additionally, lower water quality may be associated with high stocking densities due to excess accumulation of toxic metabolic waste, such as ammonia, combined with a higher rate of oxygen consumption (Person-Le Ruyet et al., 2008).

Correlations between low water quality and decreased food intake have been made, which negatively effects the growth and physiological functions of the fish (Pichavant et al., 2001; Lemarié et al., 2004). Important water quality parameters include alkalinity, dissolved oxygen, pH, temperature, ammonia, nitrite, and nitrate levels. Rainbow trout can survive in the temperature range of 10˚C to 25˚C, with 14˚C to 19˚C being the optimal temperature for feed efficiency and growth rates (Myrick and Cech, 2000). Water temperature may play a role in the colonization of different bacterial species in the GI tract, as bacteria are adapted to specific

growth temperatures (Zhao et al., 2020). Water salinity has also been shown to be a significant determinant of the bacterial community in the GI tract of fish (Zhao et al., 2020; Kim et al., 2021).

Pathogens and Antibiotics

Regulations in the United States

In the United States and Europe, antibiotic use in finfish aquaculture is strictly regulated regarding the control and treatment of bacterial infections, which is not the case globally. When disease is first believed to present in a fish population, a presumptive diagnosis can be made based on symptoms and visual indicators (Yanong et al., 2021). However, within the U.S., a definitive diagnosis and treatment plan is required from a licensed veterinarian under an established Veterinary-Client-Patient Relationship (VCPR) in order to be approved for antibiotic use (NOAA, 2022; FDA, 2024). Diagnosis is typically based on pathogen identification from bacterial culture isolation obtained from the internal organs of the fish (Yanong et al., 2021)

Once diagnosed, treatment with antibiotics can be done via three methods. The most common method is using medicated feed (oral administration) following an order from the Veterinary Feed Directive (VFD) under the U.S. Food and Drug Administration (FDA) (NOAA, 2022; FDA, 2024). Although much less common and only in the cases of severe infections, prescription drugs can be administered through either injection or immersion methods (Assefa and Abunna, 2018; NOAA, 2022). Injection includes direct administration of the drug into the bloodstream of the fish, whereas immersion methods involve dissolving the drug within the water column at a particular concentration (Assefa and Abunna, 2018). Due to concerns about antibiotic resistance, when antibiotics are used in food fish aquaculture a certain withdrawal

period is required prior to harvesting the fish, and it is based on the type of drug and administration method used (Yanong et al., 2021).

Intestinal Dysbiosis

Due to high stocking densities and stressors imposed by aquaculture systems on fish, an increase in both disease frequency and severity due to pathogens has been noted (Lafferty et al., 2015). Antibiotics used to treat these diseases lead to concerns about consumer safety, ecological impacts in the environment, and antibiotic resistance (Brandt et al., 2015). Additionally, antibiotics reduce diversity in the GI tract of the fish, which is one of the main entry points for pathogens (Ringø et al., 2007; Tang et al., 2020). Disease and antibiotics are the primary causes of dysbiosis, and the bacterial community of the GI tract is an important indicator of host health status (Xiong et al., 2019). Dysbiosis leads to a higher chance of pathogen invasion, contradicting the intended use of antibiotics for disease treatment (He et al., 2017).

Large shifts in the community also pose a risk of commensal opportunistic pathogens proliferating and therefore leading to more disease (He et al., 2017). As an alternative to antibiotics, modulation of the bacterial communities in the GI tract of fish has been an emerging area of research to mitigate disease (Lyons et al., 2017; Xiong et al., 2019). This may include the use of beneficial microbes known as probiotics, and nutrients that promote the growth of beneficial microbes which are known as prebiotics.

Greater microbial diversity helps protect against pathogens, with one hypothesis being that with more diversity comes a higher probability that a commensal species has antagonistic traits toward the invading pathogen (Fargione and Tilman, 2005). In the intestinal tract, the mucosal layer is the first line of defense, containing bacterial communities that compete for nutrients and block adhesion sites, therefore preventing pathogen attachment and invasion

(Kamada et al., 2013; Kim et al., 2017; Kurnianto et al., 2020). Protection via the colonization of commensal microorganisms is conferred in various other ways as well. A few examples include the acidification of the intestinal environment (Fukuda et al., 2011), production of extracellular enzymes and metabolites, such as bacteriocins and proteinaceous toxins (Xiong et al., 2019; Kurnianto et al., 2020), and release of various other antimicrobial substances (Gómez and Balcázar, 2008). Overall, it is important to understand the role of the bacterial community of fish when it comes to preventing disease and the effects of antibiotics.

Diet and Prebiotics

Prebiotics refer to non-digestible food ingredients that stimulate growth of beneficial intestinal bacteria, leading to improvements in the health of the host (Davani-Davari et al., 2019). As described in more detail above, higher stocking densities lead to a growing concern for disease outbreaks, and therefore an increased use of antibiotics as preventative measures (Hoseinifar et al., 2015). Prebiotics have been explored as an alternative measure for avoiding disease due to the health benefits that can be accrued. The indirect effect of prebiotics on the bacterial community of the intestinal tract play an important role regarding increased health benefits. Commonly, prebiotics work by increasing the number of beneficial bacteria and therefore decreasing the number harmful bacteria. The increased number of beneficial bacteria leads to protection against pathogens due to a variety of factors, such as competition for space and nutrients (Hoseinifar et al., 2015).

In aquaculture, low-level prebiotics can enhance immune response and lead to increased protection and disease resistance against pathogens (Tukmechi and Bandboni, 2014). When compared to mammals, the immune system of fish is not nearly as effective against pathogens. The main immune organs in fish are the head kidney, spleen, and thymus. As in other

vertebrates, fish such as rainbow trout possess both innate and adaptive immunity. The innate immune system includes functions such as physical barriers to mitigate entry, as well as various proteins used to destroy pathogens like the complement system and phagocytic cells (Jenik et al., 2024). The adaptive immune response in fish includes various components such as B and T cells, as well as a variety of immunoglobulins (Ig), with IgT having been first detected in rainbow trout (Mokhtar et al., 2023). However, fish lack components such as lymph nodes, M cells, and IgA secretion (Hoseinifar et al., 2015). In one study, mannan oligosaccharide (MOS) derived from the cell wall of yeast was used as a dietary supplement in rainbow trout. The study found that an MOS-supplemented diet significantly reduced mortality rates and improved various indicators of immune function in trout (i.e., antibody titer, bactericidal activity, lysozyme concentration, and complement activation) (Staykov et al., 2007). The exploration of other alternative dietary supplements and feed additives is an active area of research.

Brewing Industry Byproducts as Feed Additives

Beer is one of the most popular beverages around the globe. In 2018 alone, approximately 1.9 billion hectoliters were consumed worldwide (Marson et al., 2020). The two most prevalent byproducts in the brewing industry are brewer's spent grain (BSG) and brewer's spent yeast (BSY), consisting of 85% and 15% of the waste, respectively (Rachwał et al., 2020). These low-value waste products have the potential to be converted into sustainable, high-value feed additives in aquaculture-raised fish.

Brewer's Spent Grain

Brewer's spent grain is produced after the mashing step of the brewing process when the solids (BSG) are filtered from the wort. Wort is the liquid portion that contains the sugars for

fermentation. BSG is produced directly from the barley grain that is used initially, and mainly consists of the husk, pericarp, and seed coat (Mussatto *et al.*, 2006). As the primary byproduct of the brewing process, BSG is widely available year-round at little to no cost and must be disposed of frequently depending on brewery size (Kerby and Vriesekoop, 2017).

BSG is a lignocellulosic material that is rich in concentrated protein and fiber (20% and 70%, respectively) due to most of the starch being utilized prior in the process (Mussatto et al., 2006). Due to the high moisture content, BSG is a perishable material. However, the BSG shelf life can be improved by drying, which decreases volume, aiding in storage and shipping costs. Drying of BSG still maintains the nutrient composition while significantly increasing shelf life (Ikram et al., 2017). BSG is a source of both essential and non-essential amino acids, as well as vitamins. Additionally, BSG has natural antioxidant properties due to the presence of phenolic compounds. Studies of phenolic acids have suggested the compounds may be both anticarcinogenic and anti-inflammatory (Ikram et al., 2017).

BSG as a Substrate for Insects

BSG remains underutilized in the food industry, but it has been proven to be a low-cost substrate that can be used for the cultivation of insects, specifically the black soldier fly (BSF; Diptera *Hermetia illucens*). BSF larvae are of particular interest due to the ability to convert low-value organic waste products into high-value protein. The insects are rich in beneficial nutrients, containing up to 40% proteins and 30% lipids. Additionally, the amino acid profile (both essential and non-essential) has been shown to be similar to fishmeal (Belghit et al., 2019).

Furthermore, research has suggested that culturing BSF larvae on BSG may lead to a female-dominant population and ultimately a higher production of eggs, which is a desired attribute for mass production. Female larvae in general take longer (0.5-1 day) to develop when

compared to males, but the female prepupae and adults weigh on average 13-18% more than males (Tomberlin et al., 2009). This trend may be attributed to increased energy requirements for egg production (Jucker et al., 2019). Regarding fish feed, previous studies have proven that BSF larvae meal can replace a portion of fishmeal in the diets of Atlantic salmon (*Salmo salar*) without any significant or negative effects (Belghit et al., 2019). Cultivating BSF larvae on BSG is an efficient way to recycle waste in the food industry while simultaneously converting a low-value byproduct into a high-value source of protein that can be used in fish feed.

Brewer's Spent Yeast

Brewer's spent yeast is produced after the fermentation step of the brewing process, where it settles to the bottom of the tank and can be recovered by sedimentation. The BSY can then be reused up to six times, which is referred to as re-pitching (Marson *et al.*, 2020). The BSY must eventually be discarded to not compromise the quality of the beer. The slurry collected from the brewing process has a high moisture content, reaching upwards of 85%. The yeast extracts present in BSY gain antioxidant properties due to the absorption of polyphenolic compounds during the brewing process (Rachwał et al., 2020). As with BSG, drying methods can be used to stabilize the perishable slurry product, while still retaining the nutritional value of BSY. Yeast has been shown to be a sustainable protein substitution that can replace a portion of the fishmeal (up to 20%) present in fish feed (Øverland and Skrede, 2017; Hines et al., 2020).

The amino acid profile of yeast, especially after processing, is comparable to that of fishmeal (Øverland and Skrede, 2017). The amino acid profile is well balanced, with the most abundant amino acids being glutamic and aspartic acid, as well as the essential amino acids leucine and lysine (Marson et al., 2020). Although it varies by strain, yeast is comprised of

approximately 49% proteins, 40% carbohydrates, 7% vitamins and minerals, and 4% lipids (Rachwał et al., 2020).

The primary obstacle that needs to be overcome during the conversion of BSY into a high-value feed additive is the low digestibility of the BSY. The main reason being that the cellular components are protected by the cell wall of yeast, which has high rigidity and low permeability. The strength of the cell wall can be attributed to the presence of ß-glucans, chitin, and mannoproteins, which are molecules involved in cell structure (Marson et al., 2020). Although digestibility of unprocessed BSY can be similar to fishmeal in certain fish species, it is significantly lower in other species such as salmonids (Øverland and Skrede, 2017). By processing the BSY with extracellular proteases, such as chitinase, the bioavailability of nutrients can be significantly increased (Hamid et al., 2013).

Research Plan

As the aquaculture industry continues to grow and expand, maximizing production efficiency while simultaneously enhancing the health and welfare of the animals has become increasingly important. Additionally, improving sustainability remains an essential goal moving forward. In order to address these factors in tandem, the work in Chapter Two explores the use of brewing industry byproducts, BSY and BSG-fed insects, as possible prebiotic feed additives for use in aquaculture. Transforming low-value waste products into a high-value commodity benefits both food industries involved and leads to more sustainable practices. Not only have feed additives been shown to improve growth rates in aquaculture-raised fish, specifically rainbow trout, it is hypothesized that additives also may provide health benefits to the fish. Increasing animal health and using alternative prophylactic treatments can lead to both the indirect and

direct reduction of antibiotic use. The role that a robust microbiome plays in the host is essential for a myriad of reasons, making it a promising element to target and study.

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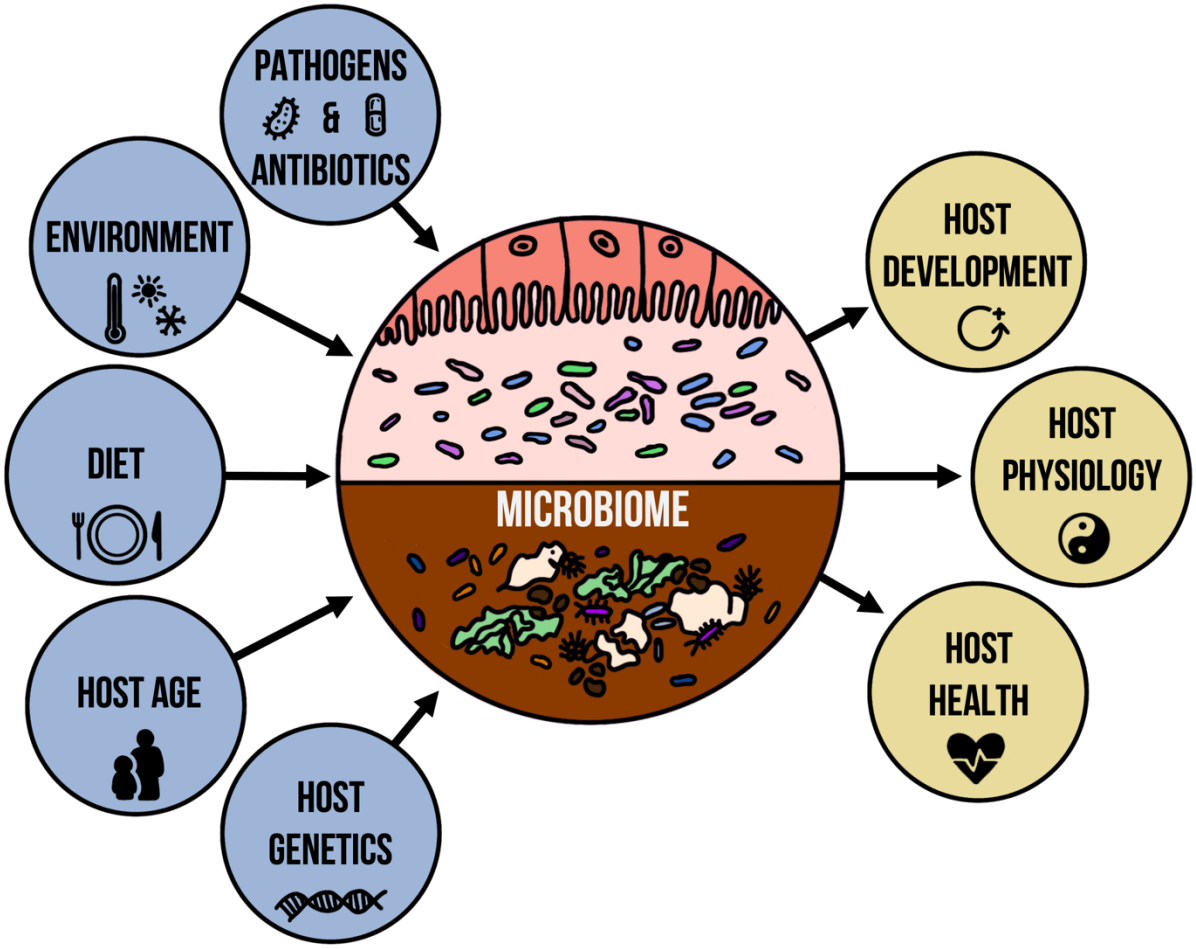

Figure 1.1. Factors affecting the microbiome and the subsequent influence of the microbiome on the host. The intrinsic and extrinsic factors shown in blue, on the left, represent factors affecting the structure and function of the host microbiome. The factors shown in yellow, on the right, represent the influence of the microbiome on host function.

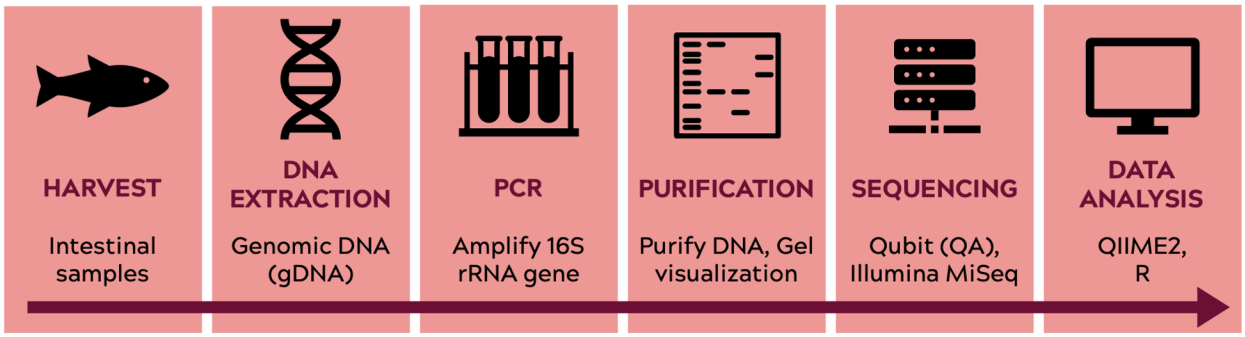

Figure 1.2. Overview of the method used to sequence and analyze the DNA of fish intestinal mucosa samples for bacterial community analysis.

CHAPTER 2

Impact of Brewing Industry Byproducts Used as Feed Additives on Aquaculture-Raised Rainbow Trout (*Oncorhynchus mykiss*) Under Thermal Stress Conditions

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Attributions

A. Rayne Layton wrote the manuscript, contributed to the study design, performed the experiment and data analysis, and reviewed the manuscript. Jason D. Pough II contributed to the study design and data analysis, performed the experiment, and reviewed the manuscript. David D. Kuhn wrote the manuscript, contributed to the study design and data analysis, performed the experiment, and reviewed the manuscript. Ann M. Stevens wrote the manuscript, contributed to the study design and data analysis, and reviewed the manuscript.

Abstract

The aquaculture industry is an essential alternative to wild-caught fisheries as it helps meet the demands of feeding seafood to a rapidly growing human population. To increase the sustainability of aquaculture practices, a collaboration was formed with Anheuser-Busch (Switzerland) of the beer brewing industry and Maltento (South Africa), a functional ingredient company. Two prevalent byproducts of brewing beer, brewer's spent yeast (BSY) and brewer's spent grain (BSG), were converted into high-value feed additives and used in the diets of aquaculture-raised rainbow trout (*Oncorhynchus mykiss*). To increase nutritional value of the brewery waste products, BSY was processed with extracellular proteases and BSG was used to cultivate black soldier fly (BSF; *Hermetia illucens*) larvae. A seven-week trial was conducted in a recirculating aquaculture system to explore the effects of the feed additives on juvenile trout growth production, biometrics, and the intestinal bacterial community. Intestinal DNA samples were purified and the V4 region of the bacterial 16S rRNA gene was amplified and sequenced. Due to a malfunction in the chiller used to maintain cool water temperatures, temperature and dissolved oxygen became substantial stress factors near the end of the trial. Clear shifts in the structure of the bacterial community were observed in the fish between the start and end of the study, including the emergence of aquaculture pathogens due to dysbiosis of the bacterial community. Results suggest that fish fed the experimental diets benefitted by having higher survival rates and growth rates when subjected to stress.

Introduction

Aquaculture, the cultivation of aquatic organisms in a controlled environment, is an important alternative to wild-caught fisheries that can be used to meet the demands of a growing human population. Fish are an important protein source for humans, providing nutrients including, but not limited to, omega-3 fatty acids, vitamin D_3 and B_{12}, and minerals such as iron and zinc (Khalili Tilami and Sampels, 2018). In the past thirty years, the annual output of global aquaculture production has increased by 609 percent, and fish consumption has doubled to over 20 kg per capita (FAO, 2022b). Rainbow trout (*Oncorhynchus mykiss*), a freshwater fish constituting 97% of farmed trout production globally, is one of the most popular farmed-fish species as they are easy to spawn, fast growing, and highly tolerant to a range of environmental conditions (Verdile et al., 2020; D'Agaro et al., 2022; FAO, 2023). The reliance on aquaculture continues to grow rapidly, therefore, it is important to conduct research on methods that can improve efficiency, sustainability, food security, and animal welfare in the industry (FAO, 2022a). The influence of nutrition on the fish intestinal bacterial community and overall animal health is one active area of research, including the use of sustainable feed additives.

Beer is one of the most popular beverages worldwide, but it poses various environmental challenges in the beer brewing industry, especially concerning waste production (Olajire, 2020). Two of largest byproducts of the beer brewing process are brewer's spent grain (BSG) and brewer's spent yeast (BSY; *Saccharomyces cerevisiae*), consisting of 85% and 15% of the waste, respectively (Rachwał et al., 2020). BSG, produced after the mashing step of the brewing process, is available year-round at little to no cost (Kerby and Vriesekoop, 2017) and composed mostly of fiber (Mussatto et al., 2006). However, it can be used as a low-cost substrate to cultivate black soldier fly (BSF; *Hermetia illucens*) larvae. In return, BSF larvae convert the low-

value waste product into a high-value aquaculture feed additive rich in lipids and protein (St-Hilaire et al., 2007; Makkar et al., 2014; Henry et al., 2015; Belghit et al., 2019; Jucker et al., 2019).

Brewer's spent yeast is another promising protein source and has been shown to be able to replace a portion of fishmeal in fish feed (Øverland and Skrede, 2017; Hines et al., 2020). Fishmeal is the main protein feed ingredient that relies on wild-caught fish (Kaushik and Troell, 2010). The primary obstacle in converting BSY into a high-value feed additive is the low digestibility of the yeast cells to the fish; this can be attributed to the high rigidity and low permeability of the cell wall of the yeast, which is made of various macromolecules including ß-glucans and chitin (Marson et al., 2020). By further processing the BSY byproduct with extracellular proteases such as chitinase, the bioavailability of nutrients to the fish can be significantly increased (Hamid et al., 2013). In addition to recycling food waste generated by the food industry, the use of BSY and BSF as feed additives in aquaculture may also provide important health benefits to the fish, such as having prebiotic effects, thereby serving as a viable alternative to the traditional application of antibiotics in aquaculture.

As the use of antibiotics remains prevalent across various industries including aquaculture, concerns about consumer safety, ecological impacts on the environment, and antibiotic resistance continue to grow (Brandt et al., 2015). Antibiotics are known to reduce bacterial diversity and induce dysbiosis in the gastrointestinal (GI) tract, one of the main entry points for pathogens (Ringø et al., 2007; Xiong et al., 2019; Tang et al., 2020). Large shifts in bacterial community composition have been shown to be a prominent risk factor for pathogen invasion (He et al., 2017). As an alternative prophylactic treatment to antibiotics, modulation of

the intestinal bacterial community of fish has been an active area of research to help mitigate disease, including the use of prebiotics (Lyons et al., 2017; Xiong et al., 2019).

Prebiotics refer to host non-digestible food ingredients that stimulate growth of beneficial intestinal bacteria, leading to improvements in the health of the host (Davani-Davari et al., 2019). Commonly, prebiotics are thought to work by increasing the number of beneficial bacteria, therefore decreasing the risk of harmful bacteria proliferating due to factors such as competition for space and nutrients (Hoseinifar et al., 2015). Research has shown that mannan oligosaccharide (MOS) used a dietary supplement in rainbow trout, derived from the cell wall of yeast, significantly reduced mortality rates and improved indicators of immune function (Staykov et al., 2007). In addition to MOS, various *in vivo* and *in vitro* studies have proved that nutrients such as ß-glucans (Iliev et al., 2005; Løvoll et al., 2007), lipopolysaccharides (Iliev et al., 2005; Fierro-Castro et al., 2013), and yeast extract (Tukmechi and Bandboni, 2014) can improve rainbow trout immune function. Both the indirect and direct effects of prebiotics on the bacterial community of the intestinal tract play an important role in host health.

The goal of this study was to determine the effects of BSY and BSF (cultured on BSG) used as feed additives on the growth production and intestinal bacterial composition of rainbow trout. However, due to an unexpected malfunction in the chiller used to maintain cool water temperatures, thermal stress became an important factor towards the end of the study in the form of both chronic stress and an acute stress event. During the trial, water temperature exceeded the optimum growth range (14-19˚C) of rainbow trout, and ultimately surpassed the temperature range for survival (10-25˚C) (Myrick and Cech, 2000). Results suggest that the feed additives may have had a positive impact on the overall fish health and survival.

Materials and Methods

Fish and Animal Husbandry

Experimental methods were approved by the Virginia Tech Institutional Animal Care and Use Committee (IACUC) following the protocol guidelines and regulations under IACUC #20-084. Juvenile rainbow trout (*Oncorhynchus mykiss*), approximately three to four months of age, were acquired from the Wytheville State Fish Hatchery (Max Meadows, VA) (Time point 0, T0). The fish were acclimated to a recirculating aquaculture system (RAS) for three weeks before beginning the experimental trial (Time point 1, T1). The cold-water RAS included 30 170-liter polyethylene tanks and was equipped with a sand filter and two bead filters for solid filtration and nitrification, a dual 40-watt UV unit for disinfection, and a chiller to maintain cool water temperature. Throughout the duration of the trial, the system was exposed to halogen overhead lighting on an approximate 12:12 hour (light:dark) cycle with no natural light. Water quality parameters monitored throughout the trial included temperature, pH, dissolved oxygen, total ammonia-N, nitrite-N, and nitrate-N (Bridgewater et al., 2012). During the acclimation period, all fish were fed a commercial feed (Ziegler Bros., Finfish Silver Semi-Floating, Gardner, PA).

Feed Preparation

Experimental diets consisted of five treatment groups with the dietary supplement top coated on the commercial feed; the commercial feed was used as the control diet. Powdered yeast and insect products derived from byproducts of the brewing industry were individually resuspended in deionized water according to the desired feed additive concentration (see below) and placed into a polypropylene wash bottle (Thermo Fisher Scientific, Waltham, MA). Commercial feed was loaded into a 3.5 ft^3 portable cement mixer (Central Machinery, Moses

Lake, WA) where the experimental products were then sprayed in intervals until distributed evenly onto the feed and left to tumble for a minimum of 15 min after all the liquid product was applied. The feed was left to dry at room temperature with a fan blowing across it for 24-48 hr before being stored at -20˚C for the remainder of the trial.

The powdered feed additives were resuspended in dH_2O based on the amount of dry product needed to reach the desired final percent concentration based on the total mass of feed pellets used for each diet. Therefore, the final concentration refers to the percent concentration of the additives present on the coated commercial feed (w/w). Brewer's spent yeast (BSY; Anheuser Busch, Risch-Rotkreuz, Switzerland) was coated on the feed for two diets at a 2% and 0.75% concentration (BSY1 and BSY2). Additional experimental diets were coated with two different insect products (Maltento, Cape Town, South Africa), denoted "old" (from a previous trial, Pough et al., 2024b) and "new", at a 3% and 0.75% concentration, respectively (BSF3 and BSF4). Both insect products consisted of black soldier fly (BSF, *Hermetia illucens*) larvae cultured on brewer's spent grain (BSG). The last diet was a combination of BSY2 and the BSF4 insect product top coated at a 1.5% concentration (mixed at 0.75% for each of the diets, 1.5% total).

Experimental Design

After excluding exceptionally large or small fish, fish of average size were randomly separated into 12 fish per tank two days before the start of the experimental trial, with four replicates for each of the six diets (48 fish per diet). In total, 24 of the 170-liter tanks were used, with diets randomly assigned to each. The remaining six tanks housed extra fish not included in the experimental trial. Survival rates were recorded daily, and fish were weighed every two

weeks throughout the duration of the trial to track growth rates, adjust feed amounts, and calculate feed efficiency at the tank level. Fish were fed at a rate of 2.5% body weight per day at the tank level based on modeled linear growth over each two-week period during the entire trial.

Just prior to the start of the experimental diet trial (T1), following the three-week acclimation period on control feed, eight fish were harvested directly from the RAS to collect controls for bacterial community analysis. The fish were humanely euthanized using a 250 mg/L Syncaine (MS-222, tricaine methanesulfonate; Syndel, Ferndale, WA) water bath buffered with sodium bicarbonate and dissected immediately.

After approximately seven weeks (Time Point 2, T2), following an unforeseen acute stress event due to chiller malfunction, all remaining live fish were euthanized following the same euthanasia procedure as T1. All T2 fish were separated by tank into large, plastic zipper-lock storage bags (SC Johnson, Racine, WI) and stored at -20˚C for three days until the animal tissues could be processed. The day before a tissue harvest, fish were separated one-by-one into individual plastic zipper-lock storage bags (Food Lion, Salisbury, NC; CVS, Woonsocket, RI). Approximately nine hours prior to dissection, the fish were moved from -20˚C to 4˚C to thaw overnight.

Initially, eight T2 fish of approximately equal size were used to collect production data and obtain intestinal samples for bacterial community analysis for four of the six diets (Control, BSY2, BSF3, and Mix). Those three experimental diets were chosen based on the highest survival rate during the acute stress event. Approximately two weeks later, six additional fish from the original four diets, drawn randomly, were used to collect production data only. In addition, for the production data from the remaining two diets (BSY1 and BSF4), eight fish of

approximately equal size and another six were drawn randomly to maintain production and biometric data consistency across all diets (n=14 total).

Production Data Acquisition and Statistical Analysis

Due to the unexpected chiller malfunction, the growth data (weight gain, FCR) presented in the results accounts for just six of the seven weeks of the trial. Week six is when the last tank-level weigh-in occurred prior to the mortality event at week seven. The biometric data (fillet yields, VSI, HSI, Fulton's condition factor) were calculated after the harvest using week seven fish.

During each of the tissue harvests, the fish were measured and dissected individually using ethanol flame-sterilized surgical tools. The dissection area of each fish (anus to gills) was first rinsed with phosphate-buffered saline (PBS) for approximately five seconds. Between sampling of each fish, cutting boards, surgical tools, and gloves were all sanitized with 70% ethanol. The production factors measured were total weight, whole length (nose to end of caudal fin), body length (nose to beginning of caudal fin), viscera weight, liver weight, and fillet weight. From the production data, biometrics such as fillet yield, VSI, HSI, and condition factor were calculated using standard equations (Stoneham et al., 2018).

Fish production data including weight gain, feed conversion ratio (FCR), fillet yields, viscerosomatic index (VSI), hepatosomatic index (HSI), Fulton's condition factor, and survival rates were all analyzed in Microsoft Excel (v.16.76.1, Redmond, Washington) using a one-way analysis of variance (ANOVA) test from the Analysis ToolPak add-in. No post-hoc testing was performed on production data since the ANOVA results indicated no statistically significant differences ($P > 0.05$) of any metrics.

Bacterial Community Sampling

At T1, 16 intestinal swab (2/fish) and eight fecal (1/fish) samples from eight fish were collected to be used as controls during the bacterial community analysis of T2 samples. The same sampling procedure was followed at T2 for eight fish from four of the six diets (Control, BSY2, BSF3, and Mix). However, sufficient fecal material was unable to be recovered from the fish sampled at T2. For the fish used for bacterial community analysis sampling, a portion of the intestinal tract was removed, consisting mostly of the midgut and hindgut. The segment was then cut open lengthwise and laid flat in order to perform two swabs of the mucosal layer. A nylon flocked swab, flexible mini-tip, in a sterile peel pouch was used for all swab samples (Copan Diagnostic, Murrieta, CA). Half the intestinal tract was swabbed lengthwise while rotating the swab with gentle pressure, and the other half swabbed following the same procedure to obtain two swabs per fish. Each of the swabs were transferred individually into 1.5 ml polypropylene microcentrifuge tubes (USA Scientific, Ocala, FL) and flash frozen in a 70% ethanol dry ice bath before short term storage at -20˚C, then subsequently at -80˚C for long term storage after the completion of the harvest. Any fecal material present (T1 only) was recovered by using a metal spatula to gently scrape the material off the mucosa, which was then stored following the same conditions as the swabs.

DNA extractions were performed within a three-week period after each harvest. Water column samples were collected (~2.5 L each; n=2) from the RAS at the start (T1) and end (T2) of the trial, stored at 4˚C, and processed within 24 hours using vacuum-filtration (Corning 0.22 μm filter, Corning, NY). A sterile swab was used to scrape the filter retentate before being placed into a PowerBead tube (Qiagen, Germantown, MD) and stored at -20˚C. A swab of the

plastic storage bags, a sterile swab, and beads from the PowerBead tube were collected as negative controls. Lastly, samples of the control feed and each feed additive were collected in 15 ml conical tubes and then distributed by weight directly into four PowerBead tubes per each feed sample group (n=4, 16 total). Sample weights for DNA extraction were as follows (mean ± SEM): control feed pellets (12.58 ± 0.77 mg), powder yeast product (11.93 ± 0.60 mg), "old" liquid insect product 1 (19.54 ± 3.46 mg), and "new" liquid insect product (19.09 ± 3.02 mg).

DNA Extraction

Genomic DNA (gDNA) was extracted from all samples using the Qiagen DNeasy PowerLyzer PowerSoil Kit following the manufacturer's protocol with the following alterations: 10-min incubation at 72˚C after addition of C1, addition of 50 µl of dH_2O before the elution step including a 5-min incubation at 72˚C. Swabs, feces, and feed additive samples were added directly into the PowerBead tubes. After the extraction of DNA, sample quality was analyzed via total gDNA concentration and purity (A_{260}/A_{280}, A_{260}/A_{230}) using a nanospectophotometer (Implen, Westlake Village, CA). Samples were stored at -20˚C until polymerase chain reaction (PCR) amplification.

PCR Amplification

The 16S rRNA gene V4 region was amplified via PCR in a thermal cycler (Bio-Rad Laboratories, Hercules, CA) from each gDNA sample in triplicate with dH_2O used as a negative control. A positive control of *Vibrio parahaemolyticus* RIMD 2210633 gDNA template at 50 ng/µl was used for each sample set. An experimental PCR reaction consisted of 12.5 µl of Q5 High-Fidelity 2X Master Mix (New England Biolabs, Ipswich, MA), 1.25 µl of barcoded 515

forward and 1.25 µl of 806 reverse universal primers (500 nM; Caporaso et al., 2011), 120 ng of gDNA template, and a variable amount of dH_2O to bring the final volume to 25 µl (Hines et al., 2023b). The universal primer concentration was doubled (1000 nM) for four mucosa samples (two from BSY2 and two from BSF1) where PCR was initially unsuccessful under the previously stated conditions. Additionally, the gDNA template was decreased in half (60 ng) for one fecal sample (Control, T1) for the same reason. The thermocycling conditions were as follows: initial denaturation at 98˚C for 30 sec, 30 cycles of denaturing at 98˚C for 10 sec, annealing at 55˚C for 20 sec, and elongation at 72˚C for 15 sec, as well as a final elongation at 72˚C for two min.

Following PCR, the triplicate products were combined, totaling 75 µl. An aliquot of 10 µl was visualized using gel electrophoresis on a 1% agarose gel and imaged (Bio-Rad ChemiDoc). The remaining 65 µl of PCR products were stored at 4˚C for short-term storage until being purified using the Qiagen QIAquick PCR Purification Kit following the manufacturer's instructions with the following alterations: incubation at 72˚C for 5 min prior to elution with dH_2O. The purified PCR products were subsequently stored at -20˚C for long-term storage before preparation for sequencing. A nanospectophotometer was used to evaluate the same parameters recorded for the initial DNA extractions. For further accuracy of purity and yield, amplicons were also analyzed via Qubit fluorometry at the Genomics Sequencing Center (GSC) of the Fralin Life Sciences Institute at Virginia Tech (Blacksburg, VA).

Sequencing

Purified V4 amplicons were pooled at a 10 nM concentration to be further processed, cleaned, and sequenced by the GSC. The pool was first filtered using Solid Phase Reversible

Immobilization (SPRI) beads to remove unwanted DNA and to concentrate the V4 region amplicons. The amplicons were then purified again using Pippin Prep (Sage Science, Beverly, MA) to remove contaminating host 18S rDNA and further select for the V4 target region. The processed pool was analyzed by TapeStation for quality control and then sequenced on the Illumina MiSeq platform using 500 cycles of 250 bp paired-end sequencing at a concentration of 10pM with phiX spiked in at 25%.

Bioinformatics

Due to very low-quality reverse reads (Run 2) from the paired-end sequencing, only the forward reads (Run 1) were utilized in a single-read manner for bacterial community analysis. The Quantitative Insights Into Microbial Ecology (QIIME2 v. 2020.2, Bolyen et al., 2019) bioinformatics pipeline was used to analyze the generated amplicon sequences for bacterial diversity and identification. All steps using QIIME2 were performed using high-throughput computing clusters provided by the Advanced Research Computing (ARC) team at Virginia Tech (Blacksburg, VA). DADA2 (Callahan et al., 2016) was used to denoise the sequences and generate amplicon sequence variants (ASVs) and the sample-wise abundances of each ASV. The sequences were filtered using DADA2 to remove ASVs from eukaryotic-associated sequences (host DNA contamination) and low frequency (< 0.001% of total) ASVs (Hines et al., 2023b). The sequences were assigned taxonomy using a pre-trained 16S V4 region-specific classifier from the SILVA database, version 138.1 (Quast et al., 2013; Yilmaz et al., 2014; Glöckner et al., 2017).

Filtered and taxonomically-assigned ASVs were then analyzed using R v.4.3.2 (R Core Team, 2023) with various packages including: qiime2R v.0.99.6 (Bisanz, 2018), phyloseq

v.1.46.0 (McMurdie and Holmes, 2013), vegan v.2.6-4 (Oksanen et al., 2022), ggplot2 v.3.4.4 (Wickham, 2016), ComplexHeatmap v.2.18.0 (Gu et al., 2016), and dplyr v.1.14 (Wickham et al., 2023). Phylum-level bar plots were created using the 100 most abundant ASVs, representing over 99% of the total bacterial community of the mucosal samples from each treatment group. The same representation (99%) was true for all other sample groups except for the pellets of control feed (89%) and the T2 water column (96%) samples.

Bacterial Community Statistical Analyses

Alpha and beta diversity analyses were performed in R using files imported and generated from QIIME2. For beta diversity, the R packages phyloseq v.1.46.0 (McMurdie and Holmes, 2013), vegan v.2.6-4 (Oksanen et al., 2022), and pairwiseAdonis v.0.4.1 (Pedro, 2020) were used as well. Alpha diversity was analyzed using three metrics: Shannon diversity index, Pielou's evenness, and observed ASVs. The Shannon metric includes species abundance and evenness, Pielou's evenness accounts for species diversity and richness, and observed ASVs is a count of the number of unique sequences present. Alpha diversity metrics were analyzed for statistically significant differences ($P < 0.05$) using the non-parametric Kruskal-Wallis test followed by Dunn's post-hoc test with Bonferroni correction for pairwise comparisons.

Beta diversity non-metric multidimensional scaling (NMDS) plots were creating using the weighted and unweighted unique fraction (UniFrac) distance, which is a distance metric that accounts for phylogenetic information. The unweighted UniFrac distance only looks at presence and absence of species, whereas the weighted UniFrac distance incorporates species abundance data as well. Statistical significance ($P < 0.05$) was calculated using the permutational

multivariate analysis of variance (PERMANOVA) with 999 permutations, followed by a pairwise comparison with Bonferroni correction for multiple testing.

Differential abundance was determined on the family level using analysis of compositions of microbiomes with bias correction 2 (ANCOM-BC2) (Lin and Peddada, 2023) from the R package ANCOMBC v.2.4.0 (Lin and Peddada, 2020; Lin et al., 2022). Significant differences ($P < 0.05$) in intestinal bacterial composition were determined using time or diet as the covariate of interest for multi-group comparisons. The Bonferroni correction was used to control for possible error caused by multiple testing. For multiple pairwise comparisons against a pre-specified group, the function within ANCOMBC for Dunnett's type of test was used (Dunnett, 1955; Dunnett and Tamhane, 1991, 1992). Dunnett's test corrects for both the p-value and multiple comparisons, making it a conservative approach to prevent false positives. ANCOM-BC2 was performed with the sensitivity filter enabled to assess the effect of pseudo-counts on zeros. Pseudo-count methods are widely used to deal with the presence of zeros in data, however, the various approaches may influence the results (Kaul et al., 2017). Therefore, the sensitivity analysis determines if the statistical significance of the taxon was sensitive or not to the addition of psuedo-counts.

Results

Fish Production and Biometric Data

The average weight of the fish at the beginning of the study (Time Point 1, T1) was 22.15 ± 0.24 grams (mean ± standard error [SEM]). At the six-week point in the growth trial, no significant differences in weight gain were observed between BSY1 (2% yeast) or BSY2 (0.75%) compared to the control, despite BSY2 having a 10% increase in weight gain over the

control (Table 2.1). BSY1 had a 1% decrease in weight gain compared to the control. The average FCR for each of the three diets was equal at 1.35. There were no significant differences in the remaining biometric measurements. Fillet yields ranged between 50.7-54.8%, and the VSI and Fulton's condition factor were approximately equal for all treatments averaging about 9.4 and 1.7, respectively. The HSI ranged between 1.78 and 2.08. Survival rates during six weeks of the trial were high, with the control and BSY1 at 100%, and BSY2 at 98%.

The production and biometric data for the BSF diets (Table 2.2) followed a similar trend to the BSY diets when compared to the control. No significant differences were found regarding weight gain, with BSF3 (old, 3%) having a 12% increase over the control and BSF4 (new, 0.75%) being 4% lower than the control. Average FCR values were approximately equal with the control and BSF4 at 1.35, and BSF3 at 1.34. No significant differences were observed in the remaining biometric measurements. Fillet yields were very similar across the three treatments, ranging from 54.3-55.6%, and Fulton's condition factor was approximately equal with an average of about 1.7. The VSI values were between 9.33-9.61, and HSI values were between 1.78-2.01. Survival rates for BSF3 and BSF4 were 96% and 100%, respectively.

Production and biometric data for the mixed diet (BSY2 and BSF4 combination, 1.5%) also showed no significant differences with regard to weight gain, with the mix having a 7% increase over the control (Table 2.3). The average FCR values were also close, with the control at 1.35 and mix at 1.32. No significant differences were found in the remaining biometric data. Fillet yields were 52.8% for the mix, and 54.8% for the control. Both diets were approximately equal for average VSI, HSI, and Fulton's condition factor (9.4, 1.8, and 1.7, respectively). The mix diet had a 92% survival rate.

Stress and Survival Rates in Relation to Environmental Conditions

The chiller used to maintain the water temperature of the RAS slowly began to decrease in functionality after a few weeks into the trial. Additionally, the ambient temperature in the laboratory where the RAS was located (Blacksburg, VA) began to increase as the weather changed seasons from early to late spring. The decrease in chiller effectiveness, compounded with an increase in ambient temperature, led to an unexpected rise in water temperatures (associated with a decrease in dissolved oxygen) after week four (Table 2.4). This progressed for approximately two more weeks, causing chronic stress to the fish as the average temperature (21.2 ± 0.3 ˚C) was above the optimal growth range for rainbow trout (Jiang et al., 2021). Additional water quality parameters remained in a safe range throughout the duration of the trial and were as follows (average ± SEM): 7.05 ± 0.07 pH, 0.42 ± 0.12 mg/L ammonia-N, 0.03 ± 0.01 mg/L nitrite-N, and 9.04 ± 1.06 mg/L nitrate-N (Bridgewater et al., 2012; Saoud, 2014).

The feed efficiency of the fish across all diets decreased over the course of the study as represented by the FCR, which increased drastically from an average value of 1.02 to 3.31 when the animals were under chronic stress. Despite maintenance on the chiller in an attempt to fix it, the water temperatures continued to rise. The study was initially planned to be twelve weeks, but the timeline was cut short at week seven when the chiller ceased to function effectively. Water temperatures and dissolved oxygen reached a lethal range (27˚C and 4 mg/L, respectively; (Myrick and Cech, 2000) overnight, causing an acute stress event for the fish with high mortality rates. The total survival rate of all fish was 60%, but there were noticeable difference in the survival rate across the different diets (Figure 2.1).

Fish fed three of the five experimental diets showed higher survival rates on average when compared to the control (55%). The mixed diet had the highest survival rate at 78%,

followed by BSF3 at 71% and BSY2 at 69%. The two experimental diets with lower survival rates than the control were BSF4 and BSY1 at 45% and 44%, respectively. Despite BSY2, BSF3, and the mixed diet having 14-23% higher survival rates on average compared to the control, no statistical significance ($P > 0.05$) was found following an ANOVA test. There was a large amount of variance seen in the survival rates on a tank-by-tank basis for each diet. The standard error (±) for average survival rate across all diets were as follows: 10% (Mix), 14% (BSY2), 15% (BSF4), and 16% (Control, BSY1, BSF3).

Phylum-level Relative Abundance in the Bacterial Communities

The total number of sequences generated prior to filtering the data was 7,231,114 reads. The resulting number of sequences after filtering was 5,354,452 reads, with 1,183 unique ASVs identified. The phylum-level relative abundance bar plot of the control fish was determined (Figure 2.2A). Fish fed the control diet were sampled at both T1 (Control(T1)) and T2. The sampling at T2 included fish that were euthanized (Control(T2)), and fish that underwent a natural death during the acute stress event just prior to the T2 harvest (Control(ND)). Control(T1) exhibited a very large dominance of Firmicutes, followed by a lower abundance of Spirochaetota. A major shift in dominant phyla is observed between Control(T1) and Control(T2), with Actinobacteriota and Proteobacteria becoming the dominant phyla at T2, with lower levels of the Firmicutes and Spirochaetota. Fusobacteriota became the overwhelmingly dominant phylum for the Control(ND) animals. The abundance of Proteobacteria noticeably increased in both T2 samples compared to T1. Additionally, Actinobacteriota decreased drastically between Control(T2) and Control(ND).

Next, the phylum-level relative abundance bar plot of each of the experimental diets sampled at T2 was generated (Figure 2.2B). Similar to the Control(T2) results, the dominant phylum across all experimental diets was Actinobacteriota, followed by Proteobacteria. A high abundance of Fusobacteriota was seen in BSF3 relative to the other experimental diets. Overall, the Firmicutes were greatly diminished across all diets at T2 compared to what was observed in Control(T1).

Since the feed additives could have contributed to changes in the fish intestinal bacterial community, a phylum-level relative abundance bar plot of the control feed and feed additive ingredients was created to enable a comparative analysis (Figure 2.2C). The three feed additive samples consisted solely of the raw ingredients. Two dominant phyla were seen in the control feed pellets, Firmicutes and Proteobacteria. The powdered yeast product was mostly comprised of the unassigned phylum category, followed by a relatively equal abundance of Firmicutes and Proteobacteria. The old insect product (used for BSF3) and new insect product (used for BSF4 and the mix) were highly dominated by Firmicutes. Overall, there does not appear to be any strong correlation of the patterns between the dominant phyla of the raw feed additive ingredients and those of the intestinal mucosa samples from fish fed the associated diet.

Lastly, water column samples were taken to compare the external bacterial environment to the internal bacterial environment of the fish intestines (Supplementary Figure A.1). The water column samples from T1 did not contain enough bacterial DNA to be processed or sequenced. Two dominant phyla were present in the T2 water sample, Proteobacteria and Bacteroidota. Proteobacteria appears to be the only similarity between the fish mucosa samples and the water column, as it was prevalent across all T2 mucosa samples (Figure 2.2B). Bar plots of each

individual mucosa sample within the various diet groups can be found in Supplementary Figure A.2.

Family-level Relative Abundance in the Bacterial Communities

A heatmap was made of the top 25 most relatively abundant ASVs, log-transformed at the family level (Figure 2.3). The samples taken at T1 consist of feces (Feces(T1)) and mucosal samples (Control(T1)) from the control diet-fed fish after the three-week acclimation period. A high abundance of *Streptococcaceae* and *Clostridiaceae* was seen in both samples. A lower, yet relatively equal, abundance of *Peptostreptococcaceae* and *Peptostreptococcales-Tissierellales* was seen in both feces and the mucosa as well. However, the mucosal samples had a higher abundance of *Brevinemataceae* and a lower abundance of *Fusobacteriaceae* and *Moraxellaceae* in comparison to the feces. Overall, the mucosal samples and the feces share similar trends in their composition at the family level.

The most abundant ASVs at the family level for T2 (Figure 2.3), are relatively similar across all mucosal samples besides the natural death fish (Control(ND)). The two most dominant families across all the experimental diets were *Mycobacteriaceae* and *Mycoplasmataceae*. The most dominant family in Control(ND) was *Fusobacteriaceae*, which is seen across all other sample groups at a lower abundance, though higher levels in BSF3, comparatively. Furthermore, *Xanthobacteraceae* and the order Rhizobiales were also present at a higher relative abundance across the experimental groups, but essentially nonexistent in Control(ND). *Aeromonadaceae* was seen at higher levels in all T2 samples except Control(T2), in which it was noticeably lower.

Drastic shifts in the bacterial community between T1 and T2 were observed across almost every family where relative abundance was not equal to zero across all mucosal samples.

The most abundant families in T1 were diminished at T2, and dominant T2 families such as *Mycobacteriaceae* were drastically lower or nonexistent at T1. The composition of the bacterial families present in the T1 versus the T2 mucosal samples is quite distinct.

Differential abundance analysis was performed on the control diet group using ANCOM-BC2 with Dunnett's type of test to determine significant differences in taxa at the family level across timepoints. Both sample groups from T2 (Control(T2) and Control(ND)) were compared to the sample group from T1 (Control(T1); reference group), and the log fold-change (LFC) in abundance of taxa is presented in a heatmap (Figure 2.4). Two of the most abundant taxa at T1, *Streptococcaceae* and *Clostridiaceae*, both from the phylum Firmicutes, tremendously decreased in abundance at both T2 samples. Other prominent changes in organisms that passed the sensitivity test include a significant increase of *Mycobacteriaceae* in Control(T2) and *Fusobacteriaceae*, *Enterobacteriaceae,* and *Aeromonadaceae* in Control(ND). Various other taxa were identified as decreasing or increasing, too. Control(T2) and Control(ND) followed similar trends regarding increases and decreases in taxa when compared to Control(T1), though having some distinct differences between the degree of the LFC. ANCOM-BC2, using the global test function, was also performed on the experimental groups sampled at T2, but no significant differences in abundance were determined according to diet.

The water column sample from T2 had a considerably different bacterial composition when compared to the mucosal samples. Although, highly abundant families from Proteobacteria in the mucosa samples do appear to be present in the water column at extremely low levels, and vice versa. Families from the phylum Bacteroidota were highly abundant in the water column, but not present in the mucosa, including *Flavobacteriaceae*, *Chitinophagaceae*, and *Saprospiraceae*. Overall, the water column had a very different bacterial composition at the

family level when compared to the mucosal samples from T2, suggesting that it had little impact on the internal bacterial community of the fish.

The family-level relative abundance for each of the feed and feed additive samples had a distinctive pattern (Figure 2.3). The most abundant ASVs from the control feed pellets were in the families *Fusobacteriaceae*, *Vibrionaceae*, and *Streptococcaceae*. The powdered yeast product was comprised mostly of the unassigned phylum members. The three most abundant assigned families were *Bacillaceae* (also high in both insect products), *Enterococcaceae*, and *Enterobacteriaceae*. Similar levels between the yeast and insect products are also observed regarding the abundance of *Clostridiaceae* and *Planococcaceae*. However, the most dominant families for the old insect and new insect products were all from the phylum Firmicutes, with *Bacillaceae* being highly abundant in both. Between the two insect feed additives, *Paenibacillaceae* was higher in the old insect product, whereas the new insect product had higher levels of *Clostridiaceae* and *Peptostreptococcales-Tissierellales*. The insect products also differed regarding the presence of *Moraxellaceae* and *Enterococcaceae*, with lower abundance in the old insect product. A relatively equal abundance was seen for most other families. There is not an evident relationship between the most abundant families in the raw feed additives and those abundant in the T2 intestinal mucosa samples from fish fed the associated diets.

Alpha Diversity Analysis of the Bacterial Communities

Three alpha diversity analyses (Shannon, Evenness, and observed ASVs) were performed to measure the bacterial community diversity within each sample group (Table 2.5). For the Shannon index, higher values indicate higher diversity. Regarding evenness, the closer the value is to one, the more evenly distributed the community composition is. Observed ASVs just

represents a count. Within the control diet group, there were no statistically significant ($P > 0.05$) differences. However, there was a decreasing trend in alpha diversity values from Control(T1) to Control(T2), with Control(ND) having the lowest value for each metric. The largest decline is seen in the number of observed ASVs, with Control(ND) having over a 70% decrease (17.13 ± 13.63) compared to Control(T1) (66.17 ± 45.84). Control(T2) had an almost 50% decrease (36.75 ± 22.96) when compared to Control(T1).

When comparing the experimental diets sampled at T2, BSF3 had the lowest diversity across all metrics. Control(T2) had the highest Shannon diversity, though relatively similar to BSY2, followed by the mixed diet. The same pattern is seen for observed ASVs, with Control(T2) having the most and BSY2 and the mixed diet having lower values. Evenness was approximately equal for all diets aside from BSF3, which was lower. No statistical significance ($P > 0.05$) was observed in any of the metrics. However, the evenness metric was initially significant following the one-way Kruskal-Wallis test ($P < 0.05$) prior to Dunn's post-hoc test, but no significance was found after the pairwise comparisons due to the conservative nature of the Bonferroni correction which aims to prevent false positives.

The alpha diversity of the feed and feed additive samples showed some statistically significant differences across various samples and metrics (Supplementary Table A.1). Overall, differences were observed between the raw feed additives and the control feed pellets, but not between any of the raw feed additives themselves.

Beta Diversity Analysis of the Bacterial Communities

Beta diversity analysis, which provides insight into the level of dissimilarity between bacterial communities, was performed between treatment groups using the UniFrac distance

(Lozupone et al., 2011). Non-metric Multidimensional Scaling (NMDS) plots representing (1) unweighted and (2) weighted UniFrac distances were generated (Figure 2.5). Statistical differences were calculated using PERMANOVA, followed by a pairwise comparison with Bonferroni correction. For fish fed the control diet, both the unweighted (Figure 2.5A1) and weighted (Figure 2.5A2) NMDS plots help visualize that the bacterial community of Control(T1) is distinct ($P < 0.01$) when compared to both Control(T2) and Control(ND). In the unweighted NMDS plot, there is a large amount of overlap between Control(T2) and Control(ND), indicating similar community compositions ($P > 0.05$). However, when using the weighted UniFrac distance which accounts for abundance, the two groups show a significant level ($P < 0.01$) of dissimilarity.

Diversity analysis of the experimental diets sampled at T2 shows a large amount of overlap in the unweighted NMDS plot between all four groups (Figure 2.5B1), demonstrating a large degree of similarity between the bacterial communities. The same is seen for the weighted UniFrac distance (Figure 2.5B2), though BSF3 appears to have larger range, comparatively. The PERMANOVA results revealed highly similar bacterial communities ($P=1$) for both the unweighted and weighted UniFrac distances across all treatments at T2.

For the feed and feed additive samples (Figure A.3), the initial PERMANOVA results indicated significant differences ($P < 0.001$), however, the pairwise comparison showed no significance between any of the groups when accounting for multiple testing (Table A.3). Though, prior to factoring in error correction accounting for multiple testing, the pairwise comparisons did show that the bacterial communities of the feed groups are all distinct ($P < 0.05$) amongst each other.

Discussion

During this trial, no significant differences in production data were observed between fish fed the five experimental diets compared to the control. Although, when looking at the average weight gain and FCR from week six of the trial, all the experimental diets performed at least approximately equally if not better, on average, than the control (Tables 2.1-3). Biometric data (fillet yields, VSI, HSI, and Fulton's Condition Factor) collected at week seven of the trial suggested no negative impacts of the feed additives on the physiology of the fish, though, the addition of BSY and BSF as feed additives did not significantly increase overall growth efficiency.

Previous research on the use of yeast and BSF in aquaculture feeds has often focused on using these protein sources as fishmeal (FM) replacements, as opposed to feed additives. Studies have found that high percentages (40%+) of yeast used as a FM replacement in feed significantly decreased growth in species such as Atlantic salmon (Øverland et al., 2013) and rainbow trout (Hines et al., 2020). Conversely, when used in lower concentrations (≥ 20%), growth performance of rainbow trout increased or remained neutral (Barrows, 2014; Hines et al., 2020). When used as a feed additive, opposed to a FM replacement, concentrations of yeast as low as 2-5% greatly increased growth in rainbow trout (Pough et al., 2024a; Hajimoradloo, 2009). Results of BSF as a FM replacement in feed for salmonids vary greatly, with studies reporting increased, comparable, or decreased growth performance; though the majority of studies found no negative health effects (St-Hilaire et al., 2007; Sealey et al., 2011; Henry et al., 2015; Belghit et al., 2019; Pough et al., 2024b). Overall, the use of both BSY and BSF as solely feed additives, as opposed to partial or complete FM replacements, is much less common.

The results of the current trial were affected by the increase in water temperature and associated decrease in dissolved oxygen, which were unplanned variables that arose after week four of the trial. Additionally, the trial was ended five weeks early because of the acute stress event, limiting the amount of data that was obtained. Though the stress factors were not controlled for, all fish were subjected to the same conditions throughout the duration of the study due to the nature of the RAS. Notable trends in per-diet feed efficiency appear when looking at the data separated into biweekly increments (Table 2.4). In the first two weeks under optimum water quality (W0-W2), the control fish gained the most weight, which may be attributed to not having to acclimate to a new feed. However, the following two weeks (W2-W4), all diets gained approximately equal weight, and some of the experimental diets exhibited lower FCR values than the control. Most importantly, during the last two weeks (W4-W6) under chronic thermal stress, all the experimental diets outperformed the control diet (though not statistically significant, $P > 0.05$) regarding both weight gain and FCR, apart from the FCR for BSF4 which was higher. If the duration of the trial had not been cut short, and the trends observed in Table 2.4 remained, the fish fed the experimental diets may have continued to further surpass the growth of the control fish, in turn leading to significant differences in production efficiency. Overall, the results indicate that the fish were better able to utilize the feed coated with feed additives while experiencing stress, when compared to the control.

When rainbow trout are subjected to thermal stress, a physiological stress response is induced in the fish that is associated with reduced food intake, growth inhibition, and severe damage to the intestinal tract (Zhou et al., 2022). High-temperature stress is also known to cause metabolic disorders and decreased immune function in rainbow trout, negatively impacting fish physiology and ultimately leading to death in extreme temperatures (Huyben et al., 2019; Zhou

et al., 2019). Regarding stress-related mortality, three of the experimental diets (BSY2, BSF3, Mix) exhibited higher survival rates (over 14-23% greater) compared to the control during the acute stress event, although not statistically significant ($P > 0.05$; Figure 2.1). The same three diets with the highest survival rate from the acute stress event also gained the most weight after six weeks of the trial. The results from this study indicate that the feed additives (both BSY and BSF) may have had a positive impact on feed efficiency and production of rainbow trout when under stress conditions, possibly by increasing the health and robustness of the fish via immunoregulation (Staykov et al., 2007, 2007; Fierro-Castro et al., 2013) or increased feed palatability, leading to higher feed intake (Jones, 1990).

The bacterial composition of the feed additives, largely dominated by Firmicutes (particularly *Bacillaceae*), did not appear to correlate with the changes in the intestinal bacterial community observed in the fish at T2, nor did the water column (Figures 2.2 and 2.3; Supplementary Figure A.1). Highly abundant families from the water column, within the phylum Bacteroidota and Proteobacteria, have been found as common isolates from water sources.

Beta diversity analysis revealed that the intestinal mucosa-associated bacterial communities of the rainbow trout were vastly different between time points, T1 and T2 ($P < 0.01$), but not between diets ($P > 0.5$). The observed timepoint differences likely reflect the effects of thermal stress (Figure 2.5). Significant differences ($P < 0.01$) were also found in the weighted diversity analysis between Control(T2) euthanized versus Control(ND) natural death fish, which accounts for bacterial species abundance on top of just presence/absence. Alpha diversity analysis, which looks at bacterial diversity within sample groups, found no significant differences across time points or diets (Table 2.5). However, a trend was seen in the control diet group in which a decline of all alpha diversity metrics occurred as time point progressed and

health declined. This agrees with previous research where less diversity, richness, and number of taxa in rainbow trout were observed under thermal stress conditions compared to normal rearing temperatures (Huyben et al., 2018; Zhou et al., 2022).

Interestingly, although BSF3 was found to have the lowest alpha diversity metrics across all the experimental diets, it exhibited the highest weight gain of the experimental diets both during chronic stress (W4-W6) and total (W0-W6). Although lower diversity typically has negative connotations, the results may indicate that high levels of a possibly beneficial microorganism could have led to out-competition of various other potentially harmful microorganisms, reducing the overall bacterial diversity. The ASV table (Supplementary Table A.2) revealed that BSF3 had a very high abundance of *Cetobacterium* sp., which was 4 to 5-fold higher than the other T2 diets. Further, when compared to Control(T2), BSF3 had a 39% reduction in *Mycobacterium* sp., a potential pathogen discussed in detail below. Although commonly found in high abundance, the role of *Cetobacterium* within the intestinal tract of rainbow trout elicits further research. One study exploring the effects of diet supplementation and handling-stress in rainbow trout suggested *Cetobacterium* may be important for the metabolism of certain nutrients and vitamins, but also discussed the necessity for further investigations of the exact role this organism plays within the intestinal tract (Suhr et al., 2023).

At the phylum level, all diets sampled at T2 appeared to be extremely similar (Figure 2.2B), apart from BSF3 having a higher abundance of Fusobacteriota compared to the rest. However, clear shifts in the dominant bacterial phyla between timepoint controls (Control(T1) vs. Control(T2)/(ND)) were observed (Figure 2.2A), with evident changes being increasing abundances of Actinobacteriota and Proteobacteria, and the diminishing of Firmicutes at Control(T2). Firmicutes have commonly been found to be a core constituent of the intestinal

bacterial community in various fish species, encompassing diverse families including both beneficial lactic acid bacteria (LAB) such as *Lactococcus lactis*, as well as fish-specific pathogens (Ringø and Gatesoupe, 1998; Hovda et al., 2007; Gatesoupe, 2008; Hines et al., 2023; Pough et al., 2024ab). Proteobacteria is also very commonly reported across the literature as being a large component of the fish gastrointestinal tract, and contains diverse members that can be both beneficial or pathogenic (Hines et al., 2023a). When looking at Control(ND), the natural death fish, the same decrease in Firmicutes and increase in Proteobacteria as Control(T2) was seen compared to Control(T1), but the dominant phyla became Fusobacteriota. For both BSF3 and Control(ND), the organism *Cetobacterium* sp. constituted essentially the entirety of the Fusobacteriota phylum (Table A.2). As described above, higher *Cetobacterium* sp. levels typically correlate with increased fish health (Tsuchiya et al., 2008; Qi et al., 2023; Suhr et al., 2023). Therefore, the reason for the high abundance of *Cetobacterium* sp. in Control(ND) is unknown, especially because it appeared to be a possibly beneficial organism in the BSF3 diet. Potential pathogens such as *Aeromonas* sp. were notably higher (3-fold) in Control(ND) when compared to BSF3.

The family-level heatmap (Figure 2.3) and differential abundance analysis (Figure 2.4) revealed specific changes between time point controls (Control(T1) and Control(T2)) within the phylum Firmicutes, including families such as *Clostridiaceae*, *Streptococcaceae*, and *Mycoplasmataceae*. Interestingly, *Mycoplasma* sp. (family *Mycoplasmataceae*), very commonly seen and classified as a commensal organism in the fish intestinal tract (Hines et al., 2023a), greatly increased in relative abundance in both Control(T2) and Control(ND) compared to Control(T1) (Figures 2.3 and 2.4, Supplementary Table A.2). Increased abundance of *Mycoplasma* in stressed fish has been observed in prior research concerning thermal stress and

the rainbow trout microbiome; though, conflicting results have been seen regarding high abundances of *Mycoplasma* correlating to both healthy and unhealthy fish (Huyben et al., 2018; Zhou et al., 2022). The reasons are unknown. *Clostridiaceae* and *Streptococcaceae* significantly decreased in Control(T2) and Control(ND); both families include pathogenic and beneficial organisms. Minor decreases in many organisms from the family *Streptococcaceae* occurred, which collectively created the large family-level shift. In contrast, the shift in *Clostridiaceae* appeared to consist of the decrease of one organism, *Clostridium sensu stricto* 1 sp. (Supplementary Table A.2). Similarly, the significant increase in the family *Fusobacteriaceae* (Phylum Fusobacteriota, discussed above) in Control(ND) was also contributed to changes in only one organism as well, *Cetobacterium* sp.. *Enterobacteriaceae*, which encompasses many foodborne and zoonotic pathogens, significantly increased in Control(ND) (Janda and Abbott, 2021). Identification past the family level was not assigned by the taxonomic classifier, therefore it is unknown whether the shift occurred due to one organism or a collection of many.

The wide variety of family level shifts, many of which were not discussed, helps demonstrate the extreme dysbiosis that occurred after the fish were subjected to high water temperatures for extended periods of time. Additionally, the impact that the abundance of one organism can have on a community is exemplified by some of the significant shifts. On the flip side, many organisms within the same family can be simultaneously affected as well, likely due to similar growth characteristics and requirements. Major shifts in the intestinal community likely allowed for the proliferation of organisms that would typically be suppressed or outcompeted by the beneficial commensals. It is known that intestinal dysbiosis is associated with disease in humans and animals, with the reduction of beneficial organisms allowing for the

proliferation of pathogenic species (DeGruttola et al., 2016). An emergence of potential pathogens *Mycobacteriaceae* and *Aeromonadaceae* was observed during this study.

Differential abundance analysis confirmed statistically significant increases in the abundance of *Mycobacteriaceae* between Control(T2) compared to Control(T1) (Figure 2.4). The ASV table of the most abundant organisms revealed that *Mycobacterium* sp. (family *Mycobacteriaceae*) represented virtually the entirety of Actinobacteriota observed in the T2 diets (Figure 2.2B), with the remaining organisms within that phylum having negligible relative abundances (Supplementary Table A.2). A large range of *Mycobacterium* species are associated with pertinent disease outbreaks in many animals including fish and humans, with the most common aquatic bacterial species being the zoonotic *Mycobacterium marinum* (Delghandi et al., 2020). Mycobacterial infections (mycobacteriosis) are a major concern in aquaculture and cause mass-mortality associated with large economic losses, as *Mycobacterium* species are extremely challenging to eradicate and control (Lescenko et al., 2003; Prearo et al., 2004; Yanong et al., 2010). While the emergence and high abundance of *Mycobacterium* sp. in all sampled T2 fish is likely attributed to intestinal dysbiosis caused by chronic stress (Beran et al., 2006; Slany et al., 2014), the etiology cannot be proven in this study. Vaccination against mycobacterial infections in fish are uncommon due to ineffectiveness, and treatment using antibiotics is difficult due to the nature of *Mycobacterium* spp. compounded with the increase of antibiotic resistance; prophylactic treatment and prevention remains to be the most effective mitigation strategies (Delghandi et al., 2020).

A significant increase in *Aeromonadaceae* occurred as well between the Control(ND) fish compared to Control(T1) and was also observed in fish fed the experimental diets sampled at T2. *Aeromonas* spp. encompasses various organisms that are pathogenic to both fish and

humans, such as the prominent aquaculture pathogen of major concern, *Aeromonas hydrophila*, in which antibiotics are no longer effective at controlling (Zepeda-Velázquez et al., 2015; Abdul Kari et al., 2022). Development of alternative treatments to antibiotics such as prebiotics and probiotics offer promise.

Conclusion

In conclusion, using BSY and BSF cultured on BSG as feed additives in the diets of rainbow trout did not significantly affect production efficiency. However, thermal stress became a significant factor during the trial, occurring at both a chronic (two-week) and acute (12-24 hr) level. Significant differences in the intestinal mucosa-associated bacterial community were observed between time point T1 and T2 controls, but not between any of the diets sampled at T2. The emergence of the potential pathogen *Mycobacterium* sp. in the bacterial communities of all T2 fish was likely a result of stress-induced intestinal dysbiosis. The bacterial communities of the feed additives did not appear to correlate with the shifts in the fish intestinal bacterial communities at T2, nor did the composition of the water column. Increased growth and survival rates were observed in three of the experimental diets (BSY2, BSF3, and mix) when under stress, indicating that the feed additives may have increased the health and robustness of the fish when subjected to these conditions. Further research is needed to evaluate if the observed benefits can be attributed to possible prebiotic effects of the feed additives.

Data Availability

Raw 16S rRNA sequencing files were deposited into the NCBI Sequence Read Archive (SRA) with accession number pending.

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Table 2.1. Trout production (week 6) and biometric (week 7) data (individual level; mean ± standard error) of BSY treatment groups compared to the control.

Treatment	Survival Rate (%)	Weight Gain (g)	Difference over control (%)	FCR†
Control	100	31.8 ± 1.2	--	1.35 ± 0.03
BSY1	100	31.6 ± 1.0	-1%	1.35 ± 0.06
BSY2	98	35.0 ± 1.9	10%	1.35 ± 0.67
Pooled Error	--	2.83	--	0.11
P-value	--	0.21	--	0.99

Treatment	Fillet Yields (%)	VSI†	HSI†	Fulton's Condition Factor
Control	54.8 ± 1.4	9.41 ± 0.33	1.78 ± 0.13	1.70 ± 0.06
BSY1	50.7 ± 1.4	9.40 ± 0.25	1.90 ± 0.13	1.64 ± 0.02
BSY2	54.4 ± 1.2	9.44 ± 0.30	2.08 ± 0.15	1.70 ± 0.05
Pooled Error	5.08	1.10	0.52	0.17
P-value	0.08	1.00	0.30	0.56

†Feed Conversion Ratio (FCR), Viscerosomatic Index (VSI) and Hepatosomatic Index (HSI), respectively.

Table 2.2. Trout production (week 6) and biometric (week 7) data (individual level; mean ± standard error) of BSF treatment groups compared to the control.

Treatment	Survival Rate (%)	Weight Gain (g)	Difference over control (%)	FCR†
Control	100	31.8 ± 1.2	--	1.35 ± 0.03
BSF3	96	35.9 ± 2.8	12%	1.34 ± 0.67
BSF4	100	30.4 ± 1.4	-4%	1.35 ± 0.67
Pooled Error	--	3.86	--	0.19
P-value	--	0.19	--	0.99

Treatment	Fillet Yields (%)	VSI†	HSI†	Fulton's Condition Factor
Control	54.8 ± 1.4	9.41 ± 0.33	1.78 ± 0.13	1.70 ± 0.06
BSF3	55.6 ± 0.5	9.61 ± 0.22	2.01 ± 0.09	1.69 ± 0.05
BSF4	54.3 ± 1.2	9.33 ± 0.36	1.93 ± 0.09	1.65 ± 0.03
Pooled Error	4.21	1.15	0.39	0.18
P-value	0.71	0.81	0.30	0.75

†Feed Conversion Ratio (FCR), Viscerosomatic Index (VSI) and Hepatosomatic Index (HSI), respectively.

Table 2.3. Trout production (week 6) and biometric (week 7) data (individual level; mean ± standard error) of the Mix treatment group compared to the control.

Treatment	Survival Rate (%)	Weight Gain (g)	Difference over control (%)	FCR†
Control	100	31.8 ± 1.2	--	1.35 ± 0.03
Mix	92	34.1 ± 1.5	7%	1.32 ± 0.66
Pooled Error	--	2.71	--	0.08
P-value	--	0.27	--	0.56
Treatment	**Fillet Yields (%)**	**VSI†**	**HSI†**	**Fulton's Condition Factor**
Control	54.8 ± 1.4	9.41 ± 0.33	1.78 ± 0.13	1.70 ± 0.06
Mix	52.8 ± 1.6	9.44 ± 0.33	1.78 ± 0.16	1.65 ± 0.04
Pooled Error	5.59	1.22	0.55	0.19
P-value	0.35	0.96	0.98	0.50

†Feed Conversion Ratio (FCR), Viscerosomatic Index (VSI) and Hepatosomatic Index (HSI), respectively.

Table 2.4. Trout production data (individual level; mean ± standard error) of all treatment groups compared to the control separated by two-week increments.

	W0-W2[†]			W2-W4[†]			W4-W6[†]		
Temperature[‡]	16.1 ± 0.4			18.6 ± 0.6			21.2 ± 0.3		
DO[‡]	7.30 ± 0.16			6.64 ± 0.17			5.64 ± 0.08		
FCR[§]	0.88 ± 0.03			1.02 ± 0.03			3.31 ± 0.73		
Treatment	**Survival (%)**	**Weight gain (g)**	**FCR[§]**	**Survival (%)**	**Weight gain (g)**	**FCR[§]**	**Survival (%)**	**Weight gain (g)**	**FCR[§]**
Control	100	13.1 ± 0.9	0.75 ± 0.05	100	14.0 ± 0.9	1.05 ± 0.05	100	5.2 ± 1.1	4.51 ± 1.48
BSY1	100	10.8 ± 0.6	0.93 ± 0.05	100	14.1 ± 0.7	1.00 ± 0.04	100	7.2 ± 1.0	2.84 ± 0.51
BSY2	100	11.8 ± 1.2	0.87 ± 0.44	100	14.1 ± 1.4	1.05 ± 0.52	98	7.9 ± 0.9	2.72 ± 1.36
BSF3	100	12.1 ± 1.3	0.83 ± 0.42	100	14.0 ± 1.3	1.05 ± 0.53	96	9.2 ± 1.5	2.48 ± 1.24
BSF4	100	10.0 ± 1.3	0.99 ± 0.49	100	13.7 ± 1.2	0.97 ± 0.48	100	6.2 ± 1.6	4.96 ± 2.48
Mix	92	12.1 ± 0.6	0.89 ± 0.44	100	15.1 ± 0.9	0.97 ± 0.49	100	7.0 ± 1.3	3.26 ± 1.63
Pooled error	--	2.03	0.17	--	2.14	0.16	--	2.51	2.63
P-value	--	0.36	0.53	--	0.97	0.93	--	0.38	0.69

[†]Week 0 to Week 2, Week 2 to Week 4, and Week 4 to Week 6 of the trial, respectively.

[‡] Temperature (˚C), Dissolved Oxygen (DO, mg/L), respectively.

[§] Feed Conversion Ratio (FCR).

Table 2.5. Summary of alpha diversity analyses of intestinal mucosa samples (mean ± standard error).

	Diet/Treatment	Shannon	Evenness	Observed ASVs
Control Diet	**Control(T1)**	3.12 ± 1.73	0.52 ± 0.23	66.17 ± 45.84
	Control(T2)	2.52 ± 1.03	0.49 ± 0.15	36.75 ± 22.96
	Control(ND)	1.67 ± 0.74	0.43 ± 0.14	17.13 ± 13.63
	P-value†	0.143	0.290	0.062
Experimental Diets	**Control(T2)**	2.52 ± 1.03	0.49 ± 0.15	36.75 ± 22.96
	BSY2	2.41 ± 0.85	0.50 ± 0.14	28.43 ± 11.30
	BSF3	1.50 ± 0.75	0.35 ± 0.14	19.43 ± 10.28
	Mix	2.33 ± 0.27	0.51 ± 0.08	26.71 ± 10.77
	P-value†	0.069	0.042*	0.252

†P-value of the one-way Kruskal-Wallis test.

*No significant differences found after Dunn's post-hoc test.

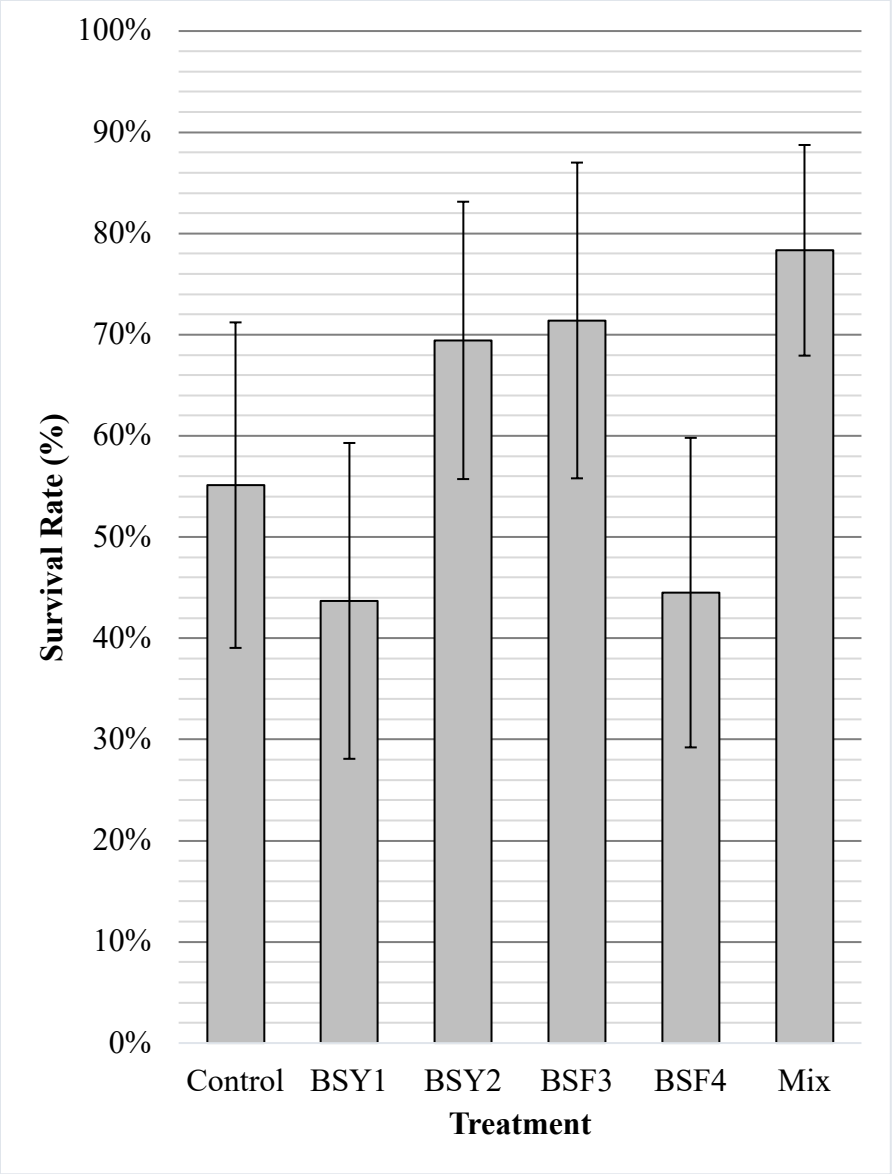


Figure 2.1. Acute stress event survival rate of all treatment groups. Average survival rates (%) were calculated based on the number of living fish the day prior to the overnight chiller failure, compared to the number of surviving fish the next day at the cessation of the trial. The number of surviving fish was recorded for each tank individually and then averaged according to diet. Error bars represent standard error of the mean (see the text for values).

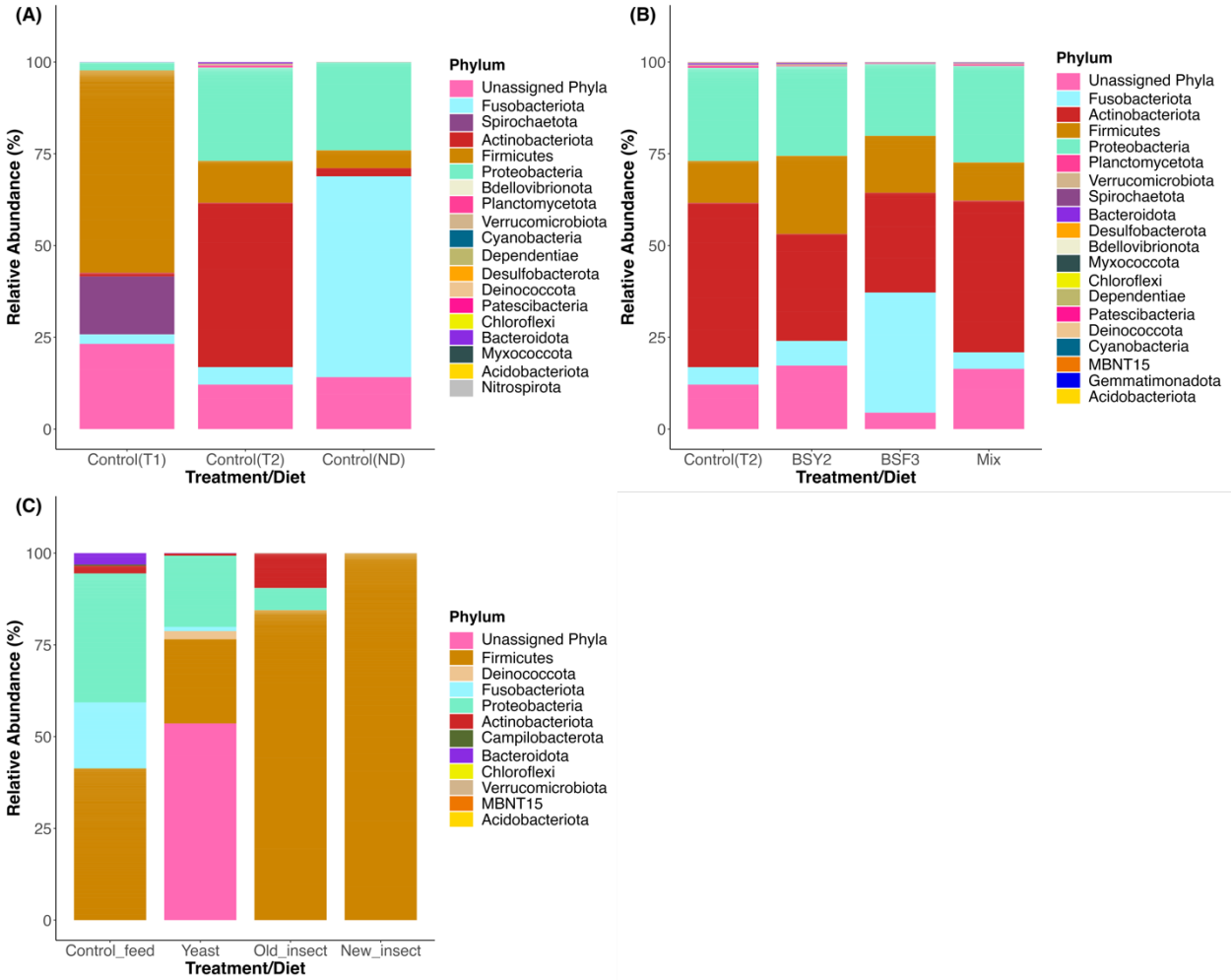


Figure 2.2. Phylum-level relative abundance bar plots of intestinal mucosa and feed bacterial communities. The 16S rRNA gene V4 region was sequenced from swab samples of the intestinal mucosa of rainbow trout, as well as from weighed samples of the control feed and feed additives. Mucosal samples from various time points of fish fed the control diet include: **(A)** time point 1 (Control(T1)), time point 2 euthanized (Control(T2)), and time point 2 natural death (Control(ND)). Intestinal samples of fish fed various diets taken at T2 include: **(B)** control, BSY2, BSF3, and mixed. Feed samples comprised of: **(C)** pellets of the control feed, the powdered yeast product, the "old" liquid insect product for BSF3, and the "new" liquid insect product for BSF4 and the mix. Bars were constructed using the average reads from the samples to illustrate relative diversity of the one hundred most abundant ASVs.

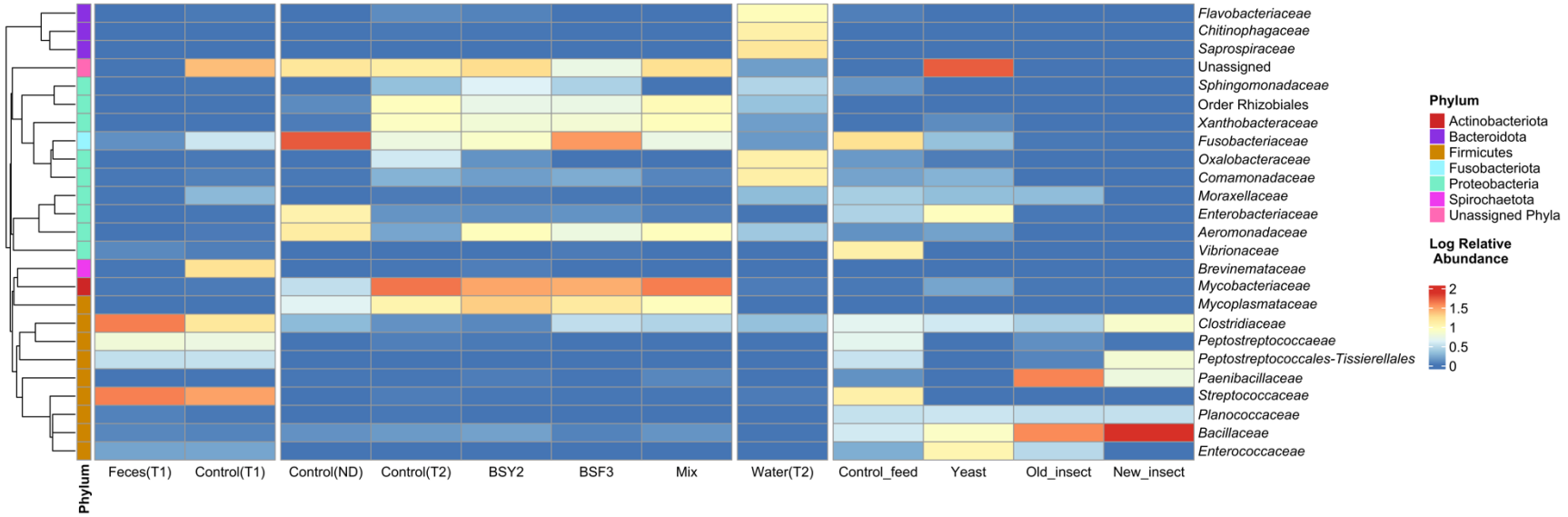


Figure 2.3. Family-level relative abundance heatmap of bacterial communities. The 16S rRNA gene V4 region was sequenced from various samples. Timepoint 1 includes: feces (Feces(T1)) and mucosa samples (Control(T1)) from fish fed the control diet. Timepoint 2 consists of: mucosa samples from fish fed the control (natural death Control(ND); euthanized, Control(T2)), BSY2, BSF3, and mixed diets, as well as a water column sample (Water(T2)). Lastly, the feed samples comprised of: pellets of the control feed, the powdered yeast product, the "old" liquid insect product for BSF3, and the "new" liquid insect product for BSF4 and the mix. QIIME2 was used to construct the phylogenetic tree on the lefthand side of the figure. The log relative abundance of the top 25 family-level taxa is plotted and sorted by phylum using the phylogenetic tree.

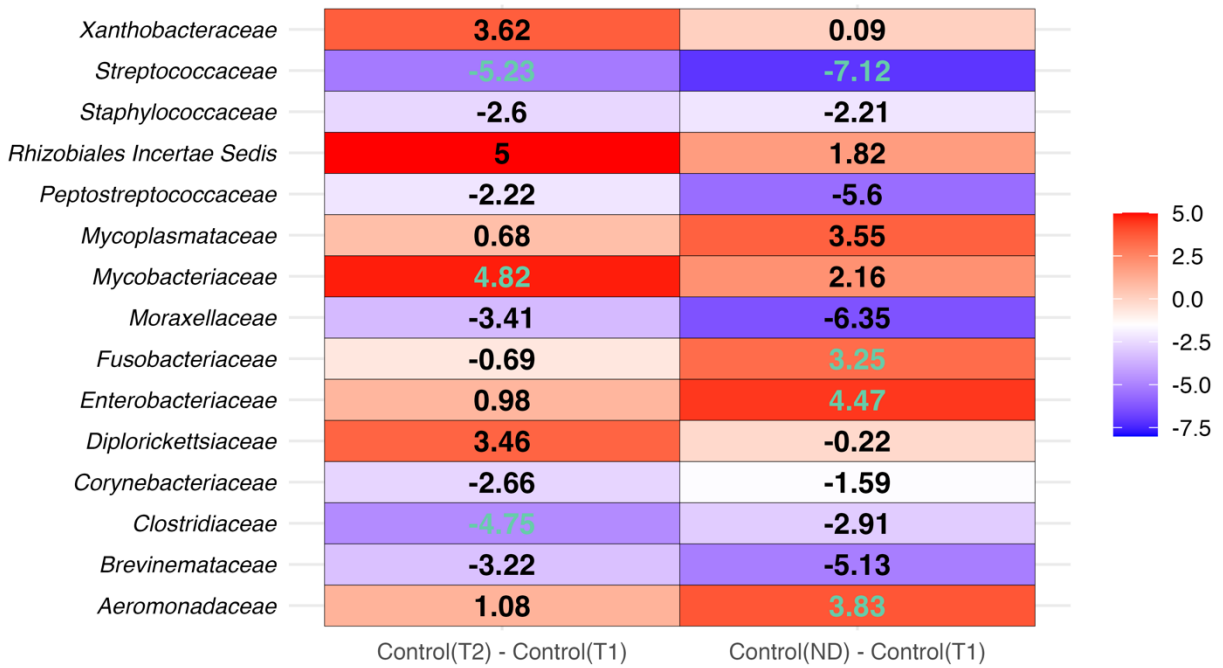


Figure 2.4. Heatmap of differential abundance showing significant taxonomic differences at the family level between intestinal bacterial communities of rainbow trout fed the control diet. Differential abundance was calculated via the analysis of compositions of microbiomes with bias correction 2 (ANCOM-BC2). The bacterial communities from the rainbow trout at T2 (euthanized, Control(T2); natural death, Control(ND)) were each compared to the bacterial community from the trout at T1 (Control(T1)) using Dunnett's type of test. The legend on the righthand side of the figure, as well as the value within each cell, represents the log fold-change (in natural log) of family-level relative abundance in comparison to Control(T1). Higher LFC values indicate larger differences between the shift in abundance compared to the reference community. Positive values (red) mean higher abundance of the family in the context of the community shift, and vice versa for negative (blue). The values presented in green represent the taxa that passed the sensitivity test regarding the addition of pseudo-counts on zero counts during the analysis.

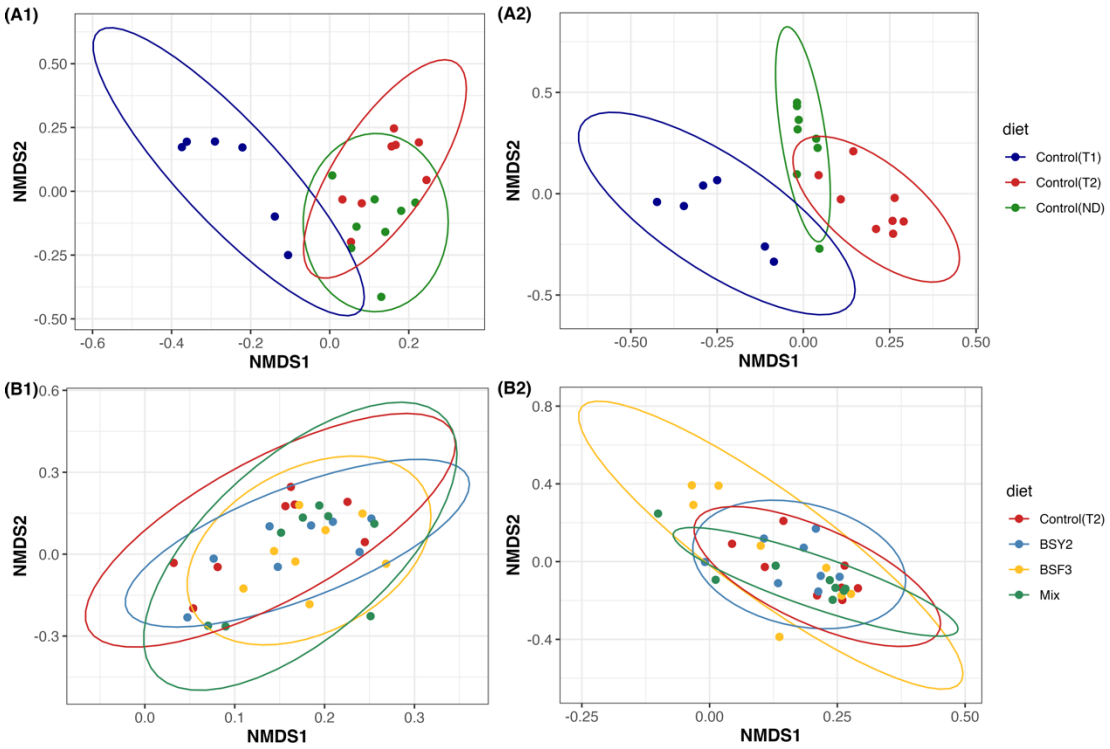


Figure 2.5. Non-metric Multidimensional Scaling (NMDS) plots representing unweighted and weighted beta diversities of intestinal bacterial communities. Dissimilarity was calculated using the **(1, left)** unweighted UniFrac and **(2, right)** weighted UniFrac distance. Intestinal mucosa samples from fish fed the control diet were sampled at: **(A)** time point 1 (Control(T1)), time point 2 euthanized (Control(T2)), and time point 2 natural death (Control(ND)). Intestinal mucosa samples of fish sampled at T2 are represented by the various diets as: **(B)** Control(T2), BSY2, BSF3, and mixed. Ellipses represent 95% confidence intervals that assume a multivariate t-distribution of: eight intestinal mucosa samples of fish sampled from each diet at either timepoint, except for Control(ND), which consisted of six.

Chapter Three

Concluding Remarks

The research presented in this thesis aimed to evaluate the efficacy of using low-value brewing industry byproducts converted into high-value feed additives in the diets of aquaculture-raised rainbow trout (*Oncorhynchus mykiss*). Particularly, the impacts of the feed additives on the production, health, and intestinal bacterial community of the fish were examined. Aquaculture is one of the fastest growing sectors of the food industry worldwide and continues to grow in importance as overfishing from wild-caught fisheries remains rampant in the environment. Natural resources have been depleted and extinction rates of various aquatic species remain high. The push toward optimizing aquaculture practices regarding environmental sustainability, food security, and production efficiency arose when the industry modernized, and large-scale production developed as a common practice. The reliance on aquaculture is ever-increasing as the human population only continues to multiply and food security remains a concern. Additionally, aquaculture is a profitable economic driver on both an industrial and local scale, providing employment and trade opportunities worldwide. Implementing sustainable practices across the entire food industry is vital, especially regarding the reprocessing of waste byproducts into valuable commodities. Research within the industry is needed in order to obtain these goals.

By partnering with Anheuser-Busch of the beer brewing industry and Maltento, a functional ingredient company, prevalent brewing byproducts (spent yeast, BSY and spent grain, BSG) of low economic value were transformed and recycled into feed additives for a use in fish feed. This was done by treatment of BSY with chitinase, which breaks down the chitin in the yeast cell wall, increasing bioavailability of nutrients to the fish. Additionally, the BSG was used by Maltento to cultivate black soldier fly (BSF, *Hermetia illucens*) larvae to convert the high-fiber byproduct into a nutritious source of proteins and lipids. Previous work within the lab found

that both BSY and BSF significantly increased growth rates of rainbow trout, which formed the basis of the current study. Finding the minimum concentration needed to observe these beneficial effects is essential in optimizing efficiency and production costs, especially because high concentrations of feed additives such as BSY, for example, have been found to negatively impact fish health. Additionally, previous research using alternative protein sources in fish feed has primarily focused on replacing fishmeal. Although that is also important, much less has been explored about the use of unconventional and reprocessed feed additives by themselves. Finding ways to recycle waste products efficiently is crucial, and positive results can spur promising movements toward sustainable practices. The goal of Chapter Two was to explore how the use of feed additives impacted growth rates and animal health, as well as the intestinal bacterial community of juvenile rainbow trout.

Within the intestinal microbiome, the bacterial community plays a major role in overall host health, influencing development, physiology, immunity, and nutrient acquisition, among other physiological traits. On the other hand, many exogenous factors such as diet, environmental conditions, and antibiotics can impact the bacterial community and therefore the host health as well. Intestinal dysbiosis can have major negative effects on host health, as large shifts in the bacterial community can create the opportunity for pathogen invasion. Antibiotic use is a major factor that can cause intestinal dysbiosis, and reducing overall use in not only healthcare but also the food industry has become a priority. The emergence of multi-drug resistant bacteria is a global concern, therefore, finding alternative prophylactic treatments is extremely important. Regulation of the intestinal bacterial community has become an emerging area of research to increase host health, as presented in this study. By using measures such as prebiotics to increase host disease resistance, antibiotic use can be reduced. The belief is that if

the host is more immune to disease, less disease would be present in the population, and therefore less antibiotics would be needed. Furthermore, antibiotics are commonly used as a preventative measure, so the replacement with other alternative treatment strategies would decrease their use as well. BSY and BSF used as feed additives could possibly act in this manner via prebiotic effects and intestinal bacterial community regulation in aquaculture-raised fish.

Both unexpected and promising results were discovered from this trial, despite no significant differences in production efficiency being observed. Inadvertently, fish health was compromised due to do an unintentional chronic period of high-water temperatures caused by equipment malfunction. The complete failure of the chiller used to maintain cool water temperatures occurred, despite maintenance and attempts to remedy the issue, causing the early cessation of the trial. The acute stress event led to a fascinating discovery that fish fed three of the experimental diets exhibited much higher survival rates compared to the control, although it was not statistically significant. Moreover, the emergence of a prominent aquaculture pathogen, *Mycobacterium* sp., was found in the intestinal bacterial community of fish from all diets sampled at the end of the trial. Although the etiology cannot be proven, the intestinal dysbiosis observed across all diets, including the control, may have been the cause of pathogens becoming dominant inhabitants within the intestinal tract. This further proves the importance of maintaining a healthy bacterial community and is a prime example of exogenous factors negatively impacting the bacterial community, and in turn, host health.

The production data, when broken into biweekly increments, also indicates that the feed additives benefitted the fish. During the two-week period of chronic stress, fish fed all of the experimental diets outperformed the control regarding weight gain, with four of the five having a lower feed conversion ratio as well. These findings suggest the fish were better able to convert

the feed containing the additives into body mass while undergoing a physiological stress response. The reproducibility of these findings and reasoning as to why the feed additives appear beneficial should be investigated further. The exact etiology of what caused the mortality event could not be proven. For example, it remains to be determined if it was caused by the high-water temperatures, the low dissolved oxygen associated with the temperature, the intestinal dysbiosis and proliferation of potential pathogens, or a combination of all these factors. A trial controlling for environmental stress conditions is needed, and exploration into the functionality of bacteria (i.e., *Mycoplasma* or *Cetobacterium*) within the intestinal tract should also occur via metagenomics, metatranscriptomics, and/or metabolomics analyses. To reevaluate the production efficacy, the trial should be repeated for a longer duration without the compounding factor of thermal stress.

Different approaches to microbial community analysis could also be applied to try and obtain higher taxonomic resolution. For example, although the V4 region of the 16S rRNA gene is widely used and agreed upon as a good gene marker for bacterial identification, reliable differentiation at the species level is challenging. As methods are constantly evolving, keeping up to date with the newest bioinformatic pipelines and databases may help solve the problem without changing pre-sequencing sample processing. However, metagenomics could provide an alternative approach for taxonomic classification, but limitations for accurate species identification would still be present. Further, due to large sample-by-sample variation of bacterial communities within experimental groups, higher statistical power could be achieved by increasing the sample size and/or determining additional bioinformatic sequence filtering methods to increase accuracy.

Though not statistically proven during this study, the use of the feed additives procured some very promising results for future experimental trials. If significant benefits can be consistently proven, widespread use of low-value waste products converted into high-value feed additives could become a common practice, leading to higher economic value, sustainability, and production efficiency within aquaculture.

Appendix A

Chapter Two Supplementary Material

Supplementary Table A.1. Summary of alpha diversity analyses of feed (mean ± standard error).

	Diet/Treatment	Shannon	Evenness	Observed ASVs
Feed Samples	**Control feed**	6.83 ± 0.06^{a}	0.86 ± 0.01^{a}	238.50 ± 7.72^{a}
	Yeast	3.49 ± 0.13^{b}	$0.72 \pm 0.03^{a,b}$	28.50 ± 0.71^{b}
	Old insect	3.62 ± 0.15^{b}	0.58 ± 0.01^{b}	75.00 ± 5.35^{b}
	New insect	$4.69 \pm 0.07^{a,b}$	$0.69 \pm 0.01^{a,b}$	$111.00 \pm 2.45^{a,b}$
	P-value[†]	0.008	0.007	0.007

[†]P-value of the one-way Kruskal-Wallis test.

[ab]Diversity metric within group significant at $P < 0.05$ following a one-way Kruskal-Wallis with Dunn's post-hoc test.

Supplementary Table A.2. Amplicon sequence variant relative abundance table (mean ± SEM) of trout intestinal samples ordered by the overall highest average read count of organisms based on Control(T2).

Phylogeny			**Relative Abundance (%)**					
Phylum	**Family**	**Organism**	**Control(T1)**	**Control(T2)**	**Control(ND)**	**BSY2**	**BSF3**	**Mix**
Actinobacteriota	*Mycobacteriaceae*	***Mycobacterium* sp.**	0.00 ± 0.00	44.21 ± 1.34	2.22 ± 0.14	28.96 ± 1.02	26.98 ± 1.52	40.99 ± 1.25
Unassigned Phyla	*Unassigned*	**Unassigned**	23.19 ± 2.72	12.11 ± 1.06	14.14 ± 1.19	17.3 ± 1.32	4.47 ± 0.48	16.41 ± 1.42
Firmicutes	*Mycoplasmataceae*	***Mycoplasma* sp.**	0.00 ± 0.00	10.32 ± 1.27	3.51 ± 0.34	20.15 ± 1.3	13.12 ± 1.49	8.06 ± 0.53
Proteobacteria	*Rhizobiales Incertae Sedis*	***Rhizobiales Incertae Sedis***	0.00 ± 0.00	7.86 ± 0.35	0.24 ± 0.01	4.22 ± 0.16	4.43 ± 0.27	8.88 ± 0.31
Proteobacteria	*Xanthobacteraceae*	***Ancylobacter* sp.**	0.00 ± 0.00	7.14 ± 0.27	0.08 ± 0.00	5.31 ± 0.16	5.45 ± 0.32	8.07 ± 0.28
Fusobacteriota	*Fusobacteriaceae*	***Cetobacterium* sp.**	2.42 ± 0.27	4.75 ± 0.29	54.72 ± 1.74	6.72 ± 0.41	32.73 ± 1.78	4.49 ± 0.35
Proteobacteria	*Oxalobacteraceae*	***Undibacterium* sp.**	0.00 ± 0.00	2.38 ± 0.30	0.00 ± 0.00	0.15 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
Proteobacteria	*Reyranellaceae*	***Reyranella* sp.**	0.00 ± 0.00	2.06 ± 0.22	0.02 ± 0.00	0.69 ± 0.06	0.41 ± 0.03	0.13 ± 0.01
Proteobacteria	*Sphingomonadaceae*	***Novosphingobium* sp.**	0.00 ± 0.00	0.83 ± 0.10	0.02 ± 0.00	2.91 ± 0.36	0.02 ± 0.00	0.00 ± 0.00
Proteobacteria	*Comamonadaceae*	***Comamonadaceae***	0.08 ± 0.01	0.72 ± 0.06	0.04 ± 0.00	0.46 ± 0.03	0.65 ± 0.08	0.19 ± 0.02
Proteobacteria	*Aeromonadaceae*	***Aeromonas* sp.**	0.00 ± 0.00	0.57 ± 0.03	12.35 ± 0.96	8.09 ± 0.98	4.71 ± 0.35	7.94 ± 0.94
Firmicutes	*Bacillaceae*	***Bacillus* sp.**	0.00 ± 0.00	0.38 ± 0.02	0.27 ± 0.02	0.12 ± 0.01	0.14 ± 0.01	0.35 ± 0.02
Firmicutes	*Clostridiaceae*	***Clostridium sensu stricto 1* sp.**	13.81 ± 1.39	0.30 ± 0.03	0.62 ± 0.07	0.13 ± 0.01	1.10 ± 0.14	0.11 ± 0.01
Proteobacteria	*Oxalobacteraceae*	***Janthinobacterium* sp.**	0.00 ± 0.00	0.30 ± 0.04	0.00 ± 0.00	0.19 ± 0.02	0.00 ± 0.00	0.00 ± 0.00
Planctomycetota	*Isosphaeraceae*	***Tundrisphaera* sp.**	0.00 ± 0.00	0.29 ± 0.01	0.00 ± 0.00	0.05 ± 0.00	0.08 ± 0.01	0.24 ± 0.01
Proteobacteria	*Sphingomonadaceae*	***Sphingopyxis* sp.**	0.00 ± 0.00	0.29 ± 0.02	0.00 ± 0.00	0.26 ± 0.03	1.64 ± 0.20	0.00 ± 0.00
Proteobacteria	*Legionellaceae*	***Legionella* sp.**	0.00 ± 0.00	0.28 ± 0.01	0.00 ± 0.00	0.13 ± 0.01	0.16 ± 0.01	0.29 ± 0.01
Proteobacteria	*Rhizobiaceae*	***Shinella* sp.**	0.00 ± 0.00	0.28 ± 0.02	0.01 ± 0.00	0.28 ± 0.03	0.00 ± 0.00	0.00 ± 0.00
Actinobacteriota	PeM15	**PeM15 sp.**	0.00 ± 0.00	0.25 ± 0.01	0.00 ± 0.00	0.08 ± 0.00	0.08 ± 0.01	0.18 ± 0.01
Proteobacteria	*Enterobacteriaceae*	***Escherichia-Shigella* sp.**	0.00 ± 0.00	0.24 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Bacteroidota	*Flavobacteriaceae*	***Flavobacterium* sp.**	0.00 ± 0.00	0.21 ± 0.02	0.00 ± 0.00	0.15 ± 0.02	0.00 ± 0.00	0.00 ± 0.00
Verrucomicrobiota	*Simkaniaceae*	***Simkaniaceae***	0.00 ± 0.00	0.20 ± 0.01	0.00 ± 0.00	0.16 ± 0.01	0.08 ± 0.00	0.21 ± 0.01
Proteobacteria	*Diplorickettsiaceae*	***Diplorickettsiaceae***	0.00 ± 0.00	0.15 ± 0.00	0.00 ± 0.00	0.05 ± 0.00	0.12 ± 0.01	0.22 ± 0.01
Proteobacteria	*Pseudomonadaceae*	***Pseudomonas* sp.**	0.00 ± 0.00	0.14 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Proteobacteria	*Enterobacteriaceae*	***Enterobacteriaceae***	0.00 ± 0.00	0.13 ± 0.01	10.62 ± 0.94	0.28 ± 0.02	0.35 ± 0.03	0.11 ± 0.01
Proteobacteria	*Acetobacteraceae*	***Acidisphaera* sp.**	0.00 ± 0.00	0.11 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.00	0.00 ± 0.00

Firmicutes	*Peptostreptococcaceae*	***Peptostreptococcus* sp.**	4.46 ± 0.26	0.11 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Proteobacteria	*Caedibacteraceae*	***Caedimonas varicaedens***	0.00 ± 0.00	0.11 ± 0.01	0.00 ± 0.00	0.07 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
Proteobacteria	*Rhodobacteraceae*	***Rhodobacter* sp.**	0.00 ± 0.00	0.10 ± 0.01	0.00 ± 0.00	0.07 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
Proteobacteria	*Beijerinckiaceae*	***Methylobacterium-Methylorubrum* sp.**	0.00 ± 0.00	0.10 ± 0.01	0.01 ± 0.00	0.13 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
Proteobacteria	*Chromobacteriaceae*	***Vogesella fluminis***	0.00 ± 0.00	0.10 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Proteobacteria	*Xanthomonadaceae*	***Stenotrophomonas* sp.**	0.00 ± 0.00	0.10 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.28 ± 0.03	0.00 ± 0.00
Proteobacteria	*Xanthobacteraceae*	***Xanthobacteraceae***	0.00 ± 0.00	0.10 ± 0.01	0.00 ± 0.00	0.16 ± 0.02	0.10 ± 0.01	0.00 ± 0.00
Proteobacteria	*Solimonadaceae*	***Nevskia* sp.**	0.00 ± 0.00	0.09 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Planctomycetota	*Pirellulaceae*	**Pir4 lineage sp.**	0.00 ± 0.00	0.09 ± 0.01	0.00 ± 0.00	0.07 ± 0.01	0.03 ± 0.00	0.05 ± 0.00
Proteobacteria	*Comamonadaceae*	***Rubrivivax* sp.**	0.00 ± 0.00	0.08 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Proteobacteria	SC-I-84	**SC-I-84 sp.**	0.00 ± 0.00	0.08 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Bdellovibrionota	0319-6G20	**0319-6G20 sp.**	0.00 ± 0.00	0.07 ± 0.01	0.00 ± 0.00	0.06 ± 0.01	0.00 ± 0.00	0.01 ± 0.00
Proteobacteria	*Chromobacteriaceae*	***Vogesella* sp.**	0.00 ± 0.00	0.07 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Planctomycetota	*Pirellulaceae*	***Pirellulaceae***	0.00 ± 0.00	0.07 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.00	0.04 ± 0.00
Proteobacteria	*Moraxellaceae*	***Acinetobacter* sp.**	0.94 ± 0.08	0.07 ± 0.01	0.00 ± 0.00	0.07 ± 0.01	0.03 ± 0.00	0.00 ± 0.00
Proteobacteria	*Alcaligenaceae*	***Alcaligenaceae***	0.00 ± 0.00	0.06 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Proteobacteria	*Rhodocyclaceae*	***Methyloversatilis* sp.**	0.00 ± 0.00	0.06 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Proteobacteria	*Xanthobacteraceae*	***Bradyrhizobium* sp.**	0.00 ± 0.00	0.06 ± 0.00	0.00 ± 0.00	0.05 ± 0.00	0.16 ± 0.02	0.02 ± 0.00
Firmicutes	*Lactobacillaceae*	***Lactobacillus* sp.**	0.26 ± 0.02	0.06 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Bacillaceae*	***Cerasibacillus* sp.**	0.00 ± 0.00	0.05 ± 0.00	0.03 ± 0.00	0.27 ± 0.03	0.00 ± 0.00	0.00 ± 0.00
Bacteroidota	*Flavobacteriaceae*	**Flavobacteriia bacterium**	0.00 ± 0.00	0.05 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Proteobacteria	*Comamonadaceae*	***Variovorax* sp.**	0.00 ± 0.00	0.05 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Proteobacteria	*Beijerinckiaceae*	***Bosea* sp.**	0.00 ± 0.00	0.05 ± 0.00	0.00 ± 0.00	0.06 ± 0.01	0.09 ± 0.01	0.03 ± 0.00
Actinobacteriota	*Microbacteriaceae*	***Galbitalea* sp.**	0.00 ± 0.00	0.05 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.00	0.05 ± 0.00
Spirochaetota	*Brevinemataceae*	**uncultured spirochete**	15.72 ± 1.69	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Streptococcaceae*	***Streptococcus* sp.**	13.48 ± 0.84	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Streptococcaceae*	***Streptococcus dysgalactiae***	8.21 ± 0.54	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Streptococcaceae*	***Streptococcus agalactiae***	2.56 ± 0.15	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Firmicutes	*Streptococcaceae*	***Lactococcus lactis***	1.88 ± 0.10	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Streptococcaceae*	***Streptococcus henryi***	1.23 ± 0.08	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Proteobacteria	*Yersiniaceae*	***Yersiniaceae***	0.78 ± 0.13	0.00 ± 0.00	0.13 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.01
Firmicutes	*Peptostreptococcales-Tissierellales*	***Peptoniphilus indolicus***	0.69 ± 0.05	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Carnobacteriaceae*	***Granulicatella* sp.**	0.64 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Enterococcaceae*	***Enterococcus* sp.**	0.55 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Peptococcaceae*	***Peptococcus* sp.**	0.55 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Peptostreptococcales-Tissierellales*	***Gallicola* sp.**	0.54 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Streptococcaceae*	***Lactococcus garvieae***	0.53 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Actinobacteriota	*Coriobacteriales Incertae Sedis*	***Raoultibacter* sp.**	0.44 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Streptococcaceae*	***Streptococcus iniae***	0.40 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Streptococcaceae*	***Streptococcus minor***	0.31 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Streptococcaceae*	***Streptococcus suis***	0.31 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Lachnospiraceae*	***Lachnospiraceae***	0.29 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Peptostreptococcales-Tissierellales*	**swine effluent**	0.26 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Streptococcaceae*	***Lactococcus raffinolactis***	0.25 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Actinobacteriota	*Atopobiaceae*	***Atopobiaceae***	0.22 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Peptostreptococcales-Tissierellales*	***Peptoniphilus methioninivorax***	0.21 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Streptococcaceae*	***Streptococcus parauberis***	0.20 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Streptococcaceae*	***Streptococcus gallinaceus***	0.18 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Peptostreptococcales-Tissierellales*	**swine manure**	0.18 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Streptococcaceae*	***Lactococcus* sp.**	0.16 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Peptostreptococcales-Tissierellales*	***Peptostreptococcales-Tissierellales***	0.14 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Streptococcaceae*	***Streptococcus pluranimalium***	0.12 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Lachnospiraceae*	***Frisingicoccus* sp.**	0.11 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Firmicutes	*Lachnospiraceae*	***Catenibacillus* sp.**	0.11 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Streptococcaceae*	***Streptococcus* sp.**	0.00 ± 0.00	0.10 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Actinobacteriota	*Corynebacteriaceae*	***Corynebacterium* sp.**	0.09 ± 0.01	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.03 ± 0.00	0.04 ± 0.00
Firmicutes	*Peptostreptococcaceae*	***Romboutsia* sp.**	0.09 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Order Lactobacillales*	**Order Lactobacillales**	0.08 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Fusobacteriota	*Fusobacteriaceae*	**Fusobacteriaceae bacterium**	0.08 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Vagococcaceae*	***Vagococcus teuberi***	0.08 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Proteobacteria	*Vibrionaceae*	***Photobacterium* sp.**	0.07 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Peptostreptococcales-Tissierellales*	***Helcococcus* sp.**	0.07 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Lachnospiraceae*	***Blautia glucerasea***	0.07 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Staphylococcaceae*	***Staphylococcus* sp.**	0.07 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.13 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Veillonellaceae*	***Veillonella* sp.**	0.07 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Peptostreptococcales-Tissierellales*	***Peptoniphilus stercorisuis***	0.07 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Vagococcaceae*	***Vagococcus fluvialis***	0.07 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Proteobacteria	*Hafniaceae*	***Hafnia-Obesumbacterium* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.35 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.21 ± 0.03
Firmicutes	*Clostridiaceae*	***Clostridium gasigenes***	0.00 ± 0.00	0.00 ± 0.00	0.26 ± 0.03	0.08 ± 0.01	1.08 ± 0.13	0.79 ± 0.10
Firmicutes	*Clostridiaceae*	***Clostridium amazonense***	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.84 ± 0.10
Firmicutes	*Clostridiaceae*	***Clostridium botulinum***	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Cyanobacteria	*Sericytochromatia*	***Sericytochromatia* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Proteobacteria	*Sphingomonadaceae*	***Sphingobium* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.00	0.06 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
Chloroflexi	RBG-13-54-9	**RBG-13-54-9 sp.**	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Paenibacillaceae*	***Paenibacillus odorifer***	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.09 ± 0.01	0.00 ± 0.00	0.19 ± 0.02
Firmicutes	*Bacillaceae*	***Oceanobacillus caeni***	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.16 ± 0.02	0.00 ± 0.00	0.00 ± 0.00
Proteobacteria	*Oxalobacteraceae*	***Massilia* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Desulfobacterota	*Desulfovibrionaceae*	***Desulfovibrionaceae***	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Bacteroidota	*Weeksellaceae*	***Cloacibacterium* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.03 ± 0.00	0.13 ± 0.02
Firmicutes	*Bacillaceae*	***Bacillus oleronius***	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Verrucomicrobiota	*Parachlamydiaceae*	***Parachlamydiaceae***	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.00

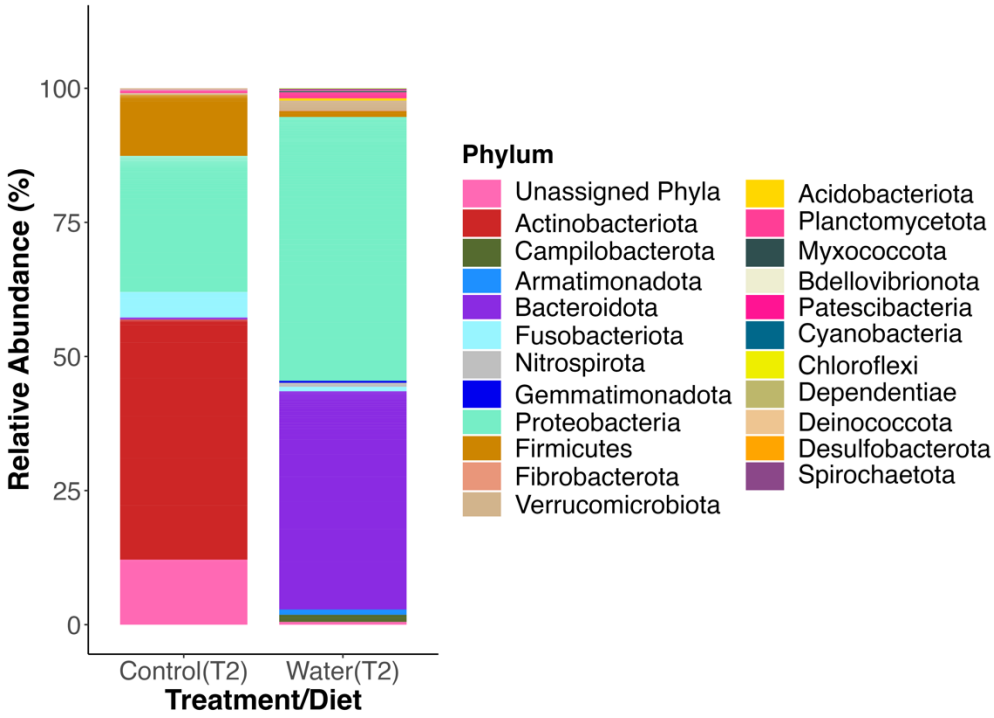
Proteobacteria	*Sutterellaceae*	***Sutterellaceae***	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.05 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Clostridiaceae*	***Clostridium argentinense***	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Proteobacteria	*Chromobacteriaceae*	***Aquitalea* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Proteobacteria	*Comamonadaceae*	***Hydrogenophaga* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.01	0.00 ± 0.00
Actinobacteriota	*Actinomycetaceae*	**bacterium NLAE-zl-C91**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Bacillaceae*	***Pseudogracilibacillus* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Deinococcota	*Thermaceae*	***Meiothermus* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Paenibacillaceae*	***Paenibacillus cookii***	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Planctomycetota	*Rubinisphaeraceae*	**uncultured planctomycete**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.04 ± 0.00
Bacteroidota	*Chitinophagaceae*	***Sediminibacterium* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Actinobacteriota	*Actinomycetaceae*	**uncultured Actinomycetales**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Verrucomicrobiota	*Rubritaleaceae*	***Luteolibacter* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.13 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
Proteobacteria	*Pseudomonadaceae*	***Azotobacter* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.11 ± 0.01	0.15 ± 0.02	0.00 ± 0.00
Verrucomicrobiota	*Rubritaleaceae*	***Verrucomicrobium* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.08 ± 0.01	0.01 ± 0.00	0.00 ± 0.00
Proteobacteria	*Rhizobiaceae*	***Rhizobiaceae***	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.01	0.00 ± 0.00	0.01 ± 0.00
Proteobacteria	*Rhodocyclaceae*	***Azospira* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.01	0.01 ± 0.00	0.00 ± 0.00
Firmicutes	*Peptostreptococcaceae*	***Paraclostridium* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.05 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
Chloroflexi	JG30-KF-CM45	**JG30-KF-CM45 sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.05 ± 0.01	0.00 ± 0.00	0.02 ± 0.00
Dependentiae	*Vermiphilaceae*	***Vermiphilaceae* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.04 ± 0.00
Bacteroidota	*Sphingobacteriaceae*	***Pedobacter* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.05 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
Firmicutes	*Carnobacteriaceae*	***Alloiococcus* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Proteobacteria	*Hyphomicrobiaceae*	***Hyphomicrobium* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.23 ± 0.03	0.00 ± 0.00
Proteobacteria	*Xanthomonadaceae*	***Pseudoxanthomonas* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.20 ± 0.02	0.00 ± 0.00
Proteobacteria	*Caulobacteraceae*	***Phenylobacterium* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.08 ± 0.01	0.00 ± 0.00
Proteobacteria	*Vibrionaceae*	***Vibrio* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.01	0.00 ± 0.00
Actinobacteriota	*Nocardiaceae*	***Nocardia* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.01	0.00 ± 0.00
Bacteroidota	env.OPS 17	**env.OPS 17 sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.01	0.04 ± 0.00

Planctomycetota	Order Planctomycetales	**Order Planctomycetales**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.00	0.07 ± 0.00
Verrucomicrobiota	cvE6	**Chlamydiales bacterium**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
Planctomycetota	*Gemmataceae*	***Gemmataceae***	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.02 ± 0.00
Proteobacteria	*Halieaceae*	**OM60(NOR5) clade sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
MBNT15	MBNT15	**MBNT15 sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
Actinobacteriota	*Microbacteriaceae*	***Microbacterium* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.00
Actinobacteriota	67-14	**67-14 sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.00
Cyanobacteria	*Gastranaerophilales*	***Gastranaerophilales* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.00
Proteobacteria	*Rhizobiales Incertae Sedis*	***Phreatobacter* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.00
Actinobacteriota	*Nocardiaceae*	***Rhodococcus* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.00
Verrucomicrobiota	*Verrucomicrobiaceae*	***Prosthecobacter* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00
Firmicutes	*Paenibacillaceae*	***Paenibacillus* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00
Proteobacteria	*Rhodobacteraceae*	***Rhodobacteraceae***	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00
Actinobacteriota	*Solirubrobacteraceae*	***Conexibacter* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00
Actinobacteriota	*Iamiaceae*	***Iamia* sp.**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00

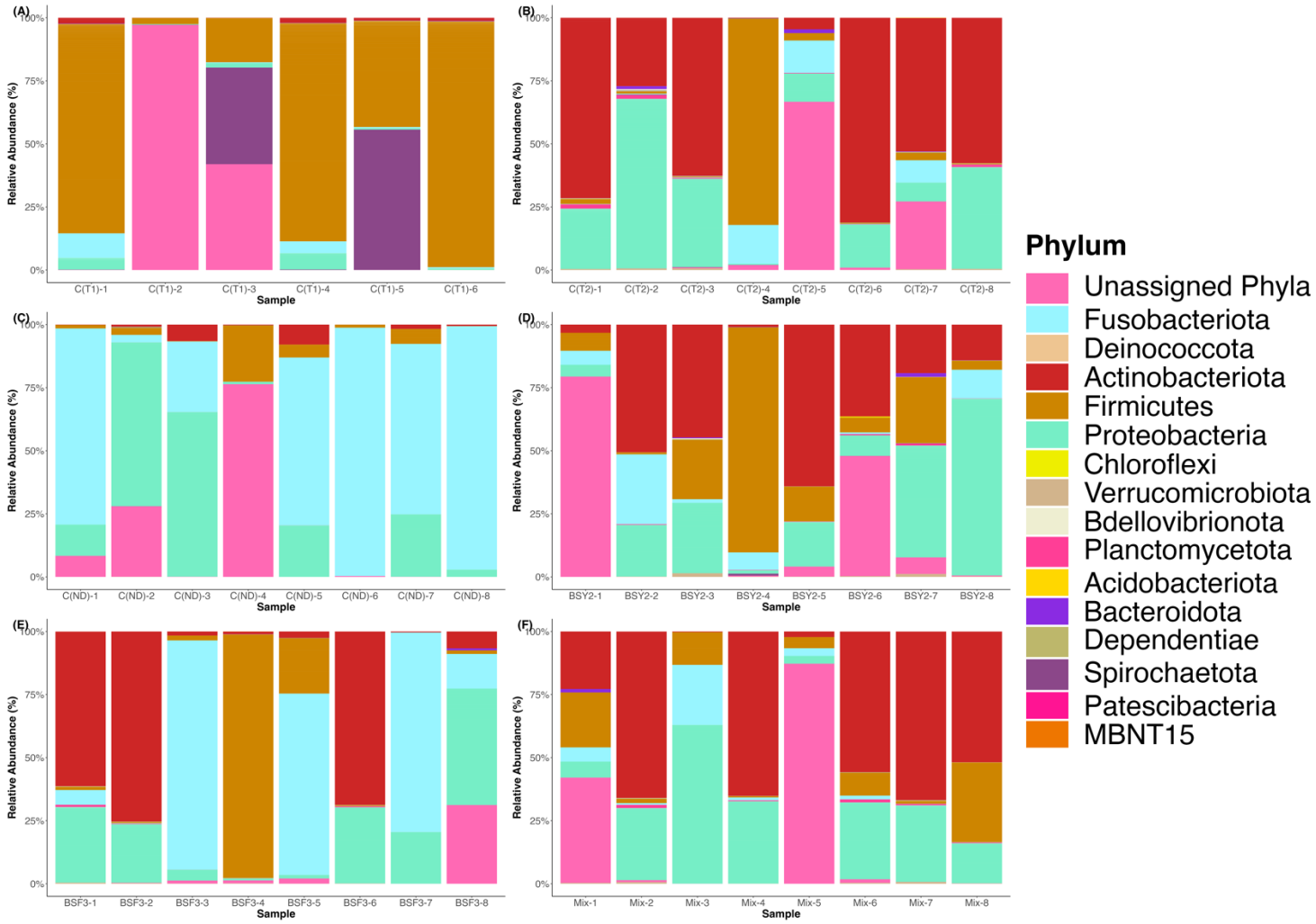
Supplementary Table A.3. Statistical summary of beta diversity analyses.

	Unweighted UniFrac			Weighted UniFrac		
	PERMA-NOVA	**Pairwise p-value**	**Pairwise p.adjusted**	**PERMA-NOVA**	**Pairwise p-value**	**Pairwise p.adjusted**
CONTROLS	**0.001 *****			**0.001 *****		
Control(T1) vs Control(T2)		0.002 **	0.006 **		0.002 **	0.006 **
Control(T1) vs Control(ND)		0.003 **	0.009 **		0.003 **	0.009 **
Control(T2) vs Control(ND)		0.018 *	0.054		0.002 **	0.006 **
T2 DIETS	**0.916**			**0.588**		
Control(T2) vs BSY2		0.914	1		0.922	1
Control (T2) vs BSF3		0.739	1		0.748	1
Control(T2) vs Mix		0.745	1		0.751	1
BSY2 vs BSF3		0.949	1		0.935	1
BSY2 vs Mix		0.489	1		0.495	1
BSF3 vs Mix		0.584	1		0.569	1
FEED ADDITIVES	**0.001 *****			**0.001 *****		
Control Feed vs New Insect		0.030 *	0.180		0.035 *	0.210
Control Feed vs Old Insect		0.024 *	0.144		0.029 *	0.174
Control Feed vs Yeast		0.032 *	0.192		0.034 *	0.204
New Insect vs Old Insect		0.035 *	0.210		0.030 *	0.180
New Insect vs Yeast		0.030 *	0.180		0.034 *	0.204
Old Insect vs Yeast		0.029 *	0.174		0.034 *	0.204

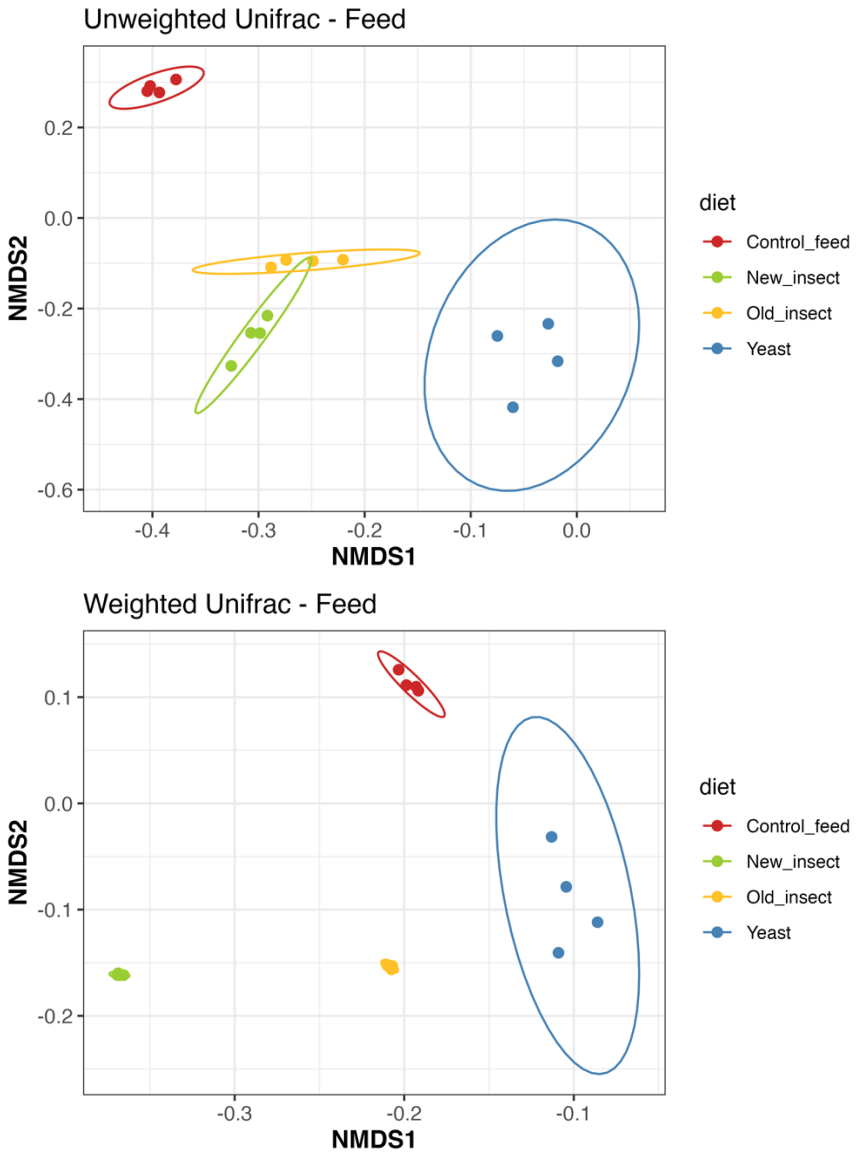
*Significant at $P < 0.05$
**Significant at $P < 0.01$
***Significant at $P < 0.001$



Supplementary Figure A.1. Phylum-level relative abundance bar plot of bacterial communities. The 16S rRNA gene V4 region was sequenced from swab samples of the intestinal mucosa of rainbow trout, as well as from the retentate of the water column produced after vacuum filtration. The bacterial community of the mucosal samples from fish fed the control diet sampled at T2 (Control(T2); n=8) are compared next to the water column sampled at T2 (Water(T2); n=2). Bars were constructed using the average reads from the samples to illustrate relative diversity of the one hundred most abundant ASVs.



Supplementary Figure A.2. Phylum-level relative abundance bar plots of individual sample bacterial communities. The 16S rRNA gene V4 region was sequenced from swab samples of the intestinal mucosa of rainbow trout. Individual mucosa samples within each diet group (n=8 for all besides Control(T1), where n=6) are presented as follows: **(A)** Control(T1), **(B)** Control(T2), **(C)** Control(ND), **(D)** BSY2, **(E)** BSF3, and **(F)** Mix. Bars were constructed using the reads from the individual samples to illustrate relative diversity of the one hundred most abundant ASVs.



Supplementary Figure A.3. Non-metric Multidimensional Scaling (NMDS) plots representing unweighted and weighted beta diversities of feed bacterial communities. Dissimilarity was calculated using the **(top)** unweighted UniFrac and **(bottom)** weighted UniFrac distance. Feed samples comprised of: **(C)** pellets of the control feed, the "new" liquid insect product used for BSF4 , the "old" liquid old insect product used for BSF3, and the powdered yeast product. Ellipses represent 95% confidence intervals that assume a multivariate t-distribution of four samples of each feed additive.