THE INFLUENCES OF DIET AND WATER SYSTEMS ON RAINBOW TROUT GUT MICROBIOME IN RELATION TO NUTRIENT UTILIZATION

by

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DEDICATION

This dissertation is dedicated to the memory of my late parents, Mr. and Mrs. Bisileko Alege. Dad, you died when I was just about 3 months old, but you left me a mom who cared so much for me. Mom, I can't be grateful enough for your courage, when the people around said you should not bother about the little one you held in your hands when dad died because "she's not going to make it". I missed your love mom!

Also, I dedicate this thesis to my family, my husband Eriola and my kids – Toni, Momi and Adeogo. Thanks guys for your encouragement and love.

Finally, "to Him who is able to do immeasurably more than all we ask or imagine, according to His power that is at work within us" the Almighty God, I also dedicate this dissertation!

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ABSTRACT

Plant protein ingredients are sustainable sources of protein that could be used to meet the demand of the growing aquaculture industry. However, feeding plant protein diets has some drawbacks in terms of reduced growth and poor feed efficiency. This dissertation evaluated the production cost of alternative protein diets for commercial production of rainbow trout. Also, it identified the microbiota and gene functions associated with alternative diets and how they differ between mid- and hind-gut sections of the rainbow trout intestine. Furthermore, it determined differences in microbial community compositions and functions in the luminal and mucosal GIT of trout when fed alternative diets, with/without prebiotics. Lastly, the significance of diet and water as environmental factors shaping the mucosal and luminal bacterial compositions in trout was investigated.

Experiment 1 demonstrated that trout growth and body indices were not affected by feeding plant protein diet (PPD). In experiment 2, shotgun metagenomics revealed predominant bacterial population in trout microbiota. Genes related to carbohydrate metabolism were increased in the hindgut intestine of those fed PPD. Experiment 3 demonstrated that replacing fishmeal with 75% GDDY did not alter growth of trout, but not feed intake and feed conversion ratio (FCR). High inclusion of GDDY in trout diet resulted in enrichment of catabolic genes involving branched chain amino acids in trout midgut region. Experiment 4 showed that rearing in a recirculating water system significantly improved trout performance compared to rearing in a flow-through water system, while feed intake and FCR increased in fish raised in the flow-through system. Water samples were more diverse than GIT samples. Bacterial diversity was greater in mucosal scrapings of the GIT than in the lumen. Water system played a major role influencing the microbial communities in trout luminal and mucosal GIT. The lumen shared similar bacteria with the rearing water.

The results of this study demonstrated that plant protein can effectively substitute for fishmeal in trout diets. It further showed that trout GIT microbiota vary between the mid- and hind-GIT. The hind-, but not the mid-GIT microbiome appears to be modulated by diet, while the mid-GIT is affected by water system.

CHAPTER ONE

INTRODUCTION TO DISSERTATION

In the last decade, the aquaculture sector has made substantial and growing contributions to global fisheries production to meet the increasing demand in human consumption of fish protein. While fish production from the capture fisheries has remained on a plateau over the last decade, contributions from the aquaculture industry have continued to increase, amounting to 157.8 million tonnes in 2012 and growing to 167.2 million tonnes in 2014 (FAO, 2016). Among aquaculture species, Rainbow trout is an economically-important fish species in North America, raised both for food fish production and for stocking recreational fisheries. Rainbow trout contributes substantially to the US economy, with production sales totalling $104 million in 2015, an increase of 1 % from 2014 (USDA, 2016). However, rainbow trout is a nutritionally-demanding fish species that requires a high-quality protein diet to meet its physiological needs. A typical commercial trout diet contains 45% crude protein on a dry matter basis (Hardy, 2002; NRC 2011).

In order to continuously expand trout and other aquaculture production, while minimizing impacts on the environment, the aquaculture industry has sought to reduce dependence on marine products by making use of alternative ingredients. Protein from fishmeal has historically been an important and substantial component of rainbow trout feed because of its highly digestible protein and balanced amino acid profile. The endogenous fish oil as energy source in trout diets has protein sparing effects and

improves production of quality of the fish fillet (Beamish and Medland, 1986; Betiku et al., 2016). However, the supply of marine ingredients to meet the growing demands of the aquafeed industry is limited, leading to research on the utilization of alternative feed sources for rainbow trout production (Gomes et al., 1995; Thiessen et al., 2004; Luo et al., 2006; St-Hilaire et al., 2007; Betiku et al., 2016). Plant proteins, in particular are considered to be more sustainable sources of protein whose utilization could also make fish products more affordable to the final consumers (Adelizi et al., 1998; Burr et al., 2012; Betiku et al., 2016). However, the successful utilization of alternative ingredients or diets in rearing fish species depends on several factors, including the digestive physiology of the fish, diet types, and diet quality, antinutritional factors as well as rearing conditions (Gomes et al., 1995; Burel et al., 2000; Carter and Hauler, 2000; Francis et al., 2001; Yamamoto et al., 2002; Chou et al., 2004; Gatlin et al., 2007). One important factor is balancing the amino acid profiles of the feed to the nutritional requirements of the fish. An effective method to achieve this is known as the 'ideal protein concept', which balances feed amino acid composition to the fish muscle profile (Boisen et al., 2000; Gaylord and Barrows, 2009). The ideal protein concept has been shown to enable trout to achieve high productivity with less total dietary protein (Boisen et al., 2000; Gaylord and Barrows, 2009).

Interactions among host, diet, and environment are important factors that can influence the utilization of any diet, particularly as it involves the complex microbial population of the gastrointestinal tract (GIT). The GIT microbiota has been proposed to be essential in digestion, development, health, immunity, and xenobiotic metabolism of

the fish (Bates et al., 2006; Nayak, 2010). Symbiotic relationships between the host and complex microbes in the GIT have significant impacts on nutrient digestion, and host immune function (Yeoman et al., 2011; Hooper et al., 2012). In fish, correlation between phenotypic observations due to interactions between microbial composition in the GIT and metabolic responses in the digestive system of the host has been suggested (Ghanbari et al., 2015). In mammalian systems, diet is an important controlling variable influencing GIT microbial composition, however, the influence of diet on fish GIT microbiota is less clear. Earlier studies on fish GIT microbiota using culture-independent methods indicated diet influenced fish GIT microbiota (Desai et al., 2012; Wu et al., 2012; Bolnick et al., 2014; Ni et al., 2014). Ingerslev et al. (2014), however, reported that only first feeding diets had significant impact in shaping the microbial community of rainbow trout. While Wong et al. (2013) reported that core rainbow trout microbiota may be resistant to variations in diet as well as rearing density in which the fish were cultivated. Another factor that is of interest in influencing fish GIT microbiota is the rearing water. The water environment contains a diverse range of microbes and these microbes are distinct from those in the other environments (Zwart et al., 2002) and are proposed to shape fish GIT microbiota (Sullam et al., 2012). Other studies have shown that host (Li et al., 2012; Wong and Rawls, 2012) is a strong factor shaping the microbiota of fish GIT.

In several fish species, the composition and numbers of microbes vary along the different parts of the GIT (Ringø et al., 1995; Apun et al., 1999; Spanggaard et al., 2000; Tengjaroenkul et al., 2000; Huber et al., 2004; Heikkinen et al., 2006; Korsnes et al., 2006; Ringø et al., 2006b). Also, depending on diets, distribution pattern of enzyme

activities and absorption capacity, differ along the GIT. For instance, studies on the morphological changes and absorption capacity in the GIT of rainbow trout and Atlantic salmon resulting from diet effects, have been reported (van den Ingh et al., 1991; Bakke-McKellep et al., 2000; Refstie et al., 2000; Krogdahl et al., 2003; Ostaszewska et al., 2005; Ringø et al., 2006a). In both species, discrete changes of the proximal and distal GIT mucosa were observed when the fishes were fed diets containing soybean products. Additionally, digestibility of carbohydrates differs in many fish species depending on the GIT structure of the host and inclusion levels as well as the source of carbohydrates and processing method employed (Krogdahl et al., 2005).

In general, carbohydrate, especially the digestible fraction from plants, is a cheaper source of energy compared to lipids and protein, but the metabolism and utilization vary in many fish species (Hemre et al., 2002). Classification of carbohydrates is contingent on the degree of polymerisation and its glycosidic linkages (Englyst and Hudson, 1996). These influence distinct carbohydrate properties including digestion, absorption, viscosity, water-binding capacity and microbial fermentation in the GIT of the host and are important in nutrition (Hemre et al., 2002; Krogdahl et al., 2005). Although all fish species have shown enzymatic activities to hydrolyse and absorb simple carbohydrate diets (Krogdahl et al., 2005), capacity for carbohydrate hydrolysis in terrestrial animals is far greater than what is obtainable in fish species (NRC, 2011). While rainbow trout has a limited ability to utilize carbohydrate, studies have shown that they have the ability to digest glucose and starch (Buddington and Hilton, 1987; Krogdahl et al., 2004). It was observed that trout prefers starch to glucose when

compared with Atlantic salmon and that spatial variation in the distribution of microbiota within GIT and carbohydrate hydrolysis, and glucose uptake differs in these regions (Krogdahl et al., 2004). It has been reported that enzyme activities and carbohydrate absorption in carnivorous fish is more active in the proximal GIT than in the distal regions (Krogdahl et al., 1999; Bakke-McKellep et al., 2000; Kamalam et al., 2017).

In addition to nutrient absorption, fermentation capacity of the proximal and the distal regions of the GIT in vertebrates and some fish species, have been reported. For example, studies on fermentation ability of the midgut in poultry (Apajalahti et al., 2004; Jozefiak et al., 2007), small intestine in swine (Imoto and Namioka, 1978; Smiricky-Tjardes et al., 2003; Molbak et al., 2007), small intestine in cattle (Calsamiglia et al., 2008) and hindgut section in sheep (Hume, 1977), lizard (Foley et al., 1992), marine turtle (Barboza and Hume 1992; 1993), freshwater turtle (Bjorndal 1997), herbivorous fishes (Rimmer and Wiebe, 1987; Mountfort et al., 2002), sea bass (Gatesoupe et al., 2014), Red seabream (Kihara et al., 1995), tilapia (Kihara and Sakata, 1997), carp (Kihara and Sakata, 2002) and salmon (Holben et al., 2002), have been investigated. Most of the information on GIT microbiota and their functions has been mostly derived from mammalian species, which represents about 10% of all the vertebrates. Information about the potential functions of GIT microbiota in rainbow trout is yet to be investigated and it is not clear whether host-microbial interactions observed in mammalian species are similar to those occurring in the trout GIT (Riesenfeld et al., 2004). Additionally, most of the culture-dependent microbial studies in fish could only account for < 1% of all the microbes (Riesenfeld et al. 2004). Hence, the use of culture-independent method is

imperative in understanding microbial diversity and their potential functions in the GIT of fishes.

Next generation sequencing technologies, especially random sequence of metagenomes, are a promising tool for understanding the complex microbial diversity and the functional capacity within microbial environments. Individual microbes living in a particular environment have a functional role, and while studies to date have simply described their taxonomic diversity ('who they are'), it is also important to determine their functional capacity ('what they are doing') so we can begin to understand their influences on host physiology. Earlier studies characterizing the microbial taxonomy in rainbow trout using 16S rRNA gene-directed approaches (Desai et al., 2012; Ingerslev et al., 2014), provided wide-ranging information about microbiota which could not be accounted for by culturing methods. It is now clear that a much more complex microbial diversity resides within the GIT environment of fishes than what was earlier reported (Ringø et al., 2016). However, the 16S rRNA gene does not provide information on the physiological role of microbiota in the fish GIT (Liu et al., 2008). On the other hand, the application of whole-genome sequencing methods can unveil both the taxonomic composition and gene functions of the microbiota (Turnbaugh et al., 2009; Qin et al., 2010). But information on the effect of diets as it relates to the distinct functions of GIT microbes in the mid- and hind-GIT of rainbow trout is rare.

Statement of Research Problem

There is the need to meet the demand of the growing aquaculture sector for

quality protein ingredients to replace conventional fishmeal for feeding carnivorous fishes including trout. There is the need to meet the demand of the growing aquaculture sector for quality protein ingredients to replace conventional fishmeal for feeding carnivorous fishes like trout. Plant protein ingredients contain anti-nutritional factors and other components, which limit their utilization and bioavailability in biological systems. This study employs the use of next-generation sequencing to understand changes in GIT microbiota compositions and gene functions with respect to feeding alternative plant protein diets to rainbow trout.

Objectives of Research

The aims of the research studies presented in this dissertation are to provide a comprehensive information on the microbial community of rainbow trout. These studies are divided into four parts:

(a) Evaluation of production cost of alternative protein diets for commercial production of rainbow trout.

(b) Identification of microbiota and gene functions associated with alternative diets and how they differ between mid- and hind-gut sections of the rainbow trout intestine.

(c) Determine if prebiotics improve utilization of alternative proteins and how this affect microbial composition and gene functions in the midgut of trout.

(d) Investigation of the significance of diet and water as environmental factors shaping the mucosal and luminal bacterial compositions in trout.

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CHAPTER TWO

LITERATURE REVIEW

Status of Aquaculture Production

Aquaculture is an important component of the world food supply and its role is expected to increase as a result of the awareness of the benefits of fish protein and oil on nutrition, health and wellbeing of the people. In 2014, the world fisheries production was 167.2 million tonnes up from 162.9 million tonnes in 2013; aquaculture accounted for 44.1% of the production in 2014 from 42.1% in 2012 (FAO, 2016). Increased production from the aquaculture industry depends upon production of quality feeds to meet the nutritional requirements of the cultured fishes. Demand for marine ingredients, fishmeal, and fish oil continues to increase in order to meet the increasing demand of the aquaculture industry. Historically, fishmeal is the conventional protein source used for production of fish diets. However, the demand for this finite resource by the aquaculture industry, especially for production of feed for carnivorous species like rainbow trout, is more than those from other animal producing sectors because of the ease to source for alternative ingredients as replacement for fishmeal in animal husbandry (Bostock et al., 2010).

Alternative Ingredients in Aquaculture

Since the current production of capture fisheries is limited, the supply of fishmeal for aquafeed production is also limited (Tacon and Metian, 2008; FAO, 2016).

Sustainability of cultured fishes like rainbow trout is of utmost importance because of its position as the second most cultured aquaculture species in the United States. Rainbow trout global production amounted to $3.6 billion in 2012 (FAO, 2016) and in 2015, the US had sales of $104 million due to the gross domestic production (USDA, 2013). This economically important species has limited ability to utilize carbohydrate for energy (Hilton and Atkinson, 1982; Buddington and Hilton, 1987; Cho and Kaushik, 1990). At present, the demand for marine ingredients, especially fishmeal, concomitant with the current low production from capture fisheries make it imperative in fish nutrition to identify reliable alternative ingredients to replace the conventional fishmeal diet. Alternative protein studies (Kaushik et al., 1995; De Francesco et al., 2004; Gaylord and Barrows, 2009; Gaylord et al., 2009; Santigosa et al., 2011) as well as studies on alternative oil sources (Caballero et al., 2002; Figueiredo-Silva et al., 2005; Betiku et al., 2016) have been conducted. It is clear that the balanced indispensable amino acid profile in fishmeal makes this ingredient a suitable protein source in rainbow trout diet (Tacon and Metian, 2008). Anti-nutritional factors in plant protein ingredients have been grouped into four major categories (Francis et al., 2001; Gatlin et al., 2007):

(i) those that affect protein digestion and utilization, including protease inhibitors, tannins and lectins,

(ii) those that reduce mineral utilization by making them unavailable or degraded, such as phytates, oxalates, glucosinolates, and gossypol,

(iii) anti-vitamins, and

(iv) other toxic substances such as mycotoxins, mimosine, nitrates, alkaloids and saponins.

Studies using plant proteins suggest the use of a combination of different plant ingredients together with supplementation of limiting nutrients such as methionine, lysine, taurine and dicalcium phosphate (Gaylord and Barrows, 2009) in order to achieve optimum growth responses.

Microbial Diversity and the External Environments

Recent reports have indicated possible alteration of metabolic pathways by dietary changes with respect to fishmeal and fish oil replacement in rainbow trout (Wacyk et al., 2012; Rolland et al., 2015). But only few studies have been directed at investigating the effect of alternative ingredients on microbial diversity and gene functions in fishes (Ringø et al., 2006; Desai et al., 2012; Ingerslev et al., 2014). For instance, microbial diversity in goldfish intestinal contents decreased with partial replacement of fishmeal with lupin (de Paula Silva et al., 2011). Specifically, soybean is being used as a source of protein in aquafeed, but information regarding the alteration of GIT microbes with respect to soybean-based diets is limited. The presence of hyper-ammonia producing bacteria associated with microbial decomposition of feed protein and amino acids have been studied in livestock because their inhibition has been linked with low ammonia production and increased productivity (Krause and Russell, 1996; Whitehead and Cotta, 2004). This information is scarce in rainbow trout nutrition, even though soybean has been identified as a less expensive alternative protein to replace fishmeal. Studies with the use of dehulled soybean meal reported reduced growth and increased mortality when

fed to rainbow trout (Merrifield et al., 2009). Moreover, soybean meal disrupts the intestinal barrier and alters membrane permeability, a condition known as enteritis (Refstie et al., 2000). However, information available on the indigenous microbiota colonizing the fish GIT are inconsistent (Nayak, 2010; Ringø et al., 2016) and the influence of specific alternative ingredients or their combinations is often not known. Understanding the complexities of the fish GIT microbiota, especially the role of fish GIT microbiomes on fish growth and how alternative feed ingredients alter the microbial composition and their functions may be of great importance in fish diet development.

Benefits of GIT Microbes as Potential Probiotics

Fish GIT microbes have great potential for exploitation in aquaculture and other animal-producing sectors. However, many of these microbes are yet to be fully identified due to limited research in this area. In humans, GIT microbes have been reported to be involved with increased energy supply from dietary carbohydrate fermentation (Hehemann et al., 2010). In fishes, digestive enzymes, including amylase, cellulase, lipase, and protease were isolated from *Bacillus species* in the GIT of silver carp, mrigal, rohu, common carp, tilapia, catla, murrel and walking catfish (Bairagi et al., 2002). In a subsequent study, the authors reported improved growth performance, lower feed efficiency and higher protein retention when the protease-producing bacteria isolated from the GIT of rohu was supplemented into its diets (Ghosh et al., 2003). Furthermore, isolated bacterial strains of *Bacillus circulans* and *Bacillus megaterium* from the intestine of tilapia and Chinese carp were suggested to have potential for use as probiotics in aquaculture (Ghosh et al., 2003), which can contribute towards improved health

management in cultured-fish. Several Gram-negative and Gram-positive bacteria are now considered to be beneficial in aquaculture. They are used as probiotics that can be included in fish feed as a live microbial supplement or part of formulated diets, with the purpose of improving growth, appetite and immune health (Brunt and Austin, 2005; Macey and Coyne, 2005). Unlike other animals in which probiotics are mainly Gram-positive lactic acid bacteria, aquatic animals use different microorganisms as probiotics. For instance, Gram-negative bacteria are not typically used as probiotics in human and terrestrial animals, but are found to be beneficial in aquaculture. In shrimp farming, Chythanya et al. (2002) reported that *Psuedomonas fluorescences* was found toxic to pathogenic *Vibrio harveyi, V. fluvialis, V. parahaemolyticus, V. vulnificus and Photobacterium damselae*. Also, in rainbow trout, *P. fluorescences* reduced mortality associated with *V. anguillaris* (Gram et al., 1999); its inclusion in rearing water reduced *Vibriosis* in rainbow trout (Spanggaard et al., 2001). Moreover, Gram-positive bacteria including *Lactobacillus rhamnosus* effectively controlled *Aeromonas salmonicidia*, leading to reduced mortalities from 53 to 19% in rainbow trout (Nikoskelainen et al., 2001). Furthermore, infection caused by *A. salmonicidia* was hindered when *A. hydrophila and Vibrio fluvialis* were fed singly or as an equi-mixture to rainbow trout (Irianto and Austin, 2002). Other organisms used as probiotics in aquaculture includes bacteriophages (*Myoviridae and podoviridae*) and yeasts (*Sacchromyces cerevisae, S. exiguous*, and *S. cerevisiae*). In the GIT of rainbow trout, *Debaryomyces hansenii* was isolated and incorporated into the diet of sea bass larvae (Tovar et al., 2002).

Benefits of Prebiotics in Aquatic Nutrition

Prebiotics are non-digestible feed ingredients that are incorporated into diets that are only metabolize by certain microorganism in the GIT, which in return confer health benefits on the host (Gibson and Roberfroid, 1995). Benefits of prebiotics have been described in humans (Roberfroid, 1997; Gibson, 1999; Cummings and Macfarlane, 2002; Macfarlane et al., 2006) and livestock (Houdijk et al., 1998; Patterson and Burkholder, 2003; Awad et al., 2009; Modesto et al., 2009). Commonly used prebiotics in animal research are Fructooligosaccharides (FOS) and Mannanoligosaccharides (MOS). Additionally, inulin, FOS and trans-galactooligosaccharide (TOS) increased butyrate concentrations and *Bifidobacteria* in growing pigs (Mikkelsen and Jensen, 2004; Loh et al., 2006). Also, in human studies, fiberous diets reduced the risk of cancer, type 2 diabetes mellitus and cardiovascular disease (Eshak et al., 2010; Lattimer and Haub, 2010; Papathanasopoulos and Camilleri, 2010).

Cultured-dependent and Molecular Methods of Characterizing Microbial Diversity

Several approaches have been used for determination of microbial composition of fishes, ranging from cultured-based to molecular methods of analyses (Trust and Sparrow, 1974; Trust et al., 1979; Mansfield et al., 2010; Desai et al., 2012; Navarrete et al., 2012; Ingerslev et al., 2014). However, the molecular methods or next-generation sequencing techniques have more recently gained recognition due to their high sensitivity and specificity over the culture-dependent methods (Roque et al., 2009). Major bacterial communities in both freshwater and marine fishes are *Vibrio*, *Aeromonas*, *Acinetobacter*,

Enterobacteriaceae, *Flavobacterium*, *Plesiomonas*, *Micrococcus*, *Clostridium*, *Bacteroides* and *Pseudomonas* (Nayak, 2010), and may vary due to differences in species, diet, and environmental factors. In a review by Cahill (1990), it was reported that microbial variations occurred between fish species and that the majority of bacteria commonly found in fish skin, gills, and GIT are related to their habitats. Assessment of a tropical freshwater pond found patterns of bacterial colonization that are similar between conventional- and experimental pond-raised fish (Apun et al., 1999). Similar observations were made from bacterial counts isolated from the skin, gill, spleen, intestine, kidney, and liver of rainbow trout from two farms (Diler et al., 2000) The latter authors associated these bacteria with those found in the rearing water. However, Grisez et al. (1997) reported fluctuations in the microbial community of sea beam and sea bass larvae from two hatcheries as a result of live feed ingested during the developmental stages of the fishes. Similar reports were presented on dietary manipulation of the microbial composition in the GIT of Arctic charr (Ringø and Olsen, 1999). But microbial composition in Grass carp resembled that of the culture water and sediments; and diet was said to also have significantly influenced this gut composition (Wu et al., 2012). There seems to be little agreement among these findings, therefore there is the need for a comprehensive study to further investigate the influence of the external environment on the indigenous microbial diversity in fish species. In such future studies, consideration must be given to the different rearing habitats in which fishes exist. Moreover, identification of microbial gene functions may provide information that is not available with the 16S rRNA gene-targeted sequencing approach. Shotgun metagenomics sequencing offers advantages over 16S rRNA gene analysis including unbiased

surveilling of archaeal and eukaryotic microbial populations, and providing information on microbial gene functions. However, the application of shotgun metagenomics to cultured-fish, especially rainbow trout is lacking.

Importance of GIT Microbes in Fish Nutrition

GIT microbes play important roles in the nutrition of fish, but their functions and mechanisms are yet to be defined (Nayak, 2010) due to the complexity of the GIT microbiota of fish species. Hence, there is the need for research in this area in order to ascertain the importance of these microbes to fish nutrition. However, several studies have suggested possible links of GIT microbes to the nutrition of fishes. For instance, some authors have described vitamin B12 production by intestinal microbes in tilapia (Lovell and Limsuwan, 1982; Sugita et al., 1991a; Nayak, 2010); in rainbow trout (Sugita et al., 1991b) and carp (Sugita et al., 1991a).

Conclusion and Implication

There remains a scarcity of knowledge on the microbial composition and functional potential of the fish GIT microbiome. Yet studies on the GIT microbiomes of humans and other animal studies outline the potential importance of the fish GIT microbiome to nutrition and health. Understanding these relationships and how factors like diet, and rearing water shape this relationship potentiate economic benefits to aquaculture industry. Detailing the mechanisms governing microbial community in fishes, most especially cultured species, could help in development of feeding strategies

through dietary manipulation that will better impact fisheries production and health in order to meet the demand for fish food and products.

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CHAPTER THREE

EVALUATION OF PROTEIN REDUCTION AND ESSENTIAL AMINO ACIDS SUPPLEMENTATION OF PLANT- AND ANIMAL PROTEIN-BASED DIETS ON PRODUCTION OF RAINBOW TROUT (*Oncorhynchus mykiss*)

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Contributions: Design the experiment and feed formulation. Data analysis and interpretation. Assisted in manuscript preparation.

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Contributions: Design the experiment, guided in fish culture, data interpretation and assisted in manuscript preparation.

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Evaluation of protein reduction and essential amino acids supplementation of plant- and animal protein-based diets on production of rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Rainbow trout is an important aquaculture species. Alternative protein sources to reduce fishmeal inclusion rates in diets have been widely studied with rainbow trout and other fish species with varying success. The current study investigated the performance of rainbow trout fed two experimental protein blend diets: animal- (APD) and plant- (PPD) based protein blends, formulated to contain 40% digestible protein (supplemented with lysine, methionine and threonine) compared to a commercially produced 45CP diet. A total of 675 rainbow trout with initial average body weight (BW) of 125 ± 3.2 g were stocked at a rate of 75 fish/2,230 L tank with three replicate tanks per diet. Fish were fed twice daily to apparent satiation in a recirculating water system. Response variables were weight gain, feed intake, and feed conversion ratio (FCR). The results presented evidence that reducing dietary protein levels and incorporating APD or PPD protein blends in feeds did not reduce growth and feed efficiency of rainbow trout. Feed intake ($P = 0.5787$) and average gain per fish were not significantly different between the treatments ($P = 0.0604$). FCR ($P = 0.0315$) was significantly affected by diets. The results of this work demonstrated that reducing dietary protein level and using alternative protein blends may lead to a sustainable rainbow trout production.

Introduction

The International Food Policy Research Institute projected an annual increase in seafood products consumption of 1.5 kg/person by year 2020 (Delgado et al. 2003; Diana 2009). Moreover, the global consumption of seafood is also expected to increase from 111,697 thousand metric tons in 2006 to 151,771 thousand metric tons in 2030 (World Bank 2013). Due to overexploitation of the wild stocks, capture fisheries production is expected to remain stable at 93 million metric tons between 2010-2030, while aquaculture production is projected to increase from 154 million metric tons in 2011 to 186 million metric tons in 2030 (World Bank 2013).

Rainbow trout (*Oncorhynchus mykiss*) is a fast-growing cold-water fish species. In 2010, the global production of rainbow trout was 728,448 tonnes up from 550,000 tonnes between 2001-2005 (FAO 2010). In the United States, trout production accounted for a total value of $79.7 million USD in 2012. Quality feed is a necessity for rainbow trout culture as well as a major portion of the total production cost. A typical commercial trout diet contains approximately 45% crude protein (Hardy 2002) in order to meet the requirement for maintenance, optimum growth, and health. Fishmeal has been widely used for trout feeds because of its highly digestible protein and balanced amino acid profile. However, since the supply of fishmeal is limited, substantial research has been focused on producing feeds utilizing alternative protein sources for rainbow trout production to safeguard the sustainability of the industry.

The use of the ideal protein concept in feed formulation has been an effective way of providing fish with less protein while still providing the balanced amino acids tailored

to mimic the fish muscle profile (Boisen et al. 2000; Miles and Chapman 2007; Gaylord and Barrows 2009). This concept, in theory, provides an estimate of the amino acids required by the animal in order to optimize performance and maximize growth. Effectiveness of this concept has been investigated in other livestock (Han and Baker 1994; Emmert and Baker 1997; Tuitoek et al. 1997; Dari et al. 2005). In fish nutrition studies, partial or total fishmeal-free diets supplemented with the essential amino acids have been shown to support equivalent growth performance as fishmeal diets (Ng and Hung 1995; Gaylord et al. 2002; Furuya et al. 2004; Gaylord and Rawles 2005).

Several studies have evaluated specific ingredients as replacements for fishmeal in order to reduce dependence on this ingredient in the aquafeed industry, but sustainability of the aquaculture industry will greatly depend on the ability to develop alternative diets that are comparable in performance and cost to fishmeal-enriched diets. Alternative ingredients studies have shown effective reduction in the use of fishmeal to about 20% in trout diets in the United States (Hardy 2010); however, more aggressive replacement of marine protein would provide greater flexibility and thus help protect the trout industry from fluctuations in production costs due to changes in ingredient availability.

Therefore, the current study evaluated the performance and cost benefit of feeding alternative plant and animal protein-based diets supplemented with essential amino acids to rainbow trout. In addition, consumer preference quality was evaluated when rainbow trout was fed the complete plant and animal protein-based diets.

Materials and Methods

Fish culture and experimental design

Earlier studies have evaluated the utilization of the similarly formulated but experimentally produced diets employed in this study in laboratory feeding trials (Craft et al. 2016; Gaylord et al. 2017). However, this present study aimed at comparing the performance of commercially produced plant protein-based feed and animal protein-based feed to a conventional commercial trout feed. Bozeman Fish Technology Centre (BFTC) was the location for the feeding trials. The commercial production and cost of the experimental diets were done by Skretting North America (Tooele, UT, USA) before they were transported to the BFTC for the feeding trials.

The dietary treatments and their composition as well as the chemical analysis are provided in Table 1. The commercial diet (CON) contains 45% crude protein and proprietary ingredient information, while the two alternative diets (APD and PPD) contained 40% crude protein (supplemented with lysine, methionine and threonine) to meet the muscle amino acid profile of rainbow trout. A complete randomized design was used. Diets were randomly allocated to tanks of fish. Rainbow trout (Troutlodge Inc., Sumner, WA, USA) with initial average body weight (BW) of 125 ± 3.2 g were stocked at a rate of 75 fish/2,230 L tank. Three replicate tanks were assigned each dietary treatment and the fish were fed to apparent satiation twice daily (8:00 am and 3:00 pm) for 16 weeks. The tanks were connected in a recirculating water system with a sand filter, biofilter, and UV disinfection. Water temperature was maintained at 15 ^{o}C over the course of the trial.

Sampling and measurements

All fish handling procedures followed regulations set by the Department of Interior, USFWS. Initial fish weight and lengths of ten randomly sampled fish were recorded at the beginning of the experiment. Feed intake was monitored weekly, while fish were bulk weighed at four-week intervals. At 16-week post feeding, five fish were randomly selected from each tank and anesthetized with tricane methane sulfonate (MS-222; 200 mg/L water). Measurements on individual fish weights, length, visera weight, fillet weight and liver weight were taken for determination of hepatosomatic index (HSI), muscle ratio (MR), and viscerosomatic index (VSI). An additional three fish per tank were euthanized and frozen for compositional analysis.

Color analysis

For fillet color determination, the remaining fish were slaughtered following the approved humane protocols of slaughter and filleting. Fillet color was determined using a hand-held Minolta Chroma Meter CR-200 (Minolta Camera Co). Epaxial muscle anterior to the dorsal fin was used for measurements. Fillet image was captured to obtain and rank color results based on lightness (L*), redness (a*), and yellowness (b*) values. Average values of L*, a*, and b* were calculated to determine the fillet color for each of the fish sampled.

Calculations

The following equations were used in calculating each of the body indices (Betiku et al. 2016):

$$WG = \left(\frac{\text{final weight - initial weight}}{\text{initial weight}} \right) \times 100 \quad (1)$$

$$SGR = \left(\text{final body mass - initial body mass} \right) \times \left(\frac{100}{\text{days}} \right) \quad (2)$$

$$FER = \frac{\text{kg body mass}}{\text{kg diet consumed}} \quad (3)$$

$$PRE = \left(\frac{\text{protein gain (g)} \times 100}{\text{protein fed (g)}} \right) \quad (4)$$

$$ERE = \left(\frac{\text{energy gain (MJ)} \times 100}{\text{energy fed (MJ)}} \right) \quad (5)$$

$$\% \text{ Heptatosomatic index (HSI)} = \left(\text{liver mass/body mass} \right) \times 100 \quad (6)$$

$$\% \text{ Viscerosomatic index (VSI)} = \left(\text{gut mass/body mass} \right) \times 100 \quad (7)$$

$$\text{Muscle ratio (MR)} = \frac{\text{fillet mass (g)} \times 100}{\text{fish mass (g)}} \quad (8)$$

$$\% \text{ Survival rate} = \left(\frac{\text{final fish number}}{\text{initial fish number}} \right) \times 100 \quad (9)$$

Statistical analysis

Data obtained were subjected to one-way analysis of variance in order to determine dietary effect on feed intake, feed conversion ratio (FCR), body weight gain (BWG), and BW using Proc GLM of SAS 9.1. (SAS Institute Inc., Cary, NC, USA), significant difference was considered at $p<0.05$.

Results

Growth performance and body indices of rainbow trout

For the period of the feeding trial, dietary treatment did not adversely affect the health of the rainbow trout, survival ranged from 96 to 100% with no significant difference between the diets. Fish weights were not significantly different ($P>0.05$); feed intake was not significantly affected across treatments (Table 2). From an initial weight of 125 g, there was over a four-fold increase in fish weight for the feeding period (Fig. 1). Reduction of protein level from 45 to 40% did not significantly affect growth of rainbow trout fed the alternative protein blend. Furthermore, FCR was not significantly affected by feeding animal and plant protein blends to rainbow trout. Across the dietary treatments, hepatosomatic index, condition index and fillet ratio were not significantly different ($P>0.05$), but visceral somatic index was significantly ($P=0.042$) higher in fish fed the animal protein diet compare to plant and fishmeal based diets (Table2).

Whole body composition and fillet color

Analysed composition of the whole body indicated that protein, lipid, and energy were not significantly ($P>0.05$) affected across the dietary treatments (Table 3). Also, protein and energy retention efficiencies were not affected by diets. However, it was observed that the fillet from trout fed the plant protein-based diet had significantly high values of L* and b* and significantly low values for a* compared with the fillets obtained from fish fed the commercial and animal protein-based diets (Fig 2).

Cost analysis

Table 4 shows cost analysis of the experimental diets. While the commercial trout diet (CON) and the alternative plant protein-based diet (PPD) costed less than the alternative animal protein-based diet (APD), cost per gain was the least for the fish fed the CON diet. Between the two alternative diets, PPD diet had lower cost per gain compare to the APD diet.

Discussion

The high cost of feed in aquafeed industry has been attributed to the limited availability of marine-derived fishmeal as a dietary protein source (FAO 2009). Several nutrition research studies have been focused on replacement of fishmeal with other alternative ingredients like plant proteins (Hardy 2002). However, development of alternative commercial diets for rainbow trout production has been challenging due to the high digestibility of fishmeal as well as its balanced amino acid profile. Identification of alternative protein blends, which can maintain performance and reduce feed cost in rainbow trout, is crucial for long-term sustainability of the trout industry. The results of this study corroborate earlier observations from the investigation on the efficiency of the ideal protein concept for diet development in rainbow trout (Gaylord and Barrows 2009), hybrid striped bass (Gaylord and Rawles 2005) and poultry (Dari et al. 2005). This study showed that reducing the protein content in the diet to 40% crude protein supported optimal growth of rainbow trout and agrees with earlier findings when these diets were fed to rainbow trout (Craft et al. 2016; Gaylord et al. 2017). Similar results have been

reported in pigs (Kerr et al. 2003) and poultry (Aletor et al. 2000) when protein level was reduced and supplemented with amino acids. The growth performance reported in this study corroborates earlier findings when alternative protein diets together with essential amino acids supplementation were fed to rainbow trout (Cheng et al. 2003; Gaylord et al. 2006; Gaylord and Barrows 2009). Supplementation of the three essential amino acids: lysine, methionine and threonine is critical in the use of the idea amino acids concept. For instance, supplementation of lysine alone was not able to provide adequate growth performance in rainbow trout when fed plant protein diet compare to the conventional fishmeal diet (Cheng et al. 2003). Higher fat deposition in the visceral that was observed in trout fed the APD diet compared to PPD and CON may be correlated to fat intake. On the contrary, similarity in body indices, whole body composition and protein and energy retention efficiencies between fish fed PPD and CON showed that the plant protein diet supported the health of rainbow trout and can successfully be used for commercial production of rainbow trout. Since product quality correlates with dietary lipid composition, it is expected that fillet compositions from this study may reflect dietary fatty acids composition. Earlier study on fillet quality of rainbow trout fed plant protein-based diet with fish oil or algae oil show no negative effect on fillet quality and consumer preference for the product (Betiku et al. 2016). However, the yellow coloration observed in fish fillet fed the PPD diet may impact consumers' choice for seafood purchase (Buttle et al. 2001), which has been shown to positively associate with xanthophyll level in corn-based diet and in the fish tissues (Lee 1987; Liu et al. 2004). But this can be avoided by substituting white corn for yellow corn or by fortifying the PPD diet with canthaxathin

rather than astaxanthin that was used in this study (Skonberg et al. 1998; Liu et al. 2004).

Considering the cost benefit analysis, the plant protein diet had the same cost/g gain as the commercial trout diet while the cost was higher in the animal protein diet. Reducing the cost of plant-based diet is complicated by a lack of cost-effective plant protein concentrations and the additional costs associated with the necessity of amino acid supplementation in order to meet trout requirement for growth and maintenance (Adelizi et al. 1998). However, this study demonstrated that a cost-effective plant protein diet is achievable using the approach outlined in this study with the published ingredient costs during the timeframe.

In summary, the results from this study provide evidence for production of a cost-effective fishmeal-free diet that supports optimum performance of rainbow trout. Adoption of this procedure for formulation of commercial diet in order to reduce dependence on marine ingredient and the cost associated with feed for trout production may lead to sustainability of the aquaculture industry.

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Table 1. Ingredients and chemical compositions of dietary treatments

Ingredients	40% Animal protein blend (APD)	40% Plant protein diet (PPD)
	g/kg dry diet	
Soybean Meal	12.39	15.96
Blood Meal	2.97	-
Poultry by-product Meal	24.28	-
Fish Oil	7.45	7.49
Lecithin Oil	0.93	0.94
Millrun	3.18	-
Choline Chloride	0.93	0.94
D L Methionine	0.67	0.67
Feather Meal	2.95	-
Astaxanthin Pink	0.04	0.043
Corn Gluten meal	8.8	21.61
L-Lysine HCl	2.67	2.94
Stable C	0.14	0.14
Whole wheat	20.62	15.76
Soy Isolate	-	16.877
Mono Cal Phos	1.21	3.09
Poultry Fat	7.56	10.34
Vitamin Premix: ARS702[1]	2.28	2.26
SC Trace Mineral Premix[2]	0.93	0.94
Compositions		
Crude protein (N*6.25) (g/kg)	428	415
Fat	152.4	220
Energy (MJ/kg	23.0	23.4

[1] Contributed in mg/kg of diet; zinc 40; manganese 13; iodine 5; copper 9.

[2] Contributed, per kg diet; vitamin A 9650 IU; vitamin D 6600 IU; vitamin E 132 IU; vitamin K3 1.1 g: thiamin mononitrate 9.1 mg; riboflavin 9.6 mg; pyridoxine hydrochloride 13.7 mg; pantothenate DL-calcium 46.5; cyancobalamin 0.03 mg; nicotinic acid 21.8 mg; biotin 0.34 mg; folic acid 2.5; inostitol 600

Table 2. Growth performance and body indices of rainbow trout fed the alternative diets

Parameter	45% Commercial diet (CON)	40% Animal protein blend (APD)	40% Plant protein diet (PPD)	$P>F^2$	SEM
	8-week growth				
g gain	234.9	229.1	240.9	0.4886	6.54
% increase	184.5	184.2	191.4	0.7218	6.96
FI	1.66	1.74	1.71	0.5465	0.05
FCR	1.20	1.27	1.22	0.2914	0.03
	16-week growth				
g gain	696.5	639.4	612.2	0.0604	19.98
% increase	546.6	514.1	486.7	0.1835	19.94
FI	1.10	1.16	1.13	0.5787	0.04
FCR	0.99	1.08	1.06	0.0315	0.03
PRE	40.7	38.5	43.9	0.4402	2.80
ERE	66.8	68.2	67.7	0.9425	2.82
	Body indices				
HIS (g)	1.0	1.2	1.0	0.105	0.05
VSI (g)	10.1	11.4	10.2	0.042	0.33
MR (g)	43.3	43.0	43.9	0.113	0.40

Table 3. Whole body composition of rainbow trout fed the alternative diets

Whole body composition	45% Commercial diet (CON)	40% Animal protein blend (APD)	40% Plant protein diet (PPD)	$P>F^2$
Protein	17.5	16.2	17.2	0.465
Lipid	16.5	18.4	18	0.274
Energy (MJ)	2641	2833	2794	0.456

Table 4. Cost analysis of the experimental diets fed to rainbow trout[1]

Dietary treatment	Ingredient cost ($/lb ingredients)	Ingredient cost/gain ($ ingredient cost/lb gain)
CON	0.656	0.646
APD	0.688	0.741
PPD	0.656	0.695

[1] Based on diet costs for a practical trout diets as provided by Skretting North America: CON, a commercial trout diet containing 45% crude protein, APD (alternative animal protein based diet) containing 38% digestible protein and PPD (plant protein based diet) diet containing 38% digestible protein.

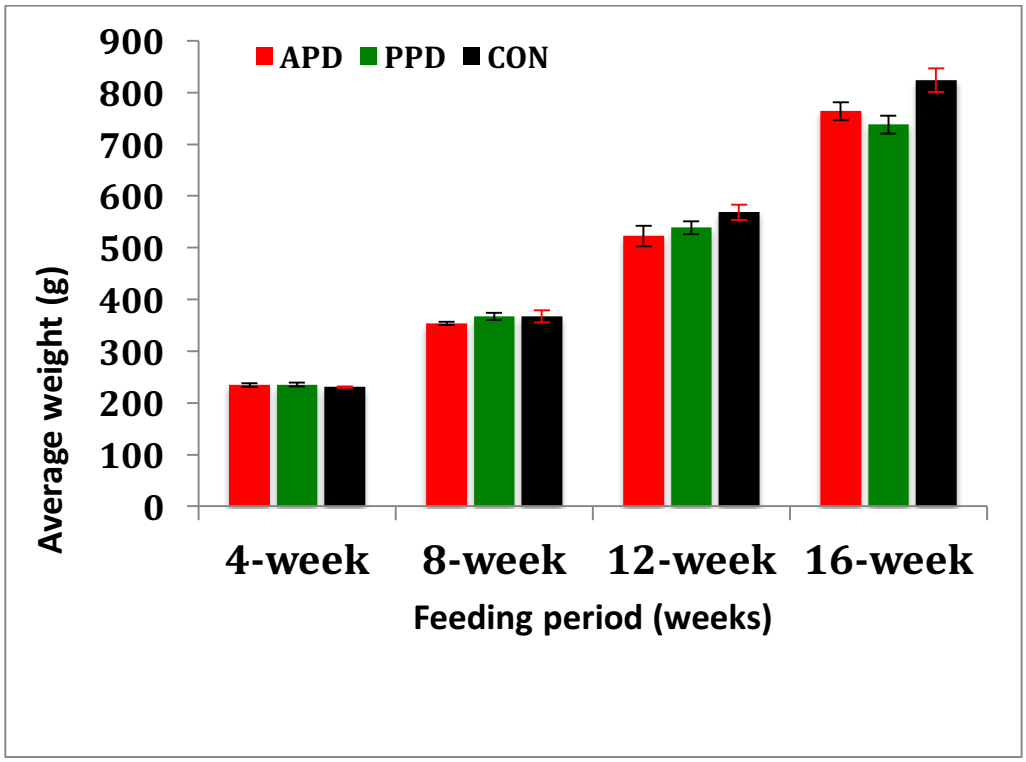

Fig 1. Monthly growth comparison of rainbow trout fed the APD (animal protein diet), PPD (plant protein diet), and CON (commercial trout diet).

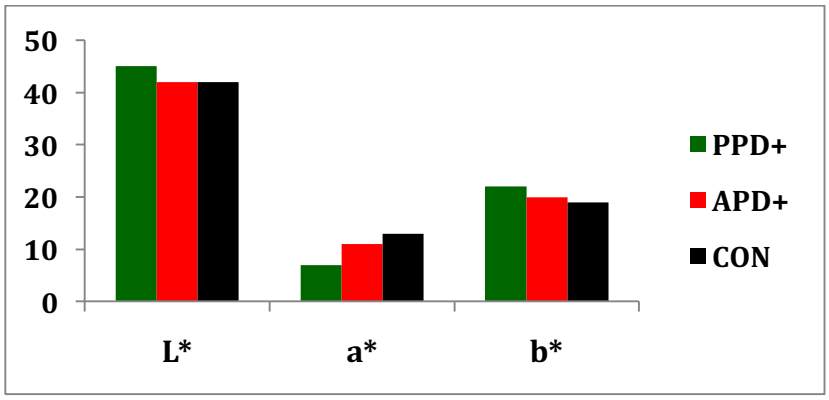

Fig 2. Fillet color measurement values of rainbow trout fed the plant protein diet (PPD), animal protein diet (APD), and the commercial diet (CON). Fillet color ranges from L* (lightness), a* (redness), and b* (yellowness) as measured by the Minolta color reader.

CHAPTER FOUR

DIVIDED NUTRITIVE FUNCTION OF THE RAINBOW TROUT (*Oncorhynchus mykiss*) MID- AND HIND- GUT MICROBIOMES

Contribution of Authors and Co-Authors

Manuscript in Chapter 4

Author: Omolola C. Betiku
Contributions: Involved in experimental design. Performed the experiments, sample collection and laboratory analysis. Data analysis and wrote manuscript with input from co-authors.

Co-Author: Carl J. Yeoman
Contributions: Involved in the design of the experiment. Data analysis and interpretation. Assisted in manuscript preparation.

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Divided Nutritive Function of The Rainbow Trout (*Oncorhynchus mykiss*) Mid- and Hind- Gut Microbiomes

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ABSTRACT

The nutritive role and ecology of gut-dwelling microbes in rainbow trout remain uncertain. The gastrointestinal tract (GIT) is divided into two regions, demarcated for this study as the mid- and hind-GIT. To improve our understanding of the rainbow trout GIT microbiome, we performed whole shotgun metagenomic analyses on the luminal content of both the mid- and hind-GIT samples from 90 trout fed one of three dietary treatments (a conventional fishmeal diet, or diets where fishmeal was replaced with either non-aquatic animal or plant protein sources). In total, we observed 3,267 unique microbial species that collectively encoded 6,054 unique annotatable gene functions. By sample, 343±130 microbial species were observed in the mid- (377±142) and hind-GIT (310±111). Good's estimates of coverage indicated we had captured >96% of species and gene functions in all samples. Microbes were predominantly bacteria (74%) and largely of the phyla Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes, Tenericutes, and Fusobacteria. Eukaryotic (25%), and Archaea (<1%) microbes mostly from the

Ascomycota and Euryarchaeota phyla were also present along with bacteriophage (1%) principally of the Caudovirales. Comparisons of species-level classifications and functional profiles revealed GIT location and location X diet relationships. Hind-, but not mid-GIT samples varied with diet. The hind-GIT microbes had a greater number of genes involved in carbohydrate, and fatty acid, lipid, or isoprenoid metabolisms. While the mid-GIT microbes were enriched for genes involved in sulfur, potassium, and aromatic compound metabolisms. These results support a nutritive role for trout GIT microbes and indicate a potential division of nutritive function between the mid- and hind- GIT microbiomes. These differences in functional division between the mid- and hind-GIT appear to be modulated by, and in some respects, adaptable to the fish diet.

INTRODUCTION

Alternative diets are critically needed for use in the aquafeed industry to reduce dependence on marine-derived fishmeal as a dietary protein source (Naylor et al., 2000). In particular, identification of alternative protein blends that can maintain or improve performance and production costs are a major focus of research of the aquaculture industry (Gatlin et al., 2007). Utilization of alternative ingredients or diets are known to be dependent on several factors including the digestive physiology of the fish, diet types and quality, anti-nutritional factors, as well as rearing conditions (Burel et al., 2000; Carter and Hauler, 2000; Francis et al., 2001; Yamamoto et al., 2002; Chou et al., 2004). As microbes colonizing the gastrointestinal tract (GIT; the GIT microbiota) are known to be important factor to the nutritive success of many animal species (Yeoman and White,

2014), it is surprising that limited information is available on the taxonomic diversity of, and gene functions encoded by microbes of the rainbow trout GIT, particularly when fed alternative diets that are completely fishmeal-free. Mutualistic relationships have been described to exist between a multitude of animal hosts and the diverse assortment of microbes that colonize their GITs, with significant impacts on nutrient digestion, nutrient availability along with normal functioning of the host immune system (Yeoman et al., 2011; Hooper et al., 2012). In better-described animal systems, the major products of microbial fermentation are short chain fatty acids (SCFAs) and microbial biomass (Mackie and White, 2012; Yeoman and White, 2014). SCFAs have been calculated to supply between 10% (i.e. for humans (McNeil, 1984) and up to 70% (i.e. for ruminants (Bergman, 1990)) of the hosts daily energy requirements, while microbial biomass can be exploited in some species as an additional protein source (Yeoman and White, 2014). In fish, the importance and contribution of SCFAs and microbial protein to host nutrition is yet to be fully established. Yet, it is noteworthy that SCFAs have been detected in studies of several other fish species (Kihara and Sakata, 2001). It may, therefore, prove critical to elucidate the role of the GIT microbiota in fish nutrition so their influence can be accounted for in efforts to optimally incorporate these alternative dietary regimens. Despite a scarcity of information on the GIT microbiota of fish species, they are believed to be essential to digestion, development, health, immunity and xenobiotic metabolism (Nayak, 2010; Ghanbari et al., 2015). Diet is an important modulating variable that has been shown in many animal systems to have strong influences on the composition of the GIT microbiota and their metabolic activities, along with profound effects on the host

immunity (Montagne et al., 2003; Ringø et al., 2006; Bakke-McKellep et al., 2007; Hooper et al., 2012; Nicholson et al., 2012). However, early 16S rRNA-based approaches studies on fish GIT microbiota have suggested diet has more limited effects in modulating the microbiota composition (Desai et al., 2012; Ingerslev et al., 2014). For example, Ingerslev et al. (2014) reported that only the first fed diets significantly impact the microbial community of rainbow trout, while Wong et al. (2013) reported that variation in diet and rearing density have only a small impact on the core GIT microbiota of rainbow trout despite significant effects on their growth performance. However, the effect of diet on the functional profile of the GIT microbiota remains enigmatic.

We therefore set out to apply whole-shotgun metagenomics to the mid- and hind-GIT locations of a cornerstone aquaculture species, rainbow trout, under a typical aquaculture setting to better understand the nutritive function of the fish GIT microbiota and the modulatory effects of commonly investigated alternative feeds. The present study is therefore first to implement a whole-shotgun metagenomic sequencing approach to examine the functional capacity of microbial communities in the proximal and distal GIT regions of rainbow trout fed a commercial fishmeal diet, or diets where fishmeal has been replaced by plant and animal protein blends.

MATERIALS AND METHODS

Fish Culture and Experimental Design

All experimental diets were commercially produced by Skretting North America (Tooele, UT, USA) before they were transported to the Bozeman Fish Technology Center

(Bozeman, MT, USA), where feeding trials were conducted. Composition and chemical analysis of dietary treatments are provided in Table 1, except for the proprietary commercial diet (CON). CON contained 45 % crude protein from fishmeal and was used despite its proprietary composition to reflect common aquaculture practices. The two alternative diets replaced fishmeal with a formulation target of 40 % digestible protein from either animal or plant protein sources. Plant protein diet (PPD) and animal protein diet (APD) were supplemented with lysine (2.4 g/kg), methionine (0.7g/kg), and threonine (1.1 g/kg) so the final diet would meet the muscle amino acid profile of rainbow trout (Hardy, 2002; NRC, 2011). Despite a report from Ingerslev and colleagues (Ingerslev et al., 2014) indicating that the first fed diet had the most significant impact the microbial community of rainbow trout, our objective in this first study was to replicate typical commercial practices, therefore trout were initially raised on a commercial starter diet until they reached an average body weight (BW) of 125 ± 3.2 g. Rainbow trout were then stocked at a rate of 75 fish/2,230 L tank with three replicate tanks per diet (in total 675 fish were maintained in 9 tanks) in a recirculating water system at 15 ^{o}C. Diets were randomly allocated to tanks. Fish were fed to apparent satiation twice daily for a period of sixteen weeks. The period of 16 weeks was the typical time fish of this size are expected to take to reach market size. GIT samples were collected from five randomly selected fish per tank at 8-week and at 16-week post feeding. Fish were euthanized by overdose of tricane methane sulfonate (MS-222; 200 mg/L water). Following euthanasia and within 10 min, fish were dissected and the entire GIT removed. The mid- and hind-GIT regions (Figure 1) were separated using a sterile scalpel and aseptic techniques and

the luminal content of each section was then squeezed into separate 1.6 ml Eppendorf tubes (Eppendorf, Hauppauge, NY, USA) and rapidly frozen in liquid nitrogen. Samples were then transferred within 30 min to a -20 °C freezer until they were processed for DNA.

DNA Extraction

Samples were thawed on ice and 0.25 g of each sample were then individually processed for total genomic DNA using MoBio PowerFecal DNA isolation kits (MoBio Laboratories, Inc., Solana Beach, CA). Manufacturer's protocols for GIT samples extraction were strictly adhered to, except that bead tubes containing samples were homogenized for 2 min using a bead beater instead of the 10 min vortex described in the protocol. In total, DNA was extracted from two GIT regions of 90 fish. Extracted DNA was quantified using a Qubit assay kit (Life Technologies, Carlsbad, CA) and quality checked using standard 260/280 absorbance ratios with an Epoch 2 microplate Spectrophotometer (BioTek, Winooski, VT). These DNAs were then pooled at equimolar concentrations into mid- and hind- GIT samples from each tank at each sample date (8- and 16- weeks) resulting in thirty-six GIT pool samples, which comprised of nine midgut pools and nine hindgut pools from both 8-week and 16-week sampling points.

Library Preparation and Metagenomics Sequencing

Extracted DNA samples were fragmented and barcoded using the Nextera XT DNA sample preparation protocol (Illumina, San Diego, CA) with the transposon at a

concentration of 1 ng/µl in 5 µl of DNA sample. Double stranded DNA was synthesized using 15 cycles of Polymerase Chain Reaction (PCR) in a 50 µl reaction volume, which contained 5 µl of each sample uniquely barcoded with distinct Illumina indexes 1 (i7) and 2 (i5). Following PCR, the isolated DNA were purified using AMPURE XP beads according to the manufacturers protocols (Agencourt, Beverly, MA) and were then normalized and pooled for sequencing. Pools were diluted to 12.5 pM and sequenced as paired 125 nt reads by Illumina Hiseq2500. Resulting sequences were trimmed if and where they fell below Q30 and removed if the trimmed read was smaller than 50 nt using fastq_quality_trimmer (Fastx-toolkit; http://hannonlab.cshl.edu/fastx_toolkitlicense/.html). Paired and unpaired reads were separated using customized Perl scripts. High quality reads were assembled de novo into contiguous sequences (contigs) using SOAPdenovo2 (Luo et al., 2012).

Data Analyses

Assembled contigs were annotated using the Metagenomics Rapid Annotation using Subsystem Technology (MG-RAST server) pipeline version 3.6 (Meyer et al., 2008). Contig functional and taxonomic annotations were performed using the M5nr and Subsystems databases, respectively. Annotations were inferred if alignments were ≥50% identity over ≥50 nt and resulted in an e-value of ≤1 e^{-5}, consistent with commonly applied metagenomic annotation thresholds (Kunin et al., 2008; Turnbaugh et al., 2009; Port et al., 2012; Appelt et al., 2014). Complete functional and taxonomic annotation data were downloaded and manually curated to remove non-microbial hits (i.e. reads

potentially deriving from the host or diet, which included reads from the phyla Chordate, Arthropoda, Echinodermata, Mollusca, Porifera, and Streptophyta). Because the coverage information of contigs generated by the SOAPdenovo assembly were imputed to the MG-RAST analysis (MG-RAST: A Technical Report and Manual for Version 3.3.6 - rev. 1 section 4.2.1) quantitative assessments of genes were enabled. Data were then analysed using various programs in R. Coverage estimates were obtained using entropart (Marcon and Hérault, 2015), while additional measures of α-diversity were obtained using SPECIES (Wang, 2011). Estimates of total richness were performed using both Jackknife, and Ace-1 (Chao and Lee, 1992) methods. Both methods provided similar estimates for each sample, however, a Jackknife estimate gave smaller standard deviations and so was used for subsequent analysis and interpretations. For comparing richness and diversity estimates between samples, data were normalized by random subsampling to minimize the effects of variations in sampling depth. Dietary effects on feed intake, feed conversion ratio (FCR), body weight gain (BWG), and BW were determined using one-way ANOVA-Proc GLM of SAS 9.4. Differences in the abundances of genes or taxa were also examined by one-way ANOVA and then corrected for multiple testing using the Benjamini-Hochberg False Discovery Rate (FDR) in R. Since the large numbers of variables at more discriminant levels of analysis often obstruct determinations of significance, we have reported those variables supported by ANOVA ($p<0.05$) but not FDR ($Q>0.05$) as trending to guide future, appropriately powered, hypothesis-driven studies and indicated those supported by FDR ($Q<0.05$) in all figures.

RESULTS

Effect of Dietary Treatments on Growth Performance

No mortality was observed for any of the diets across the trial period. At 8 weeks and 16 weeks of feeding, intake, feed conversion ratio, growth, and percent gain were not found to be significantly affected by the diets ($p>0.05$).

Overall Picture of the Rainbow Trout GIT Microbiome

In this study, 2,043,989,505 raw reads were assembled into 901,944,684 contiguous sequences, i.e. contigs (433,815 ± 372,347 per sample) with n50 of 136 ± 8 nt. Across all samples, 3,267 microbial species were identified encoding 6,054 unique annotatable gene functions. By sample, 343 ± 130 microbial species were observed in the midgut (377 ± 142) and hindgut (310 ± 111) regions of the GIT samples. Good's estimates of coverage indicated the approach employed in this investigation had 98 ± 0.03% of all GIT-located species and 96 ± 0.04% of all functions. Overall, bacteria were predominant in the trout GIT microbiome (avg. 74 ± 36% of all assignable contigs), but eukaryotic microbes (25 ± 37%), viruses (0.8 ± 1.6%), and archaea (0.3 ± 0.4%) were also evident (Figure 2). Bacteria were largely from the *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, *Tenericutes* and *Fusobacterial* phyla (Figure 2). Eukaryotes and archaea were almost exclusively from the Ascomycota and Euryarchaeota phyla, respectively (Figure 2), while viruses were predominantly bacteriophage of the Caudovirales order. Consistent with the protein-rich diets of trout, functions related to protein (8 ± 3%), amino acid (17 ± 4%) and nitrogen (0.37 ± 0.28%) metabolisms collectively represented

the highest fraction of annotated genes within the metagenomes (Figure 3). Genes dedicated to carbohydrate (15 ± 4%) and vitamin, cofactor, and prosthetic group metabolism (6 ± 1.6%) were also abundant (Figure 3). Microbiota of the mid-GIT and hind-GIT did not differ between samples collected at 8 and 16 weeks (ANOSIM $R<0.2$, $p>0.005$), but differences were observed between mid- and hind-GIT regions and within each GIT region by diet (Figure 4).

Subtle but Potentially Important Differences Between the Trout Mid- and Hind-GIT

Jackknife estimates of total microbial richness indicated that mid-GIT samples possessed more microbes than hind-GIT samples consistent with numbers observed and reported above ($p <0.05$). However, only PPD-consuming fish were estimated to have a greater number of unique functions in the mid-GIT relative to the hind-GIT. Conversely, APD-consuming fish were instead predicted to have a greater number of unique functions in the hind-GIT. Analysis of Shannon's diversity estimates did not support differences in overall taxonomic α-diversity between GIT regions (mid-GIT $H=3.32 \pm 1.01$, hind-GIT $H=2.79 \pm 1.48$, $p>0.05$). Analysis of species-level classifications by both analysis of similarities (ANOSIM $R=0.28$, $p<0.001$) and permutational multivariate analysis of variance using distance matrices (PERMANOVA $R^2 = 0.07$ $p=0.003$) indicated small but significant differences in the overall microbiota between GIT locations. These differences corresponded to similarly small differences in the overall functional profile between the two GIT locations (ANOSIM $R=0.23$, $p<0.001$, PERMANOVA $R^2 = 0.06$ $p=0.001$).

However, as outlined below, these small overall differences included functions involved in important nutritive roles. Archaea were more abundant within the trout mid-GIT (Figure 5), which concordantly corresponded to a higher number of genes related to methanogenesis (0.054 vs. 0.012%). Among the bacteria, only the Tenericutes approached significance toward being more abundant in the mid-GIT and no Eukaryotic phyla were determined to be more abundant in either GIT region. Further functional analyses indicated trends toward greater emphases on microbial-mediated sulphur (0.4 vs. 0.2%), potassium (0.48 vs. 0.21%), and aromatic compound (0.8 vs. 0.3%) metabolisms in the mid-GIT and carbohydrate (17 vs. 14.5%), and fatty acid, lipid and/or isoprenoid (2.8 vs. 1.6%) metabolisms in the hind-GIT (Figure S1). Differences in aromatic compound metabolism were largely driven by corresponding differences in genes dedicated to the degradation of xylenols and cresols by the Gentisate pathway and of p-cresol via the protocatechuate branch of beta-ketoadipate pathway (Figure S2). Differences in carbohydrate metabolism were largely driven by a near 3-fold enrichment of genes involved in polysaccharide metabolism (0.26 vs. 0.09%; $p<0.01$, $Q>0.05$) and other diet-specific enrichments of simpler carbohydrate moieties in the hind-GIT, while genes involved in the metabolism of arabinose, or acid derivatives of glucose and galactose (D-glucarate, D-galacterate) approached significance for enrichment in the mid-GIT (Figure S2). Genes involved in microbial biosynthesis of pyridoxine (vitamin B_6), and the provitamin A equivalent, carotenoids) also approached significance for greater abundance within the mid-GIT (Figure 6).

Dietary Impacts on the Mid- and Hind GIT Microbiome

While only small overall differences were seen in the microbiome between the mid-GIT and hind-GIT, stronger differences became evident when also accounting for diet (Microbiota ANOSIM R=0.32, $p<0.001$, PERMANOVA $R^2 = 0.23$ $p=0.001$; Function ANOSIM R=0.22, $p<0.001$, PERMANOVA $R^2 = 0.22$ $p=0.001$). As no significant differences were seen in GIT microbiota or function when just accounting for diet, this observation highlights an important GIT location by diet interaction (Figure 4). This may be partially explained by the finding that dietary treatments caused a significant shift in hind-GIT microbiota (ANOSIM R=0.32, $p<0.005$), but no measurable effect in the trout mid-GIT. Each dietary treatment did cause unique shifts in both the mid- and hind-GIT functional profiles. The inclusion of plant protein in the diet led to 7.5 and 60-fold increases in archaea (0.6 ± 0.04%) within the hindgut relative to CON (0.08 ± 0.07%) and APD diets (0.01 ± 0.01%), respectively (Figure 5). Among bacterial constituents, there were trending enrichments in Bacteroidetes within the hind-GIT of PPD-consuming fish, while the CON diet caused trending increases in Firmicutes relative to other dietary treatments in the hind-GIT (Figure 5). These observations mirrored measured genes annotated as being involved in Gram-negative and Gram-positive cell wall biosynthesis, which were numerically enriched in the mid- and hind-GIT, respectively in all but PPD-consuming fish (which displayed the opposite trend) (Figure 6). Consistently, the mid-GIT microbiome exhibited trending increases in peptidoglycan biosynthesis, peptidoglycan (murein) hydrolase, and N-acetylglucosamine and chitin metabolisms. The mid-GIT of PPD-consuming fish was also enriched for alkaloid biosynthetic pathways.

There were enrichments of protein metabolism genes in the mid-GIT of fish fed the CON and APD diets as well as in the hind-GIT of fish fed the PPD diet. Relative to the CON, both the APD and PPD diets led to significant increases in the mid-GIT of genes involved in protein degradation, perhaps indicating a greater role for microbes in liberating nitrogenous nutrients these non-typical protein sources. APD and CON diets approached significance for enrichment of genes involved in monosaccharide utilization within the mid-GIT relative to PPD-consuming fish (Figure 6). The hind-GIT of fish fed the CON and APD diets also approached or revealed significant differences in the utilization of di- and oligo-saccharides (fructooligosaccharide and raffinose utilization, maltose and maltodextrin, lactose and galactose) compared to PPD fed fish (Figure 6).

DISCUSSION

Previous studies employing a targeted 16S rRNA gene approach have similarly reported the dominance of bacteria of the Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes phyla (Desai et al., 2012; Ingerslev et al., 2014). Using a whole shot-gun metagenomic approach, our study supports these as the predominant taxa and indicates these phyla are dominant throughout the GIT irrespective of dietary treatments, albeit with greater microbial diversity in the proximal than the distal region of the GIT. The dominance of the Firmicutes and Proteobacteria phyla observed in this study is also consistent with observations of the luminal microbiota of Atlantic salmon (Gajardo et al., 2016). Similarly, the higher abundance of Tenericutes we observed in the trout mid-gut has also been described for adult wild salmon (Llewellyn et al., 2016). In addition to the

predominating bacteria, our approach further allowed us to survey non-bacterial microbes revealing significant numbers of eukaryotic microbes, along with smaller numbers of archaea. A review of earlier findings show agreement with the observations made in this present study that the mid-GIT of the trout housed a distinct but diverse microbiota irrespective of dietary treatments, while the population of microbes in the hindgut were responsive to dietary changes by the host. Wong et al. (2013) sampled the mid-GIT of rainbow trout and observed little variation in microbiota irrespective of dietary treatments and water environment, while the studies by Desai et al. (2012) and Ingerslev et al. (2014), which both focussed on the fish Hind-GIT, showed this region to be responsive to the dietary treatments utilized in those studies. These findings do, however, contrast with a study of juvenile Atlantic salmon by Navarrete et al. (2009) where temporal temperature gradient gel electrophoresis of 16S rRNA gene amplicons described very similar bacterial profiles throughout the stomach, pyloric caeca, and GIT. The findings of Navarrete may suggest the GIT lumen is strongly influenced by allocthonous (non-resident transient) microbes deriving from the water environment. Consequently, some researchers have gone so far as to describe the luminal microbiota of trout and salmon as entirely allochthonous (Merrifield et al., 2009; Navarrete et al., 2009). It may therefore be interesting to assess the relationship of the GIT microbiota to water samples as well as assess mucosal populations in future studies.

The whole shotgun metagenomic approach employed in the current study allowed us to describe and compare both the functional and taxonomic compositions of these two GIT locations, our data uncover greater emphases on select nutrients in the different GIT

regions. Specifically, we observe an enrichment of microbial contributions to sulphur, potassium, and aromatic compound metabolisms in the mid-GIT region, while the hind-GIT microbiome appears to have a greater focus on carbohydrate, and fatty acid, lipid and/or isoprenoid metabolisms. These delineations may reflect the order in which nutrients arrive and changes to their relative accessibility as they flow between the two GIT locations. Greater proportions of sulphur and potassium metabolizing genes in the more proximal mid-GIT may be enriched by sulphur amino acids such as methionine, and sulphur and potassium–based minerals supplemented to the diets. Substantial metabolism and absorption of these compounds in the mid-GIT may reduce their availability to the more distal hind-GIT. Studies of rainbow trout physiology have long noted active amino acid absorption in the mid-GIT (Dabrowski and Dabrowska, 1981; Krogdahl et al., 1994). Enrichments of genes involved in aromatic compound metabolism were largely driven by genes dedicated to the degradation of xylenols and cresols by the Gentisate pathway and the protocatechuate branch of beta-ketoadipate pathway. Both of these pathways utilize p-Cresol, a byproduct of the bacterial fermentation of protein (Hopper and Taylor, 1975; Harwood and Parales, 1996; Hamer et al., 2012). The availability of simpler carbohydrate sources including mono-, di-, and oligo- saccharides likely satisfied the carbohydrate requirements of mid-GIT microbes, leaving the more recalcitrant polysaccharides to pass on to the hind-GIT.

The narrow delineations of the two GIT regions appear to be further complicated by dietary impacts. Some of these diet-specific differences may reflect compositional features of the diets. For example, differences in the composition of simpler carbohydrate

sources could explain the diet-specific differences observed with genes related to the utilization of these compounds in the mid-GIT. It was observed that microbes in the hind-GIT of rainbow trout fed the plant protein diet (PPD) were enriched for genes associated with carbohydrate fermentation. Microbial-mediated carbohydrate fermentation in animal systems typically involves a broad and diverse group of microorganisms whose collective hydrolytic capabilities enable them to utilize plant structural components that are not otherwise available to the animal due to a lack of endogenously-encoded hydrolytic enzymes (Yeoman and White, 2014). In addition to an enrichment of genes associated with carbohydrate fermentation in the hind-GIT of rainbow trout fed the PPD, we also observed the greatest abundance of archaea and an increase in members of the Bacteroidetes phylum. Archaea mainly comprised members of the Euryarchaeota and Crenarchaeota phyla, specifically, *Methanobrevicater smithii*, along with species of the Methanophaera, Methanobacterium, Methanothermobacter and Methanothermus genera. These phyla comprise methanogenic archaea, which play important roles in the anaerobic fermentation of carbohydrates (Sekiguchi, 2006). Similarly, an enrichment of Bacteroidetes in fish fed the PPD diet compared to other dietary treatments is consistent with human studies where GIT Bacteroidetes have been shown to respond to oligosaccharides, particularly diverse glycans (Sonnenburg et al., 2010; Martens et al., 2011; Despres et al., 2016). Similarly, a recent study in mice, revealed increased gene expression from *Bacteroides fragilis* when oligosaccharides were supplemented in the diet (Charbonneau et al., 2016). We did not, however, observe a similar increase in members of the phylum Firmicutes. Firmicutes, in-particular members of the genera

Clostridium and Ruminococcus, have well-described roles in carbohydrates hydrolysis in vertebrate animals (Yeoman and White, 2014) including various fish species (Kihara et al., 1995; Smith et al., 1996; Mountfort et al., 2002). Instead, members of the Firmicutes phylum appeared to be enriched in the hind-GIT of fish consuming the fishmeal control diet. The proprietary nature of this diet precludes broader rationalization as to why hind-GIT Firmicutes may have been enriched by this dietary regimen, but co-observed increases in genes related to di-, and oligo-saccharide utilization may reflect the abundance of these compounds within the CON diet. Firmicutes species are broadly known to exploit a wide array of monosaccharides, disaccharides, starches, and other substrates such as whey and xylan (Gu et al., 2010).

Differences in the relative number of microbial genes related to protein metabolism between the fish fed the alternative diets and CON diet may be due to differences in the fishes endogenous ability to exploit these atypical dietary treatments. Earlier studies have shown the typical active absorption of amino acids in the mid-GIT can be partially shifted to the hind-GIT when inhibitors are included in the diet (Dabrowski and Dabrowska, 1981; Krogdahl et al., 1994). These observations indicate a need to further investigate the role of the rainbow trout GIT microbiota in protein metabolism, in particular when alternative diets are involved.

CONCLUSIONS

Overall, our study highlights a potential for nutritive contributions within the trout GIT and small but potentially important functional division between the mid- and

hind-GIT. These differences appear to be modulated by, and in some respects, adaptable to the fish diet. Diet is known as one of the potential factors modulating GIT microbiota of fishes. Available information on the rainbow trout microbial population corroborates our findings and with additional information on the distinctive nutritive roles of the GIT sections. Our data showed that diet type or variation in ingredient composition may interactively alter the structure of the GIT microbiome. Core microbiota for cultured fish species may depend on factors like the diet type and functionality, water environments, genetic composition, feeding method and periods, temperature, and management factors employed. Further studies are necessary to determine if these taxonomic and functional features observed in rainbow trout actively contribute to host energy balance and nutrition and potentially to immune function and health.

List of Abbreviations

ADP	animal protein diet
ANOSIM	analyses of similarity
ANOVA	analysis of variance
BW	body weight
BWG	body weight gain
CON	commercial diet
FCR	feed conversion ratio
GIT	gastrointestinal tract
PERMANOVA	permutational multivariate analysis of variance

PPD plant protein diet

SCFA short chain fatty acid

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AVAILABILITY OF DATA AND MATERIAL

Sequencing results are available in the metagenomics analysis server (MG-RAST) database (http:// metagenomics.anl.gov/), accession number 15046.

AUTHORS' CONTRIBUTIONS

CJY, WMS, GCD, and OCB conceived of and designed the study, OCB, GTG and WMS collected samples, OCB, CJY, AM, and SLI analyzed the data, OCB, CJY, and SLI drafted the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

CONSENT FOR PUBLICATION

Not applicable.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All fish handling procedures followed regulations set by the Department of Interior, USFWS and were approved by the Montana State University Institutional Animal Care and Use Committee (IACUC) under Protocol 2013-40.

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Table 1. Ingredients and chemical compositions of dietary treatments

Ingredients (g/kg dry diet)	40% Animal protein blend (APD)	40% Plant protein diet (PPD)
Poultry by product meal	24.28	-
Blood meal	2.97	-
Feather meal	2.95	-
Millrun	3.18	-
Soy isolate	-	16.88
Corn gluten meal	8.8	21.61
Soybean meal	12.39	15.96
Whole wheat	20.62	15.76
Poultry fat	7.56	10.34
Fish oil	7.45	7.49
Lecithin	0.93	0.94
L-Lysine HCl	2.67	2.94
DL Methionine	0.67	0.67
Threonine	0.85	0.81
Vitamin premix	1.43	1.45
Astaxanthin pink	0.04	0.04
Choline chloride	0.93	0.94
Stable C	0.14	0.14
Trace mineral premix	0.93	0.94
Mono Cal Phosp	1.21	3.09
Crude protein (N*6.25) (g/kg)	428	415
Fat (g/kg)	152.4	220.5
Gross Energy (MJ/kg)	23.0	23.4

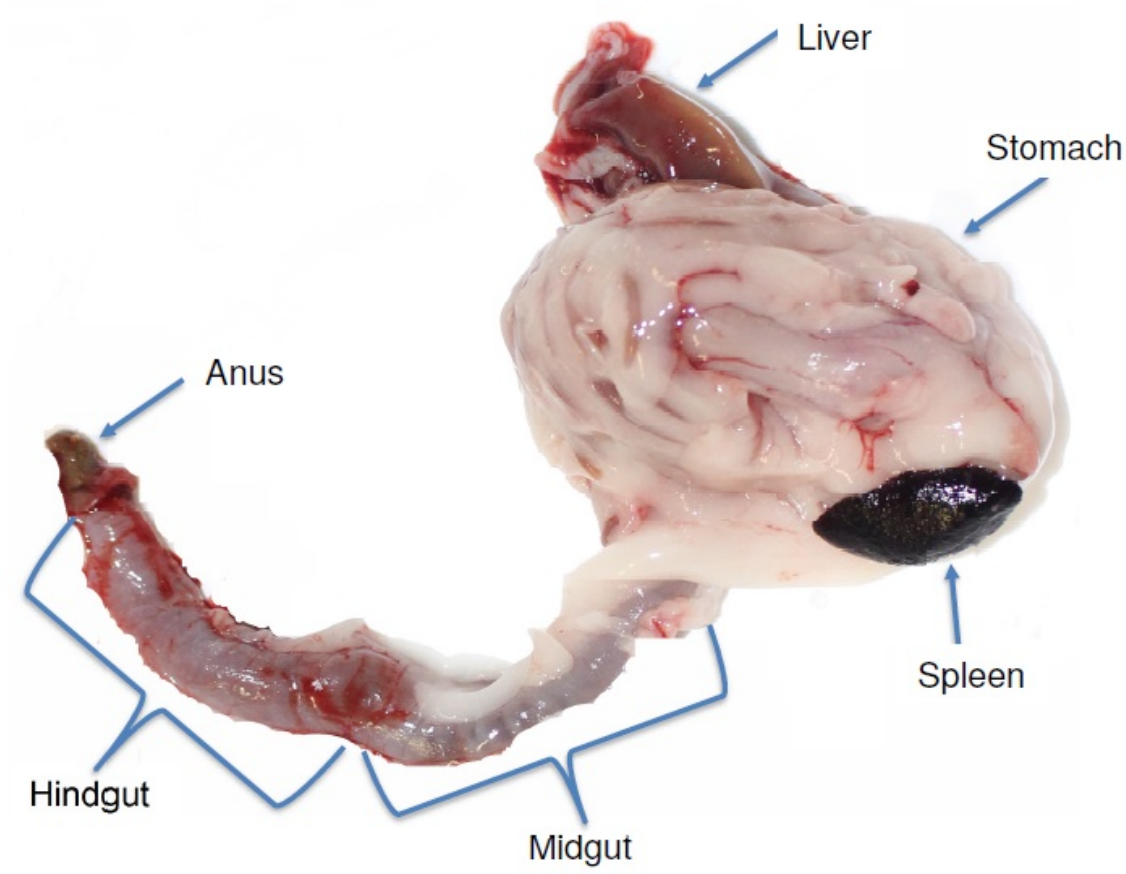

Figure 1. Gastrointestinal tract of rainbow trout.
Picture of a typical GIT extracted from a rainbow trout labelled with its main anatomical features.

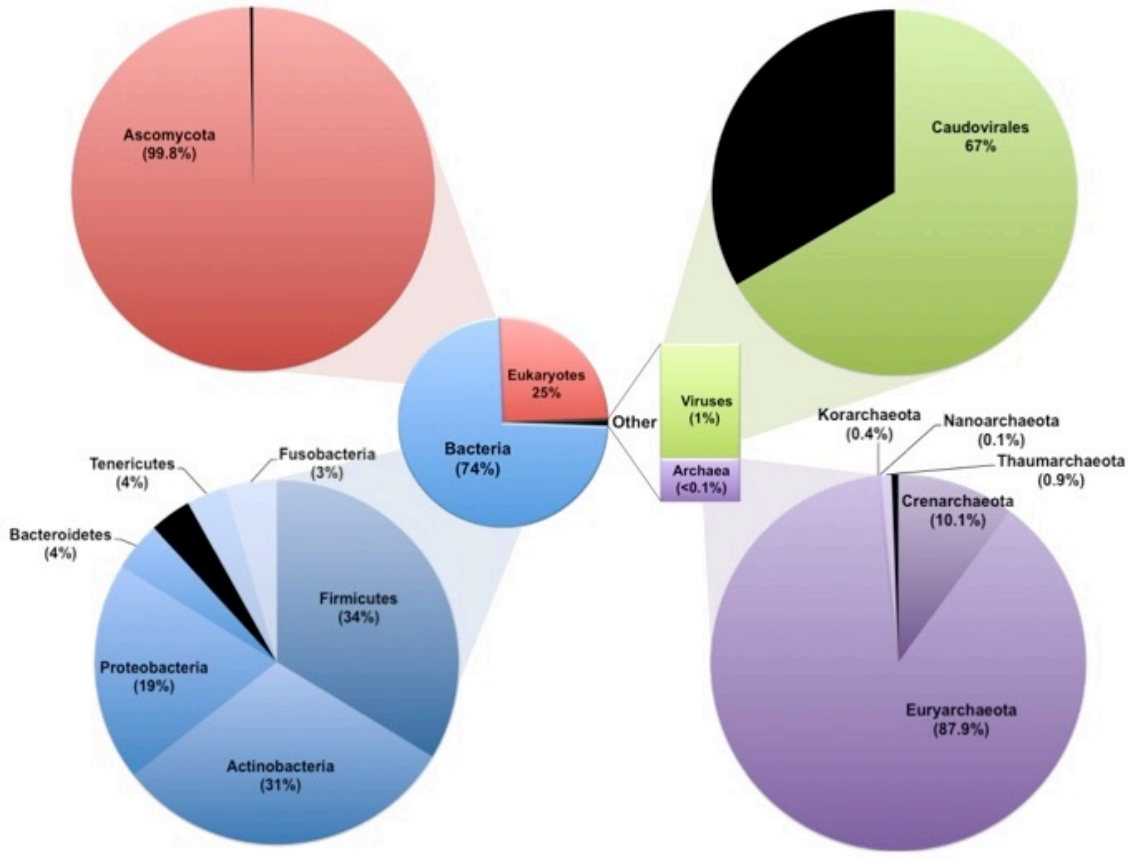

Figure 2. Overall Taxonomic Compositions of Rainbow Trout GIT Microbiota. Central pie chart shows the domain-level composition of the rainbow trout GIT, while outer pies show the major Phyla or Orders (for viruses) of each domain. Black segments of pie charts indicate all other less abundant taxa including reads that were unable to be annotated.

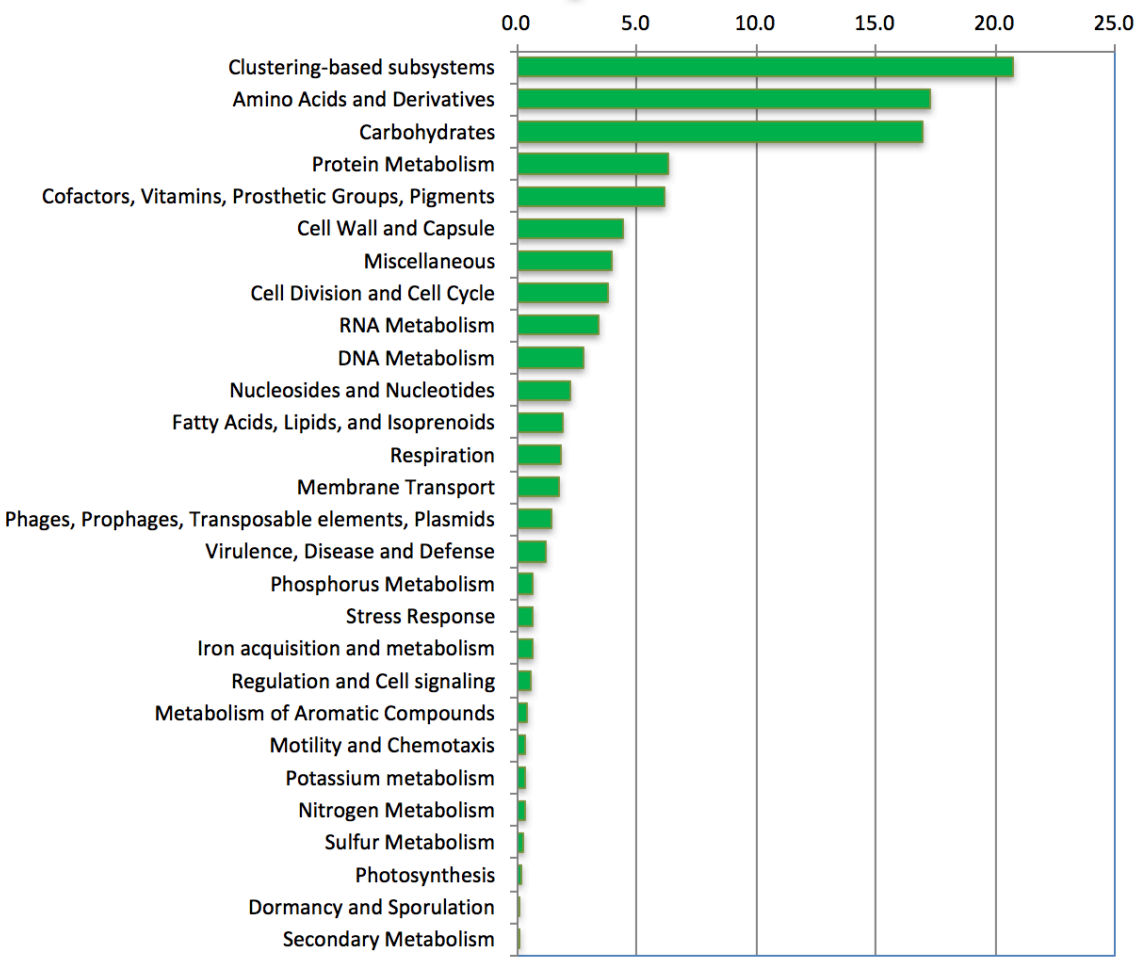

Figure 3. Overall Functional Profile of Rainbow Trout GIT
Bar chart shows the percent relative abundance of level 1 gene classifications across all fish and GIT locations.

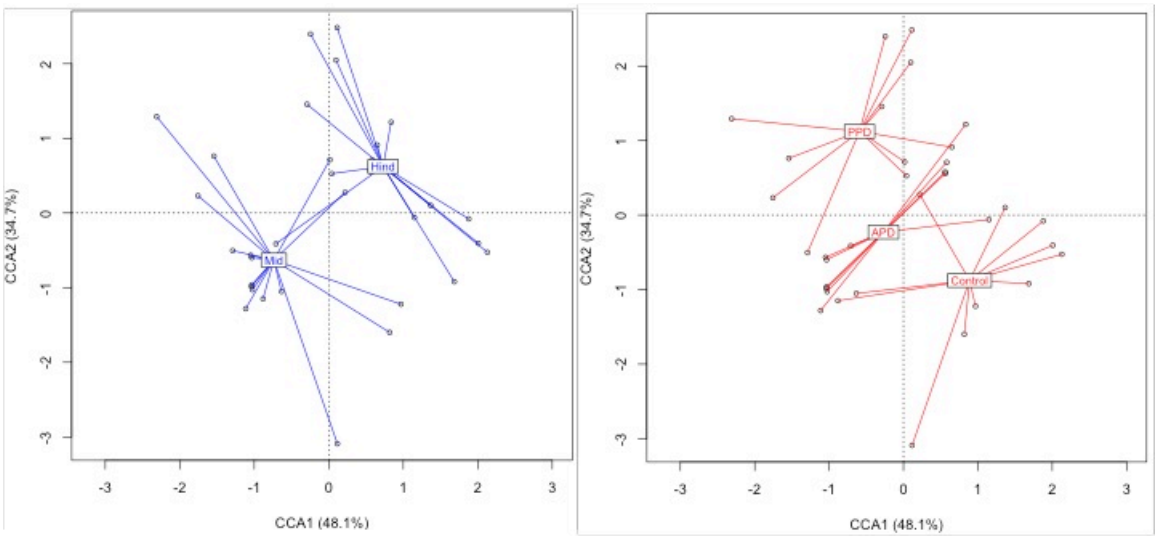

Figure 4. Contributions of GIT Location and Diet to Microbiome
Plot of constrained canonical correspondence analysis showing the small contributions of GIT location (blue, A & C) and diet (red, B & D) to the overall species-level taxonomic (A & B) and gene-level functional (C & D) profiles. Explanatory values on axes represent percent of total explanatory power (constrained proportion x eigenvalue x 100).

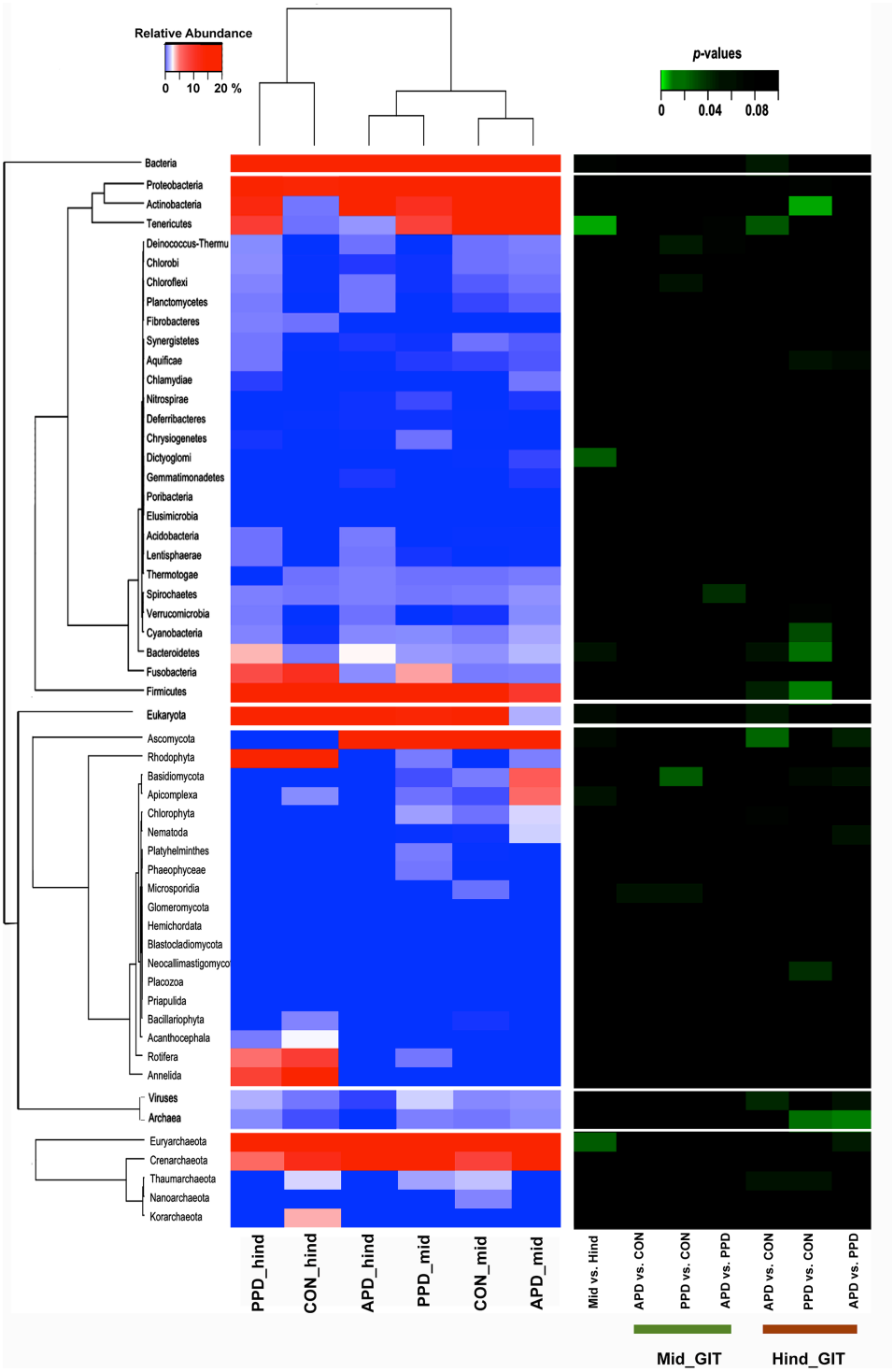

Figure 5. Phylum-level variation among rainbow trout GIT fed fishmeal-, animal- and plant-based diets. Dual heatmaps show the relative abundances of phyla by diet and GIT location (left) and the significance of any observed differences by gut location, or diet, as stratified by gut location (right).

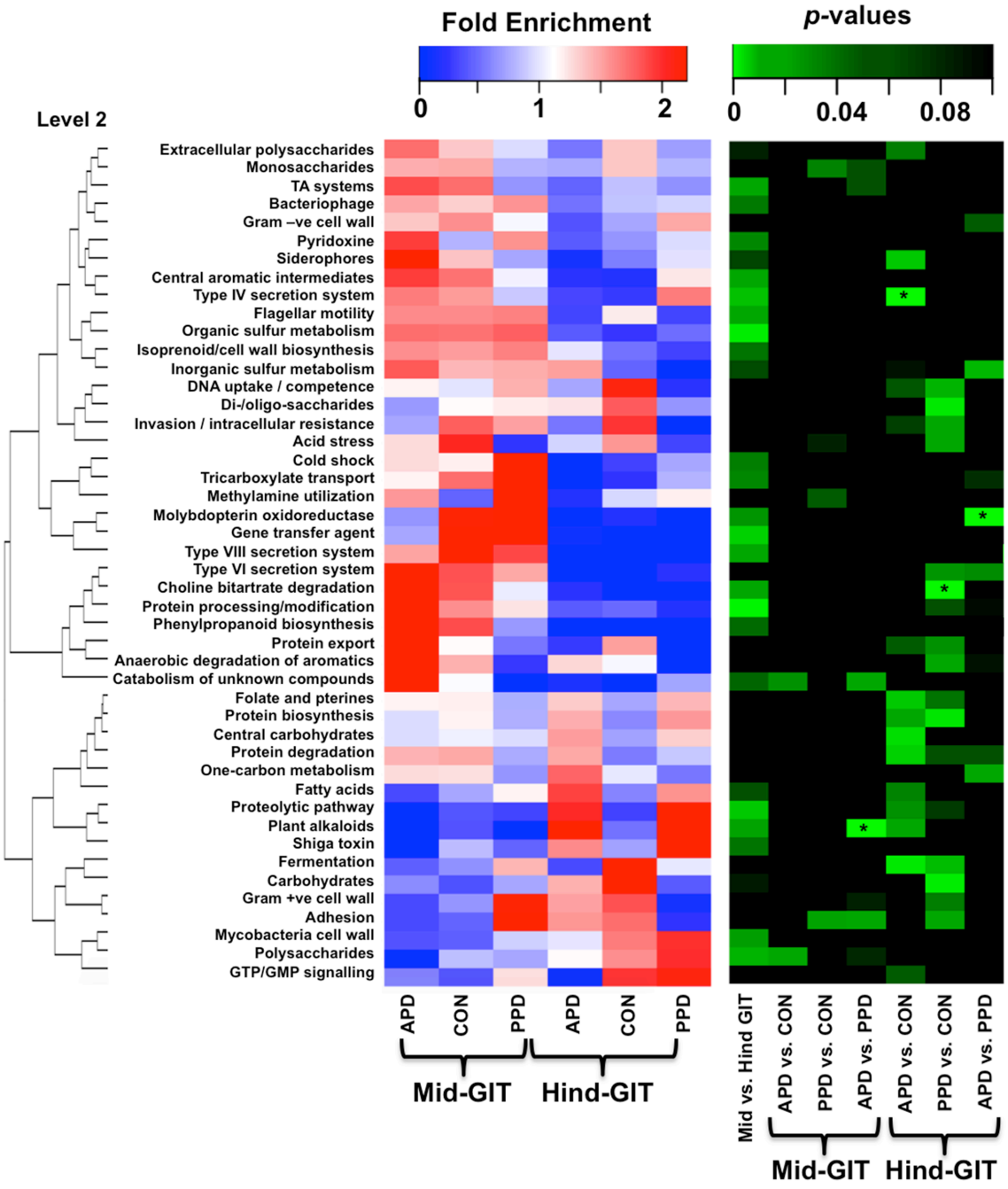

Figure 6. Functional Relationships Across Dietary Treatments and GIT Locations. Dual heatmaps show the enrichment or depletion of gene functions above or below their average relative abundances (left) and the significance of any observed differences by gut location, or diet, as stratified by gut location (right). Where significance is supported by FDR correction an asterisk appears.

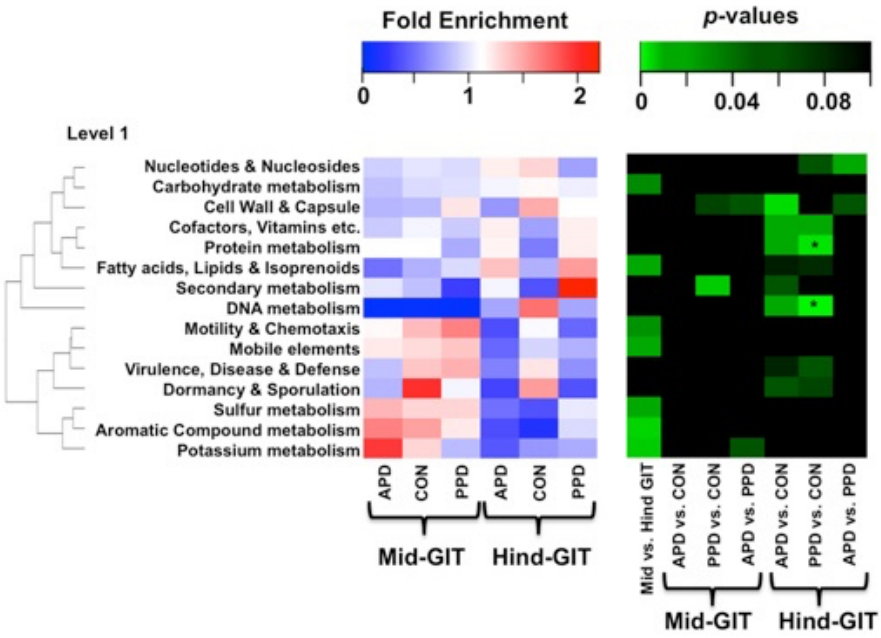

Figure S1. Level 1 Functional Relationships Across Dietary Treatments and GIT Locations

Dual heatmaps show the enrichment or depletion of gene functions above or below their average relative abundances (left) and the significance of any observed differences by gut location, or diet, as stratified by gut location (right). Where significance is supported by FDR correction, an asterisk appears.

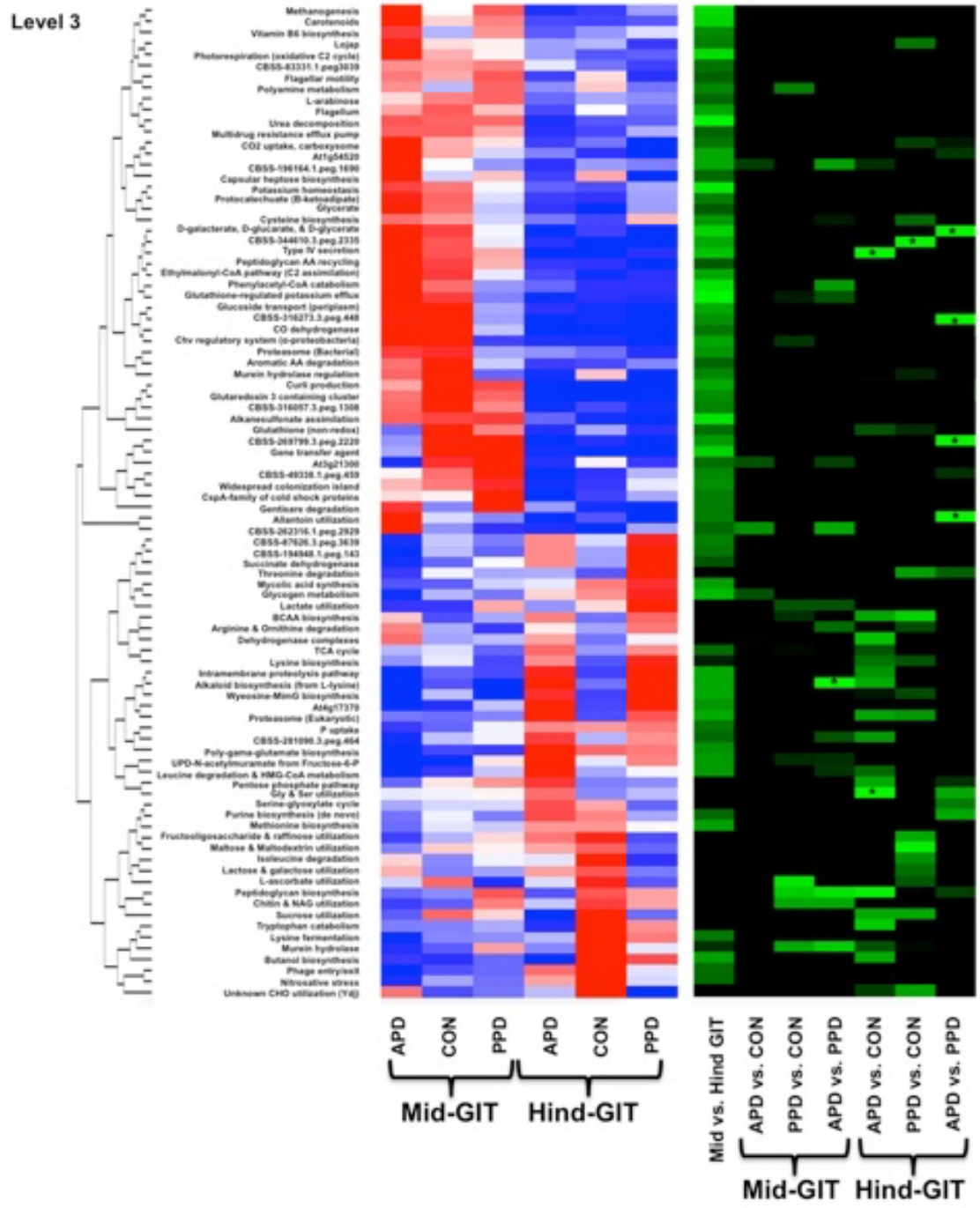

Figure S2. Level 3 Functional Relationships Across Dietary Treatments and GIT Locations

Dual heatmaps show the enrichment or depletion of gene functions above or below their average relative abundances (left) and the significance of any observed differences by gut location, or diet, as stratified by gut location (right). Where significance is supported by FDR correction, an asterisk appears.

CHAPTER FIVE

DIFFERENCES IN AMINO ACID CATABOLISM BY GUT MICROBES WITH/WITHOUT PREBIOTICS INCLUSION IN GDDY-BASED DIET AFFECT FEED UTILIZATION IN RAINBOW TROUT

Contribution of Authors and Co-Authors

Manuscript in Chapter 5

Author: Omolola C. Betiku
Contributions: Designed experiment and formulated feed. Performed the experiment and collected samples. Laboratory analyses, data analyses and interpretation. Wrote manuscript with input from co-authors.

Co-Author: Carl J. Yeoman
Contributions: Involved in the design of the experiments. Data analysis and interpretation. Assisted in the preparation of the manuscript.

Co-Author: T. Gibson Gaylord
Contributions: Involved in the design of the experiments and analysis of data. Assisted in manuscript preparation.

Co-Author: Glenn C. Duff
Contributions: Assisted in preparation of manuscript.

Co-Author: Brian Bothner
Contributions: Assisted in short chain fatty acid analysis.

Co-Author: Stephanie S. Block
Contributions: Assisted in material provision.

Co-Author: Wendy M. Sealey
Contributions: Involved in the design of the experiments. Data analysis and interpretation. Assisted in the preparation of the manuscript.

Manuscript Information Page

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Aquaculture

Differences in Amino Acid Catabolism by Gut Microbes with/without Prebiotics Inclusion in GDDY-based Diet Affect Feed Utilization in Rainbow Trout

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Abstract

There is the need to enhance feed efficiency and growth of rainbow trout in order to reduce cost associated with production costs of cultured fish. This study thus conducted a 3 x 4 factorial experiment with graded levels of grain distiller dried yeast (GDDY) protein as a substitute for fishmeal protein (0%, 50%, 75%) and four different prebiotics inclusion (0%, 0.4% mannooligosaccharides (MOS), 1% MOS and 1% GroBiotic A). The feeding trial was conducted for 12 weeks during which fish were fed daily to apparent satiation. Growth of rainbow trout was not affected by replacement of fishmeal with GDDY, but feed conversion ratio ($P<0.0001$) was greater at the highest level of GDDY inclusion. Increasing GDDY inclusion significantly increased feed intake ($P<0.00015$), which resulted in poor feed utilization. Acetic ($P=0.1994$), propionic ($P=0.8037$), butyric ($P=0.6268$), valeric ($P=0.5877$), and isovaleic ($P=0.5919$) acids profiles did not differ by diet nor with inclusion of MOS or GroBiotic A. Whole shotgun metagenomic analyses of the gastrointestinal tract (GIT) microbiota revealed enrichment in the number of fungi from *Ascomycota* and *Basidiomycota* and phylum *Actinobacteria* in the GDDY-fed fish compared to those fed the control fishmeal-based diet which may be reflective of the species endogenous in GDDY. Microbial genes involved in branched-chain amino acid metabolism (glutamate, glutamine, aspartate) ($P=0.028$) and glutamate dehydrogenase clusters ($P=0.0192$), were also elevated in the fish fed the 75% GDDY-based diet. The results from this study suggest possible catabolism of the non-essential amino acids, which significantly influence efficient utilization of dietary nitrogen in the yeast-based protein diet.

Keywords: Prebiotics, grain distiller dried yeast, alternative protein, metagenomics, amino acids, rainbow trout

Introduction

Prebiotics, which are feed ingredients that are not endogenously digestible by the host but selectively stimulate the growth of beneficial microbes in the host, have been reported to improve feed efficiency, growth rate, and weight gain in rainbow trout (Bagheri et al., 2008; Merrifield et al., 2010). Human studies have shown prebiotics can improve the production of short chain fatty acids thereby enhancing the supply of energy (Ríos-Covián et al., 2016). Positive effects of mannoligosaccharides (MOS) from *Saccharomyces cerevisiae* in fish feeding studies include improved growth, survival and immune responses (Sealey et al., 2007; Ringø et al., 2010; Mansour et al., 2012). However, reports of these benefits are inconsistent (Genc et al., 2007; Welker et al., 2007; Grisdale-Helland et al., 2008; Sado et al., 2008; Torrecillas et al., 2011; Sealey et al., 2015a). Grobiotic®-A (GroA), a mixture of partially autolyzed brewer's yeast, dairy ingredient components and dried fermentation products, is another commercially available prebiotic that is gaining attention in fish nutrition studies (Li and Gatlin, 2004; Burr et al., 2009; Sealey et al., 2015a). Although GroA is reported to lower pathogenic bacteria, information on how it specifically alters microbial compositions in the gastrointestinal tract (GIT) in these fishes is lacking (Li and Gatlin, 2004; Burr et al., 2009).

The GIT, especially, the small intestine, is the active site for digestion and metabolism of endogenous and exogenous amino acids in fish and other animals (Adibi

and Mercer, 1973; Ash, 1980; Dabrowski and Dabrowska, 1981; Attaix and Arnal, 1987; Betiku et al., 2017). The mucosa of the small intestine catabolizes non-essential amino acids (glutamine, glutamate and aspartate) for supplying oxidative fuels to the GIT mucosa and also serve as precursors for intestinal synthesis of proteins, pyrimidine, purines, proline, nitric oxide and glutathione (Reeds et al., 2000). In addition to catabolism of these non-essential amino acids, essential amino acids, including the branched-chain amino acids (BCAA), threonine, lysine and phenylalanine are also oxidized (Adibi and Mercer, 1973; Wu, 1998); and the rate of metabolism of these amino acids in the intestine is determined by enteral and parenteral amino acids availability in the arterial blood (Burrin, 2002). Fermentation of the BCAA: valine, leucine and isoleucine into short chain fatty acids (SCFA) for use by the GIT microbiota also has been observed (Ríos-Covián et al., 2016).

Although it is not fully known in fish how dietary protein sources affect amino acids catabolism and their subsequent availability for use by the GIT mucosa, this information may be of great importance in achieving improved nitrogen utilization when alternative protein ingredients are used in aquaculture (Davila et al., 2013). In addition, amino acid catabolism by the GIT mucosa plays an important role in modulating absorbed amino acids entry into the portal circulation with differences in the appearance of amino acids in the portal circulation that may not reflect their availability to the extra-intestinal tissue. In essence, amino acid catabolism plays a significant role in the status of protein turnover. Specifically in the model aquaculture fish species rainbow trout, postprandial amino acids concentrations are influenced by protein sources, resulting in differences in

appearance between essential amino acids and non-essential amino acids (Walton and Wilson, 1986; Larsen et al., 2012).

Recent evidence by Chan (2016) suggests that hepatic glutamate dehydrogenase (GDH) is the endogenous catabolism enzyme responsible for poor nitrogen retention efficiency observed in adult trout having higher concentration of this enzyme compared to the larval fish. Differences in concentration of the GDH may be associated with increased energy demand in the adult fish through increasing hormonal regulation of the digestive enzymes (Rønnestad et al., 2013). GDH is found in most living organisms including microbes, where it catabolizes a reversible transdeamination reaction of glutamate to α-ketoglutarate (Reeds et al., 2000). GDH has been detected in rainbow trout and its activity is negatively correlated with nitrogen retention efficiencies (Davies et al., 1997; Mambrini et al., 1999; Martin et al., 2003). However, the mechanistic process by which catabolized amino acids are deaminated is not fully understood, especially when there are exchange of amino acids between the GIT microbiota and the host. Since nitrogen retention involves a complex interaction between protein synthesis and protein degradation, we have employed the shotgun sequencing approach to analyze microbial diversity and gene functions associated with amino acids synthesis and catabolism within rainbow trout small intestine fed Grain distiller dried yeast (GDDY).

GDDY is a single-cell yeast-based protein ingredient with high protein content and has been successfully used as a protein source in rainbow trout nutrition, partially replacing fishmeal when supplemented with synthetic amino acids (Hauptman et al., 2014a; Hauptman et al., 2014b; Sealey et al., 2015b). However, inclusion levels above 15 - 18%

have had significant impacts on efficient feed utilization in rainbow trout suggestive of a potential nutritional stressor associated with this protein source (Hauptman et al., 2014a; Hauptman et al., 2014b; Sealey et al., 2015b). In rainbow trout, the influence of prebiotics on activity of the GIT microbial composition is yet to be determined; however, beneficial effects of prebiotics have been most often observed in nutritionally stressing diets. Therefore, the present study describes an in-depth study of the metabolic process using metagenomic technique to investigate nutrient utilization in midgut of rainbow trout and amino acids catabolic processes when fed a nutritionally stressing diet in order to provide better understanding of the mechanistic processes involve in amino acid utilization when alternative protein is employed in aquaculture

Materials and methods

Experimental design

A 3 x 4 factorial design experiment was conducted with twelve experiments diets (Table 1) to contain three increasing levels of GDDY (0, 50%, 75%) as a replacement of fishmeal and four prebiotic treatments (0%, 0.4% MOS, 1% MOS, 1% GroA). The diets were formulated at 40% crude protein and 20% crude lipid, supplemented with lysine, methionine and threonine balanced to the rainbow trout fillet ideal amino acid targets of 3.8 %, 1.3 % and 2.1%, respectively described by Gaylord and Barrows (2009). All fish were handled and treated in accordance with guidelines approved by the U.S. Fish and Wildlife Service.

Fish culture

Rainbow trout eggs were obtained from Troutlodge Inc., Sumner, Washington, USA. They were cultured at the Bozeman Fish Technology Center, Bozeman, MT before they were randomly selected and stocked into 36 experimental tanks. Fish with an average initial weight of 15.7 ± 0.4 g were stocked at the rate of 20 fish/tank in a recirculating system, with three replicate tanks per dietary treatment. The culture water temperature was maintained at 15 ºC. Fish were acclimatized to tanks for one-week prior to beginning the feeding trial. Diets were randomly assigned to each of the tank and fish were fed twice a day to apparent satiation for the period of the experiment.

Diet manufacturing

All ingredients for each of the diets were ground with an air swept pulverizer (model 18-H; Jacobson, Minneapolis, MN, USA). All the diets were manufactured using cooking extrusion (DNDL-44, Buhler AG, Uzwil, Switzerland) with an 18-s exposure to an average of 127 ^{o}C in the extruder barrel section. The die plate was water cooled to an average temperature of 60 ^{o}C. Depending on the diet, pressure at the die head was varied from 15 to 30 bar. The 3 mm pellets produced were then dried in a pulse-bed drier (Buhler AG, Uzwil, Switzerland) for 25 min at 102 ºC with a 10-min cooling period. The final moisture content in all the diets was <10%. Thereafter, oil was added to the diets by top-coating.

Growth monitoring and compositional sampling

During the 12-week growth trial, fish were weighed monthly and feed intake, feed conversion ratio (FCR) and weight gain were determined per tank. Ten fish from the initial fish population were sampled for determination of initial whole-body proximate composition. At the end of the 12-week feeding trial, three fish from each tank were sampled for determination of whole body composition. Three additional fish were sampled from each of the tank for determination of visceral somatic index (VSI), hepatosomatic index (HSI) and filet ratio (FR). Equations used to determine the body indices are displayed in Table 2.

Feces from fish in each replicate tank post sampling were obtained by manual stripping (Austreng, 1978). In brief, all fish in each tank were netted, anesthetized with MS-222 (Tricane methane sulfonate, Western Chemical Company, Ferndale, Washington, USA), dried and gentle pressure was applied to the lower abdominal region to express fecal matter into a plastic weighing pan. Care was taken to exclude urinary excretions from the collection. Fecal samples for a given tank were freeze-dried, ground with a mortar and pestle, and stored at -20 ^{o}C until chemical analyses (described below) were performed. Apparent dietary digestibility coefficients of dry matter, protein and energy were calculated according to established equations (Kleiber, 1961; Forster, 1999):

$$\text{ADCNdiet} = 100 \times \frac{(\%\ \text{marker in diet} \times \%\ \text{nutrient in feces})}{(\%\ \text{marker in feces} \times \%\ \text{nutrient in diet})} \qquad (1)$$

Histological studies

Following the 12-week feeding trial, GIT samples were obtained from three

randomly selected fish per tank. Fish were euthanized and the digestive system was dissected to obtain the distal section of the GIT. Samples were preserved in Davidson's solution prior to histological evaluation. The distal intestine was examined using the longitudinal and cross sections for each fish. Histological cellular changes and alterations in tissues related to inflammation, supranuclear vacuolization in mucosal epithelial cells, increased thickness of the lamina propria or connective tissue, stratum compactum, number of mucus cells, degeneration and necrosis of mucosal epithelial cells, and numbers of rodlet were evaluated in each of the fish tissues following the standard histological procedures (Sheehan and Hrapchak, 1983). Scored of the Cellular changes observed ranges on a scale of 0 – 4, with 0 = no changes; 1 = minimal; 2 = mild; 3 = moderate; and 4 = marked. In general, scores of 1 and 2 are considered, histologically normal or cellular changes of no significance; 3 is transitional or intermediary, moderate cellular changes that may or may not be within the normal range depending on species, age, and sex of the fish; 4 is indicative of pathological lesions (Sheehan and Hrapchak, 1983).

Short chain fatty acids analysis

To better understand whether GIT short chain fatty acid levels were altered with increasing levels of GDDY, short chain fatty acid analysis was carried out to compare only samples from 0% GDDY and 75% GDDY diets. Samples from the proximal section of the GIT were collected from three randomly selected fish from each tank. The samples collected were rapidly placed in liquid nitrogen and stored at -20 °C until analysis. Organic acids of acetic acid, butyric acid, propionic acid, valeric acid, isobutyric acid, isovaleric

acids and 1, 3-butanediol (as the internal standard, IS) were purchased from Sigma Aldrich (Sigma-Aldrich, St Louis, MO USA) and were of HPLC grade with >99% purity. All the organic acids were at 0.5% (w/v) solution and 20 µl of each were individually mixed together. A 1 µl of 0.07% (v/v) mixture of the organic acids was directly injected into the gas chromatography mass spectrophotometer (Agilent Santa, Clara, CA) and the relative response factor (RRF) of each of the volatile organic acid was determined according to Yang et al. (2001) using Eq. (2).

$$RRF_{VOA} = \left(\frac{A_{VOA}}{A_{IS}}\right) / \left(\frac{W_{VOA}}{W_{IS}}\right) = \left(\frac{A_{VOA}}{W_{VOA}}\right) / \left(\frac{A_{IS}}{W_{IS}}\right) \quad (2)$$

where, A denotes peak area and W denotes weight in milligrams.

The recovery rate of each of the volatile organic acids in the fish samples was determined by mixing 200 µl of each of the GIT sample with 10 µl of 0.5% (w/v) of the IS. Three replicates were assayed per GIT sample and 1 µl of the mixture was directly was injected into the GCMS. The recovery rate was calculated for each of the volatile organic acids. Contents of volatile organic acid in the GIT sample was calculated using Eq. (3).

$$VOA\ \left(\frac{mg}{ml}\right) = \left(\frac{A_{VOA}}{A_{IS}}\right) \times \left(\frac{W_{IS}}{RRF_{VOA}}\right) \times \frac{1}{V} \quad (3)$$

where, V denotes the volume of GIT sample in milliliters, A denotes peak area and W denotes weight in milligrams.

DNA extraction and PCR amplification

Three fish were randomly selected from each of the tank and were euthanized by overdose of tricane methane sulfonate (MS-222; 200 mg/L water). Following euthanasia and within 10 minutes, fish were aseptically dissected and the midgut luminal content and mucosal scrape samples collected (Betiku et al., 2017). The samples were stored in sterilized tubes and snap frozen in liquid nitrogen. All samples were stored in -20 °C prior to DNA extraction. Extraction of total genomic DNA from samples collected from the midgut section of the GIT was carried out using MoBio PowerFecal DNA isolation kits (MoBio Laboratories, Inc., Solana Beach, CA) following manufacturer's protocols.

To better understand changes in the functional potential of the midgut microbiota in response to increasing levels of GDDY with corresponding poor feed utilization observed with fish fed the 75% GDDY diet, shotgun metagenomic sequencing was carried out to compare only samples from 0% GDDY and 75% GDDY diets. A total of 54 samples were sequenced as twelve luminal and twelve mucosal scrape samples. Extracted DNA was quantified using Qubit assay kit (Life Technologies) and quality checked with the BioTek microplate Readers and Spectrophotometers using the standard 260/280 absorbance ratios. Polymerase chain reaction was performed using the Illumina Nextera XT kit (Illumina, Inc., San Diego, CA) following the manufacturer's instructions. After tagmentation step, the Illumina i7 and i5 indexes were used to amplify DNA genome PCR success and quality were checked Epoch 2 microplate Spectrophotometer (BioTek, Winooski, VT). The Nextera XT PCR products were cleaned using the AMPure XP beads and quantified post cleaning. The pooled library was diluted to at 12.5 pM and sequenced

on an Illumina Miseq using 150 cycle v2 kits to obtain paired 75 nt reads. The sequences obtained were trimmed using fastq_quality_trimmer (http://hannonlab.cshl.edu/fastx_toolkit/license.html). This was followed by separation of paired and unpaired reads using customized Perl scripts. By using SOAPdenovo2, high quality reads were assembled de novo into contiguous sequences (Luo et al., 2012). A total of 337,198,603 reads were assembled into contigs (n50 ranged from 129-247 nt) and annotated in Metagenomics RAST server (Meyer et al., 2008).

Analytical methods

Dry matter and ash analyses of ingredients, diets and whole body samples were performed according to standard methods (AOAC, 1995). Crude protein (N x 6.25) was determined in ingredients, diets and feces by Dumas method (AOAC, 1995) on a Leco TruSpec N nitrogen determinator (LECO Corporation, St. Joseph, Michigan, USA). Total energy was determined by isoperibol bomb calorimetry (Parr 6300, Parr Instrument Company Inc., Moline, Illinois, USA). Lipid was determined by petroleum ether extraction using an Ankom XT10 (Ankom Technologies, Macedon, New York, USA).

Statistical analyses

Proc GLM of SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) was used for factorial ANOVA, while the Tukey's means separations were used to determine differences within main effects. Effects were considered significant at $P<0.05$. Taxonomic and functional data were compared between 0% GDDY and 75% GDDY diets, and

between luminal and mucosal samples collected from the midgut GIT section. Differences in species abundance and functional capacity between diets were determined using analysis of similarity (ANOSIM) and non- parametric multivariate ANOVA (Adonis) of the Vegan package (version 2.3-5, Department of Statistics, Iowa State University, Ames, IA, USA) in R v. 3.2.2 (R Development Core Team, 2006). Similarity percentage, SIMPER was used to determine the relative contributions of each species to average similarities of the diet groups. Microbial data were then corrected for multiple testing using the Benjamini-Hochberg False Discovery Rate (FDR) in SAS 9.4. ANOVA ($P<0.05$) p-values for the analysed variables were reported and were corrected for false discovery rate [FDR ($Q>0.05$)] resulting from multiple testing and the results are reported to guide future decision.

Results

Growth performance, body condition indices, proximate composition

During the trial, fish survival ranged from 98-100% with no differences among the treatment groups. There was no significant effect of prebiotics ($P=0.5704$) or GDDY inclusion level ($P=0.4965$) or significant interactions among the prebiotics and GDDY inclusion level ($P=0.2524$) on trout growth (Table 2). Weight gain was not different between dietary treatments ($P=0.4876$) and there was no significant effect of interaction ($P=0.4530$). In contrast, feed intake was increased with increasing level of GDDY ($P<0.0001$), but was not affected by prebiotics inclusion ($P=0.5783$). In addition, FCR significantly increased with increasing GDDY level ($P<0.0001$) but no significant effect

due to prebiotics (P=0.4397) was observed. No significant difference on VSI, HSI and FR with increasing GDDY levels, prebiotic supplementation or their interaction on the body indices of rainbow trout fed these dietary treatments (Table 2).

Whole-body lipid, energy and protein observed ranged from 12.9-13.3%, 2448-2536 kcal/g and 17.7-17.9%, respectively (Table 2). No significant effect of prebiotic supplementation, GDDY inclusion level or significant interaction on response variables analyzed was observed (Table 2).

Diet digestibility results

Analyzed proximate composition (g kg) of the experimental diets for crude protein, crude lipid, and gross energy were 492 g kg^{-1}, 194 g kg^{-1}, and 23.5 MJ kg^{-1} respectively for 0% GDDY; 463 g kg^{-1}, 193 g kg^{-1}, and 23.4 MJ kg^{-1} for 50% GDDY; 452 g kg^{-1}, 201 g kg^{-1}, and 23.6 MJ kg^{-1} for 75% GDDY diets and reflected formulation targets (Table 3). Apparent dry matter, crude lipid and protein digestibility coefficients (ADCs) were 78.5%, 91.3%, and 91.7%, respectively for the 0% GDDY; 68.8%, 88.4%, and 89.4% for 50% GDDY diet; and 71.7%, 87.6% and 90.1% for 75% GDDY diet. Inclusion of prebiotics did not alter digestibility for the dietary treatments (Table 3).

Histology

The findings observed in this study were consistent among fish from the same tank but varied between tanks consuming the same dietary treatment. Cellular changes observed in the fish, irrespective of diet, were within the normal range. There were no scores

indicative of pathological lesions that were specific to tanks or treatment groups. In general, specific inflammation and increased width of connective tissue were seen in fish fed 75% GDDY diet (Fig. 1a) compared to fish fed the 0% GDDY (fishmeal) diet (Fig. 1b), but there was no significant difference in tissue inflammation, vacuolation, mucus cells, D/N ME, thick CT, and thick LP in fish fed the dietary treatments (Table 4). However, there was significant effect of interaction (P=0.0028) between prebiotics and GGDY on rodlet cells.

Response of the GIT midgut microbes to diet and prebiotic

In total, reads belonging to 2,285 microbial species and encoding 4,565 unique, annotatable gene functions were identified. Good's estimates of coverage indicated that 98 ± 0.01% of the GIT microbial species and gene functions were captured. The trout GIT microbiota were mostly bacteria (52 ± 33%), but eukaryotes (12 ± 13%), viruses (18 ± 25%), and Archaea (0.01 ± 0.03%) were also detected. Overall, the taxonomy of 13% of sequences was unable to be inferred. These unassigned sequences were mainly observed in fish consuming the GDDY-based diet.

More microbial species were observed in the 75% GDDY diet (n=278 ± 491) than 0% GDDY diet (116 ± 155) and this reflected Jackknife estimates of total microbial diversity (R<0.05). ANOSIM and Permutational Multivariate Analysis of Variance (PERMANOVA) indicated a diet X mid-GIT sections effect on microbial β-diversity (ANOSIM R=0.27, Adonis P=0.001). However, microbial species in the GIT were not significantly affected by prebiotic inclusion (ANOSIM $R < 0.05$ $P > 0.1$; PERMANOVA

$P > 0.4$) alone. The most discriminating and significant phyla as identified by Similarity Percentage Analysis (SIMPER) were mostly Bacteria from the phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Spirochaetes*, *Tenericutes* and *Verrucomicrobia*. Significant increases in the Fungal phyla *Ascomycota* and *Basidiomycota*, were detected in the GDDY diets. Although dominant phyla were consistent across diets, their relative abundances changed between fishmeal-based diet (0% GDDY) and grain distiller-rich diets. Between the two dietary groups, a greater relative abundances of the phyla *Firmicutes*, *Bacteroidetes* and *Spirochaetes* was seen in the 0% GDDY diet, while *Proteobacteria*, *Ascomycota*, *Basidiomycota*, *Actinobacteria* and *Cnidaria* were enriched in the GDDY diets (Figs. 2a and b).

Amino acids metabolism is affected by protein source and differed by GIT locations

Shotgun metagenomic sequencing of samples of fish fed the 0% GDDY and 75% GDDY diets provided information from Pearson's correlation analysis between microbial population protein metabolism and some individual amino acids genes is presented in Fig. 3. The analysis showed that protein metabolism was positively correlated with protein biosynthesis; protein degradation; lysine, threonine, methionine and cysteine; alanine, serine and glycine; histidine metabolism; transcription; RNA processing and modification; protein folding; protein processing and modification; and selenoprotein genes (Fig. 3). By contrast, genes involved in proteolytic pathways were negatively correlated with all these aforementioned genes, except transcription. Between the two diets, there was a 1.5 to 2-fold increase in genes associated with BCAA biosynthesis (P=0.028), aromatic amino acids

(P=0.047), proteolytic pathway (P=0.0617), glycine biosynthesis (P=0.0173), thiamine biosynthesis (P=0.055), lipid-derived mediator (P=0.0072), coenzyme A (P=0.0071), urea decomposition (P=0.0457), and n-phenylalkanoic (P=0.0132) in 75% GDDY diet (Fig. 4). In addition, isoleucine degradation gene (P=0.0085) was increased by 2-fold in same diet compared to fishmeal-based diet.

Genes involved in amino acid degradation had a greater relative abundance among mucosally-located microbes than those of the lumen. The relative abundance of genes related to BCAA (P=0.0299) and aromatic amino acids (P=0.0094) did not vary in luminal samples, but were approximately 2-fold greater in mucosa samples of fish consuming the 75% GDDY diet. Similarly, there was a 2-fold greater relative abundance of genes involved in isoleucine degradation (P=0.0184) in mucosal samples of fish fed the 75% GDDY diet, compared to those fed the 0% GDDY diet. The proteolytic pathway, which comprises genes related to regulatory inter-membrane proteolysis pathway (P<0.0001), had a lower relative abundance in luminal samples than observed in mucosal samples of fish consuming the 75% GDDY diet, while the reverse was observed in the microbes of fish fed 0% GDDY diet. In contrast, an interaction of diet x mid-GIT sections effect for two-related proteases gene were lower in the luminal microbes of fish fed 75% GDDY, but had a 2-fold greater relative abundance in the mucosal samples of fish fed same diet; coenzyme A gene (P=0.0002) followed same trend in same diet. Also, an interaction effect for lipid-derived mediator (P=0.0003) was increased by 2-fold in the luminal microbes of fish fed 0% GDDY diet, while for fish fed 75% GDDY, microbes showed a drastic reduction for the same gene. Furthermore, there was about 2-fold increase in GDH (P=0.0192) gene in both

the luminal and mucosa microbes of fish fed 75% GDDY diet, while a significantly low level of this gene was observed in the GIT locations of fish fed 0% GDDY diet. There was a 2-fold increase in the fish fed 75% GDDY diet of genes associated with fermentation (P=0.0466), iron acquisition metabolism (P=0.0087), membrane transport (P=0.0001), aspartate aminotransferase (P<0.0001), translation (P=0.0017), murein hydrolases (P=0.0039), fructoooligosaccharides and raffinose utilization (P=0.0001), heme uptake and utilization system (P<0.0001), creatine and creatinine degradation (P=00204), manganese transport P=0.0029), ABC transport of BCAA (P=0.0047), threonine anaerobic catabolism gene cluster, and nitrate and nitrite ammonification gene, but reverse effect was observed in these genes from the fish fed 0% GDDY diet.

Short chain fatty acids

Acetic (P=0.1994), propionic (P=0.8037), butyric (P=0.6268), valeric (P=0.5877) and isovaleric (P=0.5919) concentrations were not significantly different between the treatments. Similarly, prebiotics supplementation did not significantly affect short chain fatty acid levels of acetic (P=0.5117), propionic (P=0.9608), butyric (P=0.5617), valeric (P=0.6549) and isovaleric (P=0.4064). In addition, no significant interaction effects of GDDY inclusion level and prebiotic supplementation were observed for acetic (P=0.0637), propionic (P=0.3680), butyric (P=0.5168), valeric (P=0.5381), and isovaleric (P=0.5778) acids (Fig. 5).

Discussion

The use of alternative ingredients as protein sources in rainbow trout diets has received considerable attention (Gomes et al., 1995; Kaushik et al., 1995; Lanari et al., 1998; Gaylord et al., 2007). Consistent and confounding effects of alternative diets in trout include increased feed intake and growth retardation when high quantities of certain ingredients are used in replacing fishmeal (Gomes et al., 1995; Adelizi et al., 1998; Cheng et al., 2003; de Francesco et al., 2004; Burr et al., 2012; Hauptman et al., 2014a; Hauptman et al., 2014b; Sealey et al., 2015b). However, significant improvements have been made on utilization of alternative protein diets when additional crystalline amino acids are added to balance dietary amino acid profiles to meet the tissue requirements of the fish (Davies and Morris, 1997; Gaylord et al., 2007; Gaylord and Barrows, 2009). The present study evaluated growth performance and GIT microbiome of rainbow trout fed yeast-based diets (75% GDDY) supplemented with free amino acids and compared with a conventional fishmeal-based diet (0% GDDY). The feed ingredients incorporated in the present study were the same as those used by Hauptman et al. (2014a; 2014b), but at different inclusion levels. In the earlier studies, increased levels of GDDY significantly reduced growth of trout and affected the feed intake (Hauptman et al., 2014a; Hauptman et al., 2014b). However, in the present study, there were no significant effect of fishmeal replacement with high levels of GDDY up to 75%. This observation agrees with the findings and suggestion by Gause and Trushenski (2011) that GDDY could replace up to 75% of protein from fishmeal in Sunshine Bass. Correspondingly, no positive effects on growth efficiency was observed when MOS and GroA were included in the GDDY diets or the fishmeal-

based diet. The amino acid profile of the GDDY diets used in the present study were expected to meet the nutrient requirements of rainbow trout because the diets were formulated using the digestible amino acid composition of the GDDY protein. Moreover, since no negative effect resulted from the diet on the absorptive capacity of rainbow trout, based on histological observations and the comparable growth performance between fishmeal- and yeast-based proteins, GDDY has potential as a protein source in rainbow trout, which can replace fishmeal protein up to 75%. It is important to note that nucleic acid-based nitrogen is a substantial component of the yeast-based protein from *S. cerevisiae* used in this study (12-20%) and understanding the role of nucleic acids to rainbow trout nutrition is still in its infancy (Li and Gatlin, 2006). Our findings agree with earlier reports indicating that nucleic acids are well utilized by rainbow trout (Rumsey et al., 1991; Rumsey et al., 1992), but further work is necessary to completely understand nucleic acid metabolism in fishes. The ability of yeast to act as an immuno-stimulant has been observed in cultured-fishes (Burrells et al., 2001; Ortuño et al., 2002; Leonardi et al., 2003; Li and Gatlin, 2004). Feeding GDDY, a yeast-based protein from *S. cerevisiae* in this study enhanced the modulation of the colonic microbes for proliferation of beneficial microbiota. Specifically, 2-fold increase in fermentation genes were observed in fish fed the 75% GDDY diet and supports the ability of *S. cerevisiae* to aid fermentation for fast energy production (Otterstedt et al., 2004; Merico et al., 2007). In addition, greater relative abundances of genes associated with iron uptake and ABC transporters observed in the fish fed 75% GDDY diet may reflect increased fungal and bacterial capacity to access host iron, which plays a role in the innate immune system (Caza and Kronstad, 2013).

Greater feed intake and lower feed efficiency observed in fish fed increased levels of GDDY, as observed in this study, are in line with what have been previously reported (de Francesco et al., 2004; Hauptman et al., 2014a; Hauptman et al., 2014b; Sealey et al., 2015b). Differences in nitrogen metabolism or appetite stimulation due to the addition of crystalline amino acids from the diets may be the culprit for the increased feed intake and poor feed efficiency associated with this ingredient (Martin et al., 2003; Snyder et al., 2012). However, we rule out hyperphagic effect from amino acids supplementation on the GDDY diets since there was no such effect observed in fish fed the fishmeal-based diet.

Midgut SCFA concentrations did not vary, indicating microbial fermentation was not altered by dietary treatments. Since the concentrations of acetic, propionic, butyric, isobutyric, valeric and isovaleric acids were not different in the trout fed either fishmeal or GDDY-based diets, it is unlikely that the poor feed utilization observed in the GDDY diet was because of microbial fermentation. Hence, we sought another mechanistic explanation for the reduced feed utilization in trout.

From the metagenomics result, we hypothesize that increased amino acid catabolism resulting from genes related to GDH cluster affected the efficient utilization of the absorbed nutrients. Due to the complexity of the amino acids pathway, more studies are needed to identify the contribution of other catabolic enzymes to amino acids catabolism and how environmental factors that are important in aquaculture, influence these processes to trigger changes in dietary amino acids utilization. In the present study, we employed the knowledge of microbial gene functions to investigate amino acids metabolism pathways in rainbow trout fed GDDY-based protein.

Protein metabolism in rainbow trout involves dietary protein degradation and catabolism of amino acids in both liver and the small GIT tissues for synthesis of new proteins as well as other metabolites (Windmueller, 1982). In this study, greater numbers of gene clusters related to BCAA, aspartate amino transferase (AAT) and GDH activities in fish fed yeast-protein diet were observed, while the relative abundance of genes related to proteolysis decreased in the same fish. This observation is corroborated by earlier findings in that increased GDH and AAT enzyme activities are indicative of metabolic changes in pathways involved in the dietary utilization of alternative proteins in rainbow trout (Martin et al., 2003; Wacyk et al., 2012). Taken together, results of this study suggest catabolism of L-amino acids by AAT to glutamate increased the activity of GDH, a distinct adaptive response of amino acids deaminase enzyme to dietary protein levels (Cowley and Schmidt, 1995). This catabolic enzyme is up-regulated in microbes of fish consuming the GDDY-based diet, possibly increasing hepatic activity in the microbes for effecting the disposal of resulting amino acids from the GDDY-based diet via transdeamination. Although the contribution of nucleic acids to amino acids pool is not yet defined, we suspect an amino acid imbalance possibly from the nucleic acid content of the GDDY-based diet, which increased concentrations of GDH in fish fed this yeast diet compared to the fishmeal-based diet.

Another possible explanation is that inhibitory feedback mechanism if higher absorption rate of dietary glutamate from the yeast diet is responsible for the increased GDH enzyme activity observed in the microbial genes of fish fed this diet is should be further investigated. Similar changes in metabolic enzymes have been reported in rainbow

trout fed dietary nucleotides (Keyvanshokooh and Tahmasebi-Kohyani, 2012). One explanation for this observation may be related to different time of appearance of essential amino acids and non-essential amino acids (Calbet and MacLean, 2002), an observation that is associated with either a plant protein diet (Larsen et al., 2012) or fast absorption of free amino acids (Schuhmacher et al., 1997) in rainbow trout. Although these observations require further investigation and special attention on the BCAAs concentration is required to determine their role in this cascade of reactions involving GDH in rainbow trout, especially when alternative protein diets are used. Increased degradation of isoleucine cluster in the GIT microbes is of great interest, but we cannot ascertain why isoleucine is degraded in this study. A correlation between increased concentrations of circulating BCAA (Snyder et al., 2012) or glutamate (Burrin and Stoll, 2009) and overeating have been suggested. Possible explanations could be signals related to feed intake in the central nervous system that is regulated by changes in the concentration of circulating free amino acids (Leung and Rogers, 1973). This observation also requires further investigation.

Differences in feed utilization related to amino acid catabolism was seen in rainbow trout fed yeast-based diet. Greater numbers of microbial genes related to glutamate dehydrogenase observed in fish fed the yeast-based diet suggest microbes were competing for dietary amino acids resulting in poor utilization of high levels of GDDY in rainbow trout. The importance of the catabolic products to the host and the GIT microbiota is yet to be fully understood from the current study and should be investigated in future studies.

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Figure Legends

Fig. 1. Distal intestine of rainbow trout. (a) Trout fed 75% GDDY-based diet, (b) Trout fed 0% GDDY diet (fishmeal protein). LP = Lamina Propria.

Fig. 2a. Relative abundance of microbial populations in the mid-GIT of trout fed fishmeal and GDDY-based diets: 0% GDDY: Fishmeal-based diet; 75% GDDY.

Fig. 2b. Relative abundance of microbial populations in the mid-GIT of trout fed fishmeal and GDDY-based diets: 0% GDDY: Fishmeal-based diet; 75% GDDY with prebiotics inclusion.

Fig. 3. Correlation plot of microbial genes related to protein metabolism from mid-GIT of rainbow trout. A: Protein metabolism; B: Lysine, threonine metabolism; C: Alanine, Serine; D: Protein degradation; E: Protein biosynthesis; F: Proteolytic pathway; G:

Transcription; H: Protein folding; I: Protein processing; J: Selenoprotein; K: RNA processing; L: Histidine metabolism.

Fig. 4. Fold increase of microbial gene functions affected by protein sources in rainbow trout. BCCA=Branched chain amino acid, Cys Biosynthesis=Cysteine Biosynthesis, Proteolytic path=Proteolytic pathway, GDH=Glutamate Dehydrogenase, Ile degradation=Isoleucine degradation, Urea decomp=Urea decomposition, Pentose P pathway=Pentose phosphate pathway, Asp aminotrans=Aspartate aminotransferase, Thiamine biosyn=Thiamine biosynthesis, Aromatic AA=Aromatic amino acids, Lysine Biosyn=Lysine Biosynthesis.

Fig. 5. Concentration of organic acids from mid-GIT samples of rainbow trout fed either 0% GDDY diet (control) or 75% GDDY-based diet. Means are from three replicate samples.

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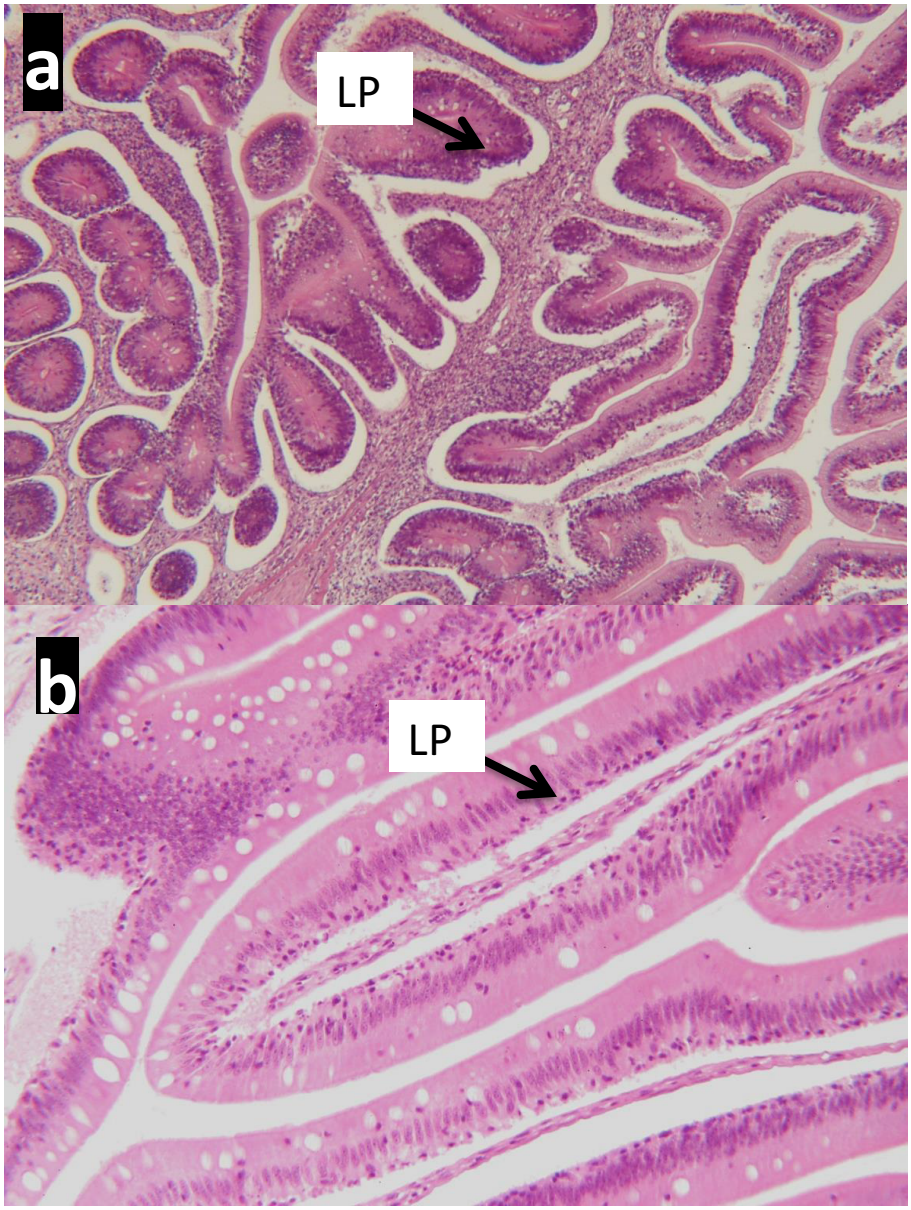


Fig. 1.

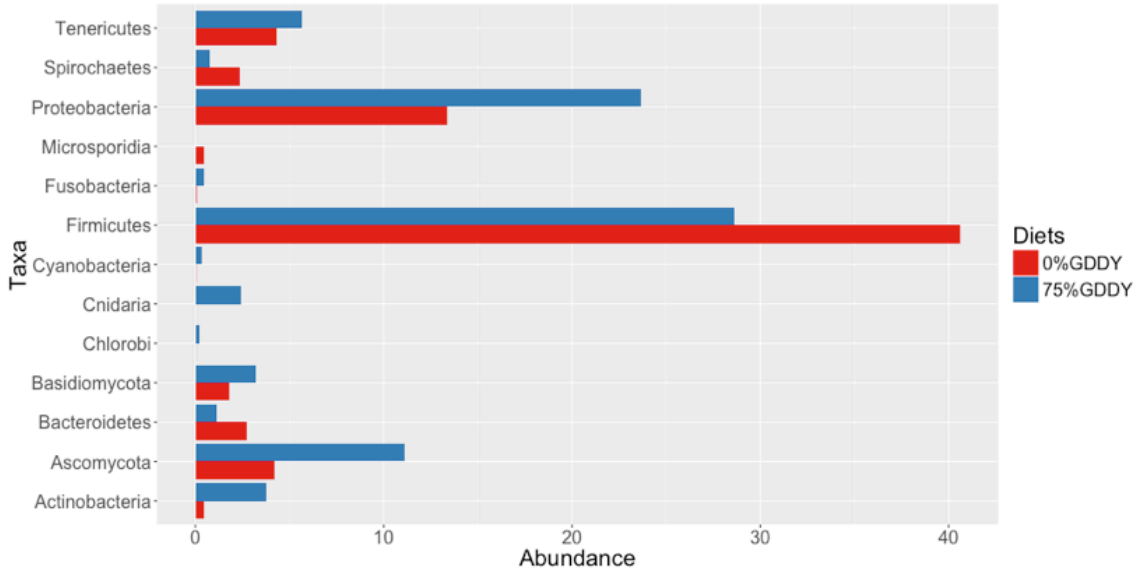

Tenericutes
Spirochaetes
Proteobacteria
Microsporidia
Fusobacteria
Firmicutes
Cyanobacteria
Cnidaria
Chlorobi
Basidiomycota
Bacteroidetes
Ascomycota
Actinobacteria
Taxa
0
10
20
30
40
Abundance
Diets
0%GDDY
75%GDDY

Fig. 2a

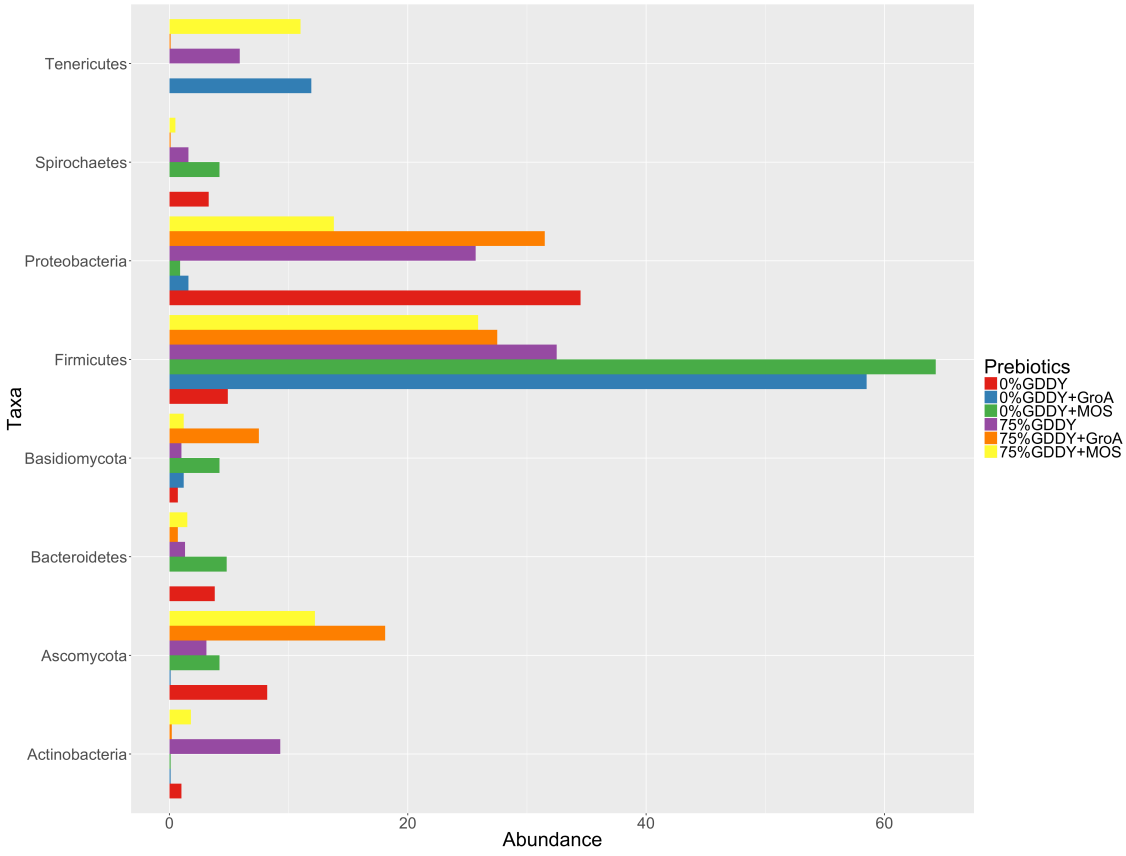
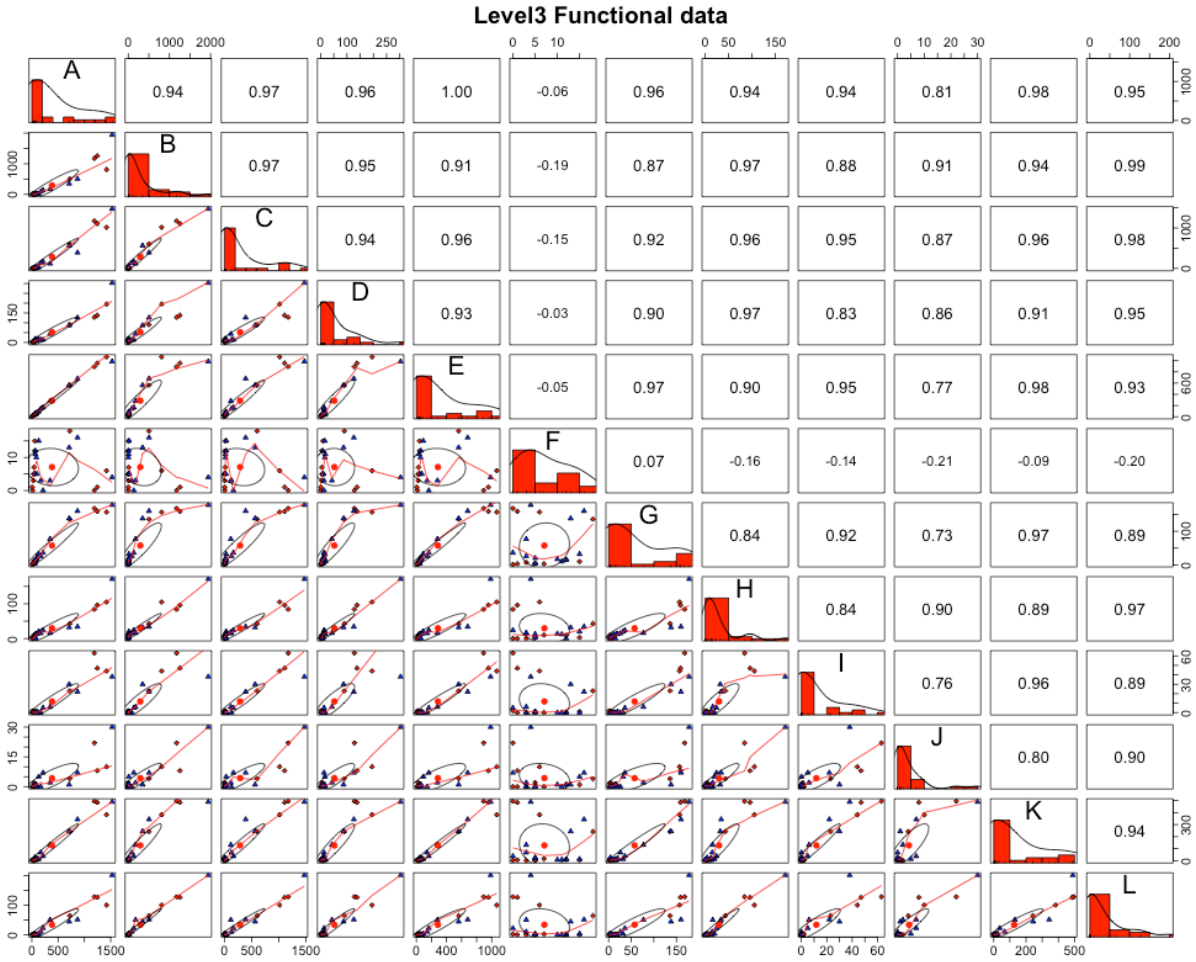

Tenericutes
Spirochaetes
Proteobacteria
Firmicutes
Basidiomycota
Bacteroidetes
Ascomycota
Actinobacteria
Taxa
0
20
40
60
Abundance
Prebiotics
0%GDDY
0%GDDY+GroA
0%GDDY+MOS
75%GDDY
75%GDDY+GroA
75%GDDY+MOS

Fig. 2b


Level3 Functional data
A
B
C
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E
F
G
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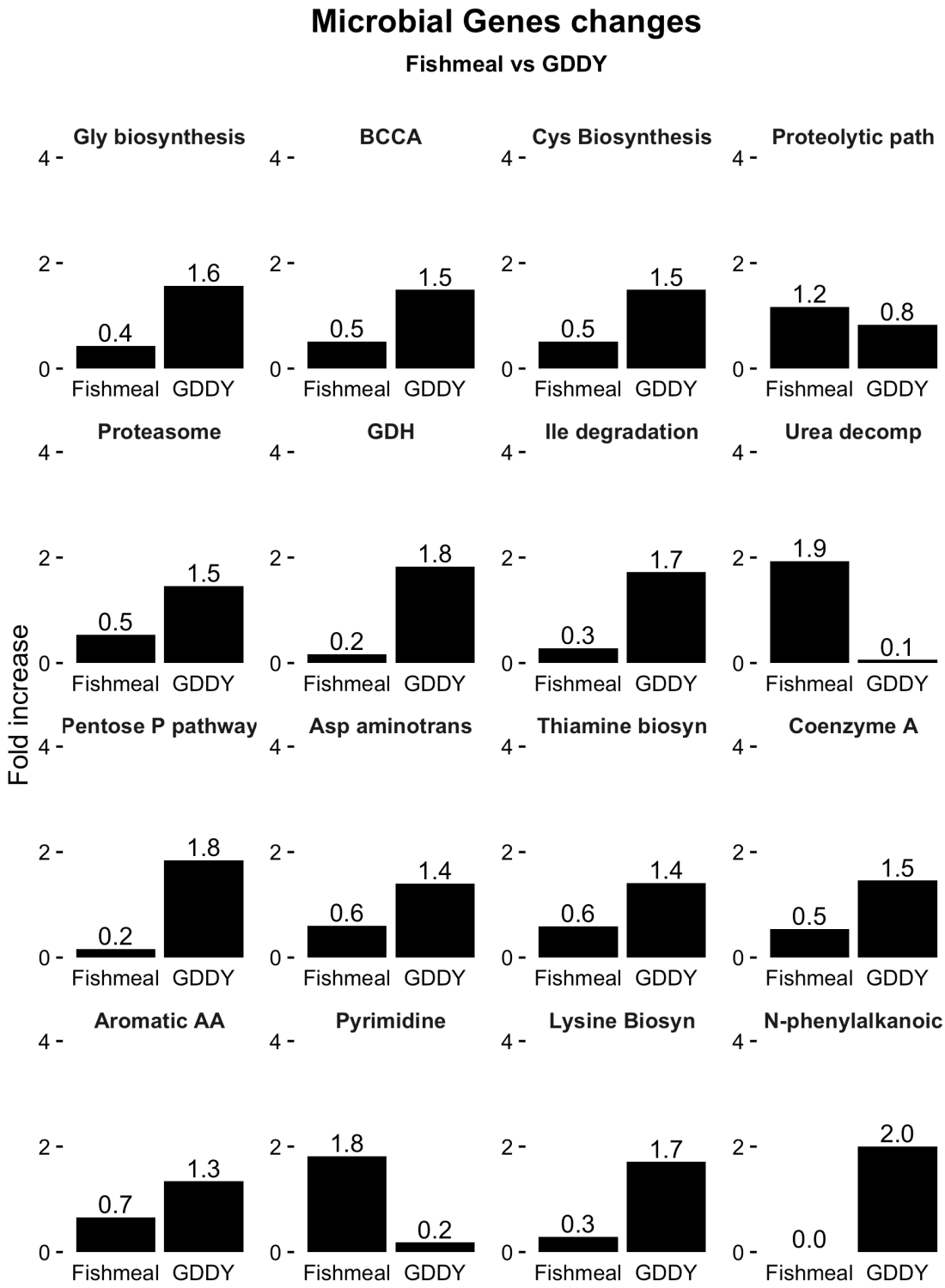
I
J
K
L
0.94 0.97 0.96 1.00 -0.06 0.96 0.94 0.94 0.81 0.98 0.95
0.97 0.95 0.91 -0.19 0.87 0.97 0.88 0.91 0.94 0.99
0.94 0.96 -0.15 0.92 0.96 0.95 0.87 0.96 0.98
0.93 -0.03 0.90 0.97 0.83 0.86 0.91 0.95
-0.05 0.97 0.90 0.95 0.77 0.98 0.93
0.07 -0.16 -0.14 -0.21 -0.09 -0.20
0.84 0.92 0.73 0.97 0.89
0.84 0.90 0.89 0.97
0.76 0.96 0.89
0.80 0.90
0.94

Fig. 3.


Microbial Genes changes
Fishmeal vs GDDY
Fold increase
Gly biosynthesis
0.4
1.6
BCCA
0.5
1.5
Cys Biosynthesis
0.5
1.5
Proteolytic path
1.2
0.8
Proteasome
0.5
1.5
GDH
0.2
1.8
Ile degradation
0.3
1.7
Urea decomp
1.9
0.1
Pentose P pathway
0.2
1.8
Asp aminotrans
0.6
1.4
Thiamine biosyn
0.6
1.4
Coenzyme A
0.5
1.5
Aromatic AA
0.7
1.3
Pyrimidine
1.8
0.2
Lysine Biosyn
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1.7
N-phenylalkanoic
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2.0
Fishmeal
GDDY
0
2
4

Fig. 4.

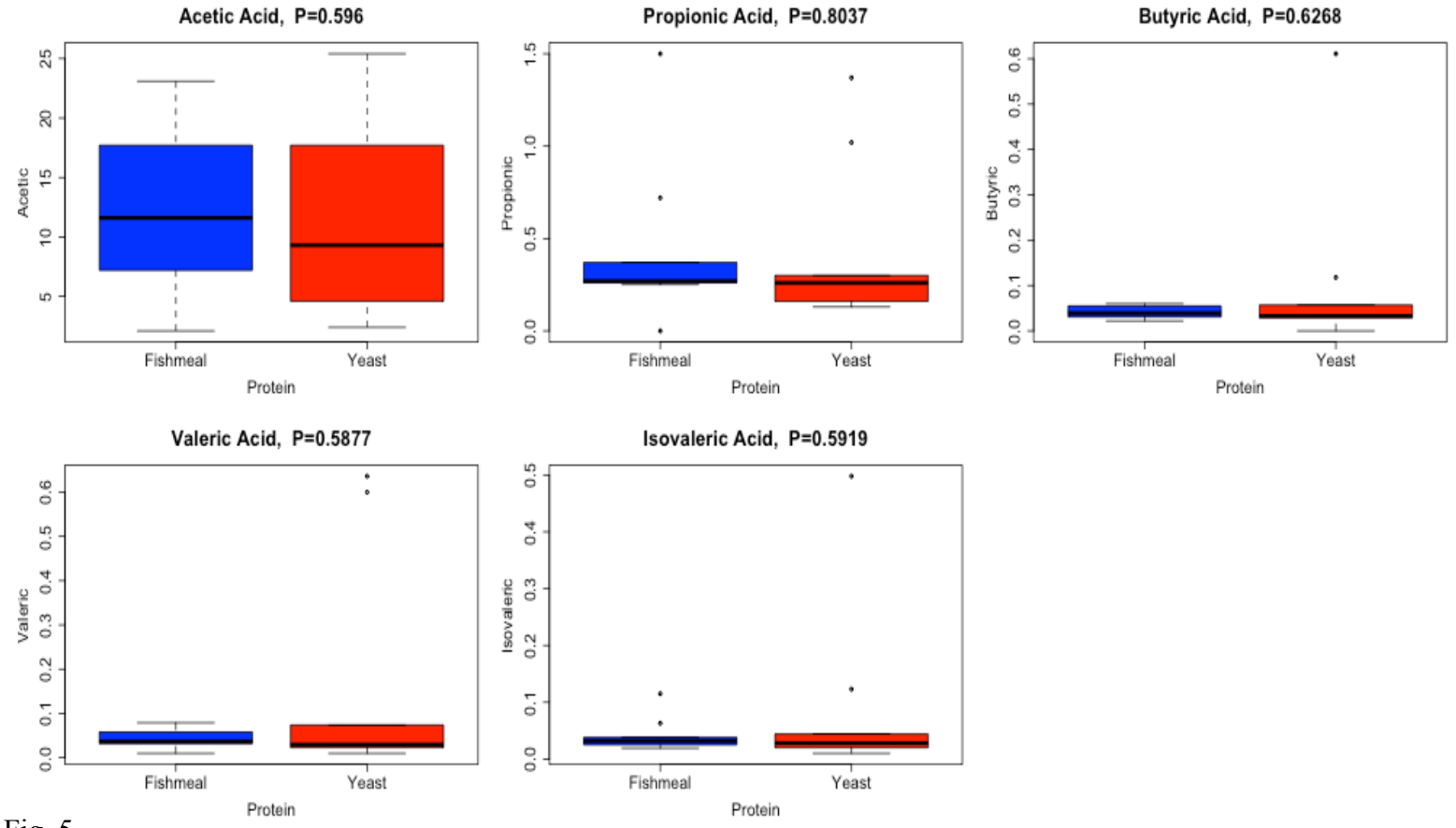
Acetic Acid, P=0.596
Acetic
Fishmeal
Yeast
Protein
Propionic Acid, P=0.8037
Propionic
Fishmeal
Yeast
Protein
Butyric Acid, P=0.6268
Butyric
Fishmeal
Yeast
Protein
Valeric Acid, P=0.5877
Valeric
Fishmeal
Yeast
Protein
Isovaleric Acid, P=0.5919
Isovaleric
Fishmeal
Yeast
Protein

Fig. 5.

Table 1: Ingredients compositions of dietary treatments

Ingredient	Diets[1]											
	0% GDDY				50% GDDY				75% GDDY			
	0%	0.4% MOS	1% MOS	1% GroA	0%	0.4% MOS	1% MOS	1% GroA	0%	0.4% MOS	1% MOS	1% GroA
Grain distiller's dried yeast[2]	0	0	0	0	16	16	16	16	26.5	26.5	26.5	26.5
Menhaden fish meal[3]	25	25	25	25	12.5	12.5	12.5	12.5	6.25	6.25	6.25	6.25
Grobiotic A[4]	0	0	0	1	0	0	0	1	0	0	0	1
MOS[5]	0	0.4	1	0	0	0.4	1	0	0	0.4	1	0
Corn protein concentrate[6]	6.22	6.22	6.22	6.22	6.22	6.22	6.22	6.22	4.50	4.70	4.50	4.50
Soybean Meal 48% CP[3]	5	5	5	5	5	5	5	5	5	5	5	5
Soy protein concentrate[3]	5	5	5	5	5	5	5	5	5	5	5	5
Blood meal US[7]	4.85	4.85	4.85	4.85	4.85	4.85	4.85	4.85	4.85	4.85	4.85	4.85
Poultry by-product, pet food[7]	4	4	4	4	4	4	4	4	4	4	4	4
Wheat flour[7]	19.4	19	18.4	18.4	16.15	15.75	15.14	15.14	14.24	13.66	13.24	13.24
Menhaden fish oil[3]	18.03	18.03	18.03	18.03	18	18	18	18	17.6	17.6	17.6	17.6
Lecithin	1	1	1	1	1	1	1	1	1	1	1	1
Stay-C 35	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Vitamin premix ARS 702[8]	1	1	1	1	1	1	1	1	1	1	1	1

TM ARS 640[9]	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
NaCl	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28
Magnesium oxide	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
Potassium chloride	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56
Monocalcium phosphate	4.2	4.2	4.2	4.2	3.4	3.4	3.4	3.4	2.9	2.9	2.9	2.9
Choline Cl 50%	1	1	1	1	1	1	1	1	1	1	1	1
DL-Methionine	0.54	0.54	0.54	0.54	0.63	0.63	0.63	0.63	0.68	0.67	0.68	0.68
Lysine HCl	2.19	2.19	2.19	2.19	2.6	2.6	2.6	2.6	2.77	2.77	2.77	2.77
Threonine	0.74	0.74	0.74	0.74	0.82	0.82	0.83	0.83	0.88	0.87	0.88	0.88
Taurine	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Yttrium oxide	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Astaxanthin	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08

[1] Percent of fishmeal replaced by grain distiller's dried yeast on a digestible protein basis.
[2] Archer Daniels Midland (Decatur, IL, USA).
[3] Nelson & Sons Inc. (Murray, UT, USA).
[4] International Ingredient
[5] Alltech, USA.
[6] Gavilon LLC, (Omaha, NE, USA).
[7] MGP Ingredients, Inc. (Atchison, KS, USA).
[8] Contributed per kg of diet: vitamin A (as retinol palmitate), 30,000 IU; vitamin D_3, 2160 IU; vitamin E (as DL-%-tocopheryl-acetate), 1590 IU; niacin, 990 mg; calcium pantothenate, 480 mg; riboflavin, 240 mg; thiamin mononitrate, 150 mg; pyridoxine hydrochloride, 135 mg; menadione sodium bisulfate, 75 mg; folacin, 39 mg; biotin, 3 mg; vitamin B_{12}, 90 µg.
[9] Contributed in mg/kg of diet: zinc, 37; manganese, 10; iodine, 5; copper, 3; selenium, 0.4

Table 2: Production performance metrics, body condition indices and proximate composition of rainbow trout fed yeast-based protein

Diet	Weight gain[3] (% increase)	Feed intake[4] (% bw/d)	FCR[5] (g feed/g gain)	Visceral index[6] (%)	Fillet ratio[7] (%)	Hepatosomatic Index[8]	Body fat (%)	Body energy (cal/g)	Body protein (%)
0% GDDY+0% Pre	3208.1	1.9[c]	0.8[c]	32.3	63.7	2.6	13.0	2394.9	17.7
0%GDDY +0.4%MOS	3262.6	2.0[c]	0.8[c]	34.0	63.5	2.9	13.0	2443.6	17.5
0%GDDY +1%MOS	3172.7	1.9[c]	0.8[c]	28.5	63.4	2.6	13.4	2470.5	18.0
0%GDDY +1%GroA	3158.7	1.8[c]	0.7[c]	29.4	62.6	2.6	13.0	2481.9	18.5
50%GDDY+0% Pre	2986.7	2.0[b]	0.8[b]	26.5	57.1	2.5	12.4	2368.3	18.0
50%GDDY+0.4%MOS	3116.3	2.0[b]	0.8[b]	34.9	62.5	2.7	12.6	2405.1	18.4
50%GDDY +1%MOS	3009.8	2.0[b]	0.8[b]	32.8	64.7	3.0	13.4	2534.9	18.0
50% GDDY +1%GroA	3120.7	2.0[b]	0.8[b]	34.1	53.0	2.6	13.4	2404.7	17.8
75%GDDY+0% Pre	2973.7	2.3[a]	0.9[a]	35.2	63.1	2.8	12.9	2533.9	17.9
75%GDDY+0.4%MOS	2795.4	2.3 [a]	1.0[a]	32.6	55.4	2.5	13.4	2583.8	17.3
75%GDDY+1%MOS	3192.7	2.2 [a]	0.9[a]	32.3	58.7	2.8	13.1	2519.8	17.9
75%GDDY+1%GroA	3413.7	2.3[a]	0.9[a]	38.4	63.5	3.0	12.3	2461.5	17.9
Pooled SEM	172.2	0.06	0.03	3.31	4.53	0.21	0.48	106.5	0.37
Main effect means									
GDDY	3117.6	2.1	0.8	32.5	60.9	2.7	12.9	2466.9	17.9
Prebiotic	3118	2.1	0.8	32.6	60.9	2.7	13	2466.9	17.9
ANOVA, Pr > F[2]	0.5858	<0.0001	<0.0001	0.5113	0.6960	0.7861	0.7597	0.9452	0.6145

[1] Initial tank weights were 15.7 ± 0.4 g with no significant differences between tanks, for all analyses means of three replicate tanks were presented (20 fish/tank).

[2] Mean determinations of nine fish per treatment; three replicate tanks per diet.

[3] Weight gain (%) = (final weight – initial weight)*100 / initial weight.

[4] Feed intake (%) = g dry feed consumed/average fish biomass (g) /culture days *100.

[5] FCR, feed conversion ratio = g dry feed consumed / g weight gained.[4] Weight gain (%) = (final weight – initial weight)*100 / initial weight.

[6] Visceral somatic index (%) = viscera mass x 100 / fish mass

[7] Fillet ratio (%) = fillet with rib mass * 100 / fish mass.

[8] Hepatosomatic index (%) = liver mass x 100 / fish mass.

Table 3: Diet proximate compositions and apparent digestibility coefficient[1]

	Analyzed proximate composition			Apparent digestibility coefficient		
Diet	Crude Protein (N*6.25)	Fat (g/kg)	Gross Energy (MJ/kg)	DM*	Fat*	Protein*
0%GDDY	492	194	23.5	78.5^{a}	91.3^{ab}	91.7^{abc}
0%GDDY +0.4%MOS	495	210	23.4	76.6^{abc}	92.5^{a}	91.4^{abc}
0%GDDY +1%MOS	488	198	23.4	76.0^{b}	90.0^{abcd}	91.9^{ab}
0%GDDY +1%GroA	489	184	23.3	77.3^{a}	92.1^{abc}	92.1^{a}
50%GDDY	463	193	23.4	68.8^{bcd}	88.4^{defg}	89.4^{cd}
50%GDDY+0.4%MOS	461	203	23.7	68.0^{cd}	89.7^{bced}	88.8^{d}
50%GDDY +1%MOS	460	198	23.5	69.0^{d}	87.9^{efg}	89.4^{d}
50% GDDY +1%GroA	458	195	23.1	71.3^{cd}	89.2^{cdef}	90.3^{cd}
75%GDDY	452	201	23.6	71.7^{d}	87.6^{fgh}	90.1^{d}
75%GDDY+0.4%MOS	451	200	23.6	68.8^{cd}	85.5^{efg}	89.9^{bcd}
75%GDDY+1%MOS	454	176	23.6	70.3^{cd}	86.2^{gh}	90.3^{abcd}
75%GDDY+1%GroA	459	183	23.6	66.9^{d}	84.4^{h}	88.7^{d}

[1] Means of duplicate analyses on a dry matter basis (mg g^{-1} sample). *Pr>F for DM (<0.0001), Protein (<0.0001), Fat (<0.0001)

Table 4: Histological data of rainbow trout fed the dietary treatments

Parameter	0% GDDY				50% GDDY				75% GDDY				P>F[2]
	0%	0.4% MOS	1% MOS	1% GroA	0%	0.4% MOS	1% MOS	1% GroA	0%	0.4% MOS	1% MOS	1% GroA	
Inflammation	2.25	1.80	2.60	1.75	2.25	1.80	1.20	1.50	2.25	1.40	2.40	2.25	0.5477
Vacuolation	1.50	2.20	2.40	2.00	1.75	1.25	2.20	1.75	1.50	1.80	2.00	2.25	0.4276
Mucus	2.50	2.80	2.60	2.67	2.25	2.25	2.40	2.50	2.75	2.60	3.00	2.75	0.6952
Cells	2.25	2.00	1.80	1.67	1.50	1.75	1.60	1.75	1.75	2.00	2.40	2.00	0.6497
DN	1.50	1.20	1.60	1.33	1.25	1.00	0.60	1.25	2.00	1.00	1.60	2.00	0.7446
Thickct	1.25	0.80	1.00	0.67	0.75	0.50	0.60	0.75	1.50	0.60	1.40	1.75	0.2003
Thicklp	1.25	2.40	1.20	1.67	1.50	1.00	1.60	2.00	1.50	2.0	1.80	1.50	0.2289
Rodletcells	27.00	18.00	20.20	30.50	26.25	23.40	20.40	22.75	23.25	18.20	22.00	25.00	0.9789

S1 – Supplementary material

Microbial genes expressed were mostly predicted to be involved in amino acid (14 ± 4%) and protein metabolism (9 ± 4%) as well as carbohydrate (17 ± 3%) utilization (Fig. S1). Other gene functions, including cofactors and vitamins (5 ± 2%), RNA metabolism (4 ± 2%), DNA metabolism (3 ± 1%), nitrogen (0.3 ± 0.5%), and sulfur metabolism (0.2 ± 0.3%) were also abundant in all samples. Replacing fishmeal with 75% GDDY led to a 1.5 to 2-fold increase in genes associated with BCAA biosynthesis (P=0.028), aromatic amino acid metabolism (P=0.047), glycine biosynthesis (P=0.0173), thiamine biosynthesis (P=0.055); and in the mucosa, membrane transport (P=0.001), fermentation (P=0.0009), coenzyme A (P=0.0071) in 75% GDDY diet. But gene related to pyrimidine utilization (0.0871), lipid-derived mediator (P=0.0072) and oxidative stress (P=0.0023) reduced in same diet. While a 1.5 to 2-fold increase were observed in fishmeal-based diet (0% GDDY), except for fermentation-related gene, which was significantly higher in 75% GDDY diet compared to the 0% GDDY diet, but it was not significant.

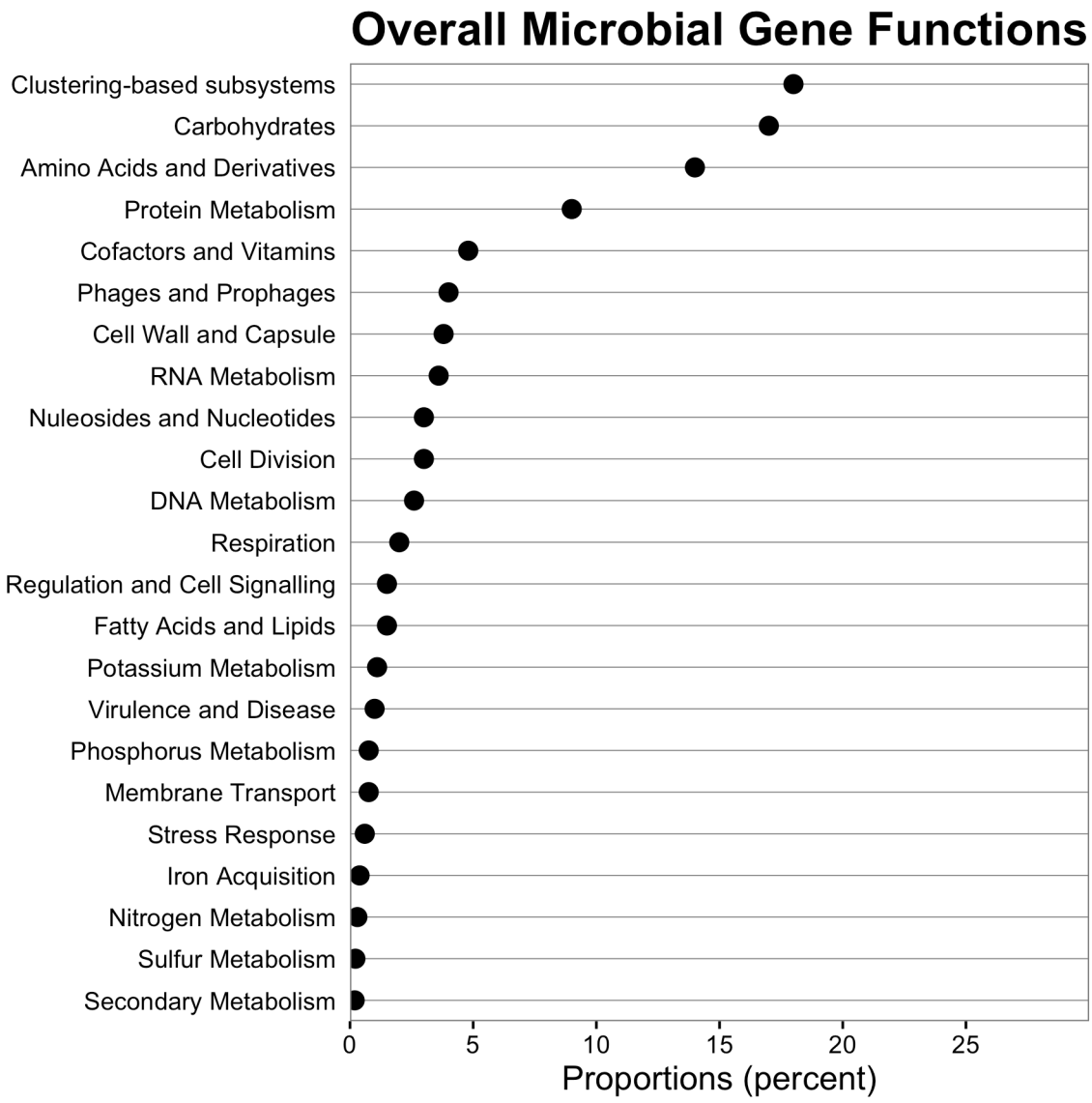

Fig. S1. Overall functional profile of rainbow trout mid-GIT fed the dietary treatments.

CHAPTER SIX

WATER SYSTEM IS A CONTROLLING VARIABLE MODULATING BACTERIAL DIVERSITY OF GASTROINTESTINAL TRACT AND PERFORMANCE IN RAINBOW TROUT

Contribution of Authors and Co-Authors

Manuscript in Chapter 6

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Contributions: Designed the experiment and formulated feed. Performed the experiments and collected samples. Carried out laboratory analyses, data analyses and interpretation. Wrote manuscript with input from co-authors.

Co-Author: Carl J. Yeoman
Contributions: Involved in the design of the experiments. Provided guidance on metagenomics analysis. Assisted in the preparation of the manuscript.

Co-Author: T. Gibson Gaylord
Contributions: Involved in the design of the experiments and analysis of data. Assisted in the preparation of the manuscript.

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Co-Author: Glenn C. Duff
Contributions: Assisted in preparation of manuscript.

Co-Author: Wendy M. Sealey
Contributions: Involved in the design of the experiments, provided guidance on fish culture and assisted in both data analysis and manuscript preparation.

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Water system is a controlling variable modulating bacterial diversity of gastrointestinal tract and performance in rainbow trout

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Abstract

A two-phase feeding study evaluating the performance of rainbow trout and comparing their luminal and mucosal gastrointestinal tract (GIT) bacterial community compositions when fed two alternative protein diets in two rearing systems was carried out. Alternative protein diets included animal protein and plant protein diets balanced with crystalline amino acids or unbalanced were fed to rainbow trout in two separate water systems (recirculating (RR) and flow-through (FF)) for a period of 16 weeks. The four diets each

contained 38% digestible protein and 20% fats were fed to rainbow trout with an average weight of 12.02 ± 0.61 g, and sorted at 30 fish/tank and 12 tanks per dietary treatment. Phase1 lasted for 8 weeks after which fish from each tank were randomly divided, with one-half moved to new tanks of the opposing system (i.e. from RR to FF and vice versa). The remaining halves were retained in their initial tank and system, and fed their original diets for another 8 weeks (phase 2). After the 16-weeks, 3 fish/tank were sampled for each of proximate analysis, body indexes and 16S rRNA analysis of GIT microbiota. Fish weight ($P=0.0008$, $P=0.003$, $P<0.001$) and body fat ($P=0.0008$, $P=0.0041$, $P=0.0177$) were significantly affected by diet, diet quality and system, respectively. Feed intake ($P=0.0008$) and body energy ($P<0.001$) were altered by system. Body indexes were not affected by dietary treatment and water systems. Compositional dissimilarities existed between samples from the rearing water and GIT locations (ANOSIM: ($R=0.29$, $P=0.001$), PERMANOVA: $R=0.39$, $P=0.001$), but not in dietary samples (ANOSIM: $R=0.004$, $P=0.314$, PERMANOVA: $R=0.008$, $P=0.454$). Bacterial were predominantly from the phyla *Proteobacteria*, *Firmicutes* and *Bacteroidetes*, and their abundance differed when compared to the water samples, more dissimilarity in the luminal samples (ANOSIM: $R=0.40$, $P=0.001$, PERMANOVA: $R=0.56$, $P=0.001$) compared to those from the mucosal intestine (ANOSIM: $R=0.37$, $P=0.001$, PERMANOVA: $R=0.41$, $P=0.001$). Bacteria generally associated with carbohydrate and certain amino acids metabolism were observed in the mucosal intestine while rearing water appeared to serve as the main route of colonization of *Aeromonas* and *Acinetobacter* in the rainbow trout.

Keywords: bacterial community, bacterial diversity, gastrointestinal tract, sequencing, recirculating water system, flow-through water system, cross design.

1. Introduction

Host-microbial symbioses are vital to life and can be commensal, pathogenic, mutualistic or parasitic [1]. In vertebrate species, the complex microbial ecosystem in the gastrointestinal tract (GIT) plays a specific and important role in the physiology and metabolism of the host [2, 3]. Composition of the GIT bacteria are crucial to maintenance of host's health and minor perturbations may lead to a shift in the microbial population, which may affect normal functioning of the host system. Most information on vertebrate GIT microbiota has been primarily focused on mammalian species [4], with little attention on the lineage of fishes that represent over half of all vertebrate species [5]. Consequently, processes that underlie the role of individual GIT microbes in nutrition and health of fishes are still poorly understood.

Early cultivation-independent descriptions of the GIT microbiota in fishes have recently emerged [6, 7]. But what constitutes 'core' microbiota in aquatic animals has not yet been defined due to differences in bacterial communities reported in the literature. It also remains to be determined how environmental factors shape the composition of fish GIT microbiota. This information is important as maintaining a healthy microbiota is likely to improve performance of cultured fishes in aquaculture. Available evidence has shown that diet [8-11] and rearing water [12] are important environmental factors that modulate the normal GIT microbiota in fishes. While the majority of fish microbiota studies have

focused on characterization of the communities of microorganisms in the GIT lumen [10, 13-15], those that adhere to the mucosal surface, which are generally believed to be important in specialized physiological functions, remain uncharacterized.

Studies have proposed that core GIT microbiota in fishes are closely related to that of the fish feed or the rearing habitat (Nyholm et al., 2000), implying a largely allochthonous microbiota. This information for fish husbandry connotes that regulation of the external environment may be important for improving health and production of aquaculture fish species. This becomes very important when complete replacement of fishmeal is the goal for production of economically important fishes such as rainbow trout with a production sales of 104 million dollars in 2015 in North America [16]. Earlier studies of the 16S rRNA gene composition of rainbow trout GIT [13, 15] suggested the possibility of colonization by microbiota from the environment before first feeding and less significant changes in due to diets. However, it is difficult to ascertain whether feeding rainbow trout a fishmeal-free diet will have the same response on the microbiota composition of the GIT since the diets used in the studies by Desai et al. [13] and Ingerslev et al. [15] contained significant amounts of fishmeal as protein source.

Therefore, the present study investigated the performance of rainbow trout fed two fishmeal-free diets (animal- and plant-based diets) and assessed the impact of feeding these diets on the bacterial diversity and composition of the rainbow trout GIT. It also focused on characterization of the GIT bacteria associated with the luminal and the mucosal sections of rainbow trout GIT and establishment of the different bacteria that exist between trout GIT and the two commonly used water environments (recirculating and flow-through

systems) in fish farming.

2. Materials and Methods

2.1. Experimental design for the study

A 2 x 2 x 2 factorial design experiment was conducted using two experimental diets: animal protein diet (APD) and plant protein diet (PPD) with two diet qualities (amino acid balanced and unbalanced), which were fed to trout in two different water systems (recirculating and flow-through). Four diets were formulated at 38% DP and 20% lipids (Table 1), supplemented with or without free amino acids: lysine, methionine and threonine to meet the reported nutrient requirements for rainbow trout [17]. All fish were handled and treated in accordance with guidelines approved by the U.S. Fish and Wildlife Service.

2.2. Fish culture

Rainbow trout eggs were obtained from Troutlodge Inc., Sumner, Washington, USA. The fish were cultured at the Bozeman Fish Technology Center, Bozeman, MT to fingerlings size before the commencement of the experiment. Prior to the start of the feeding trials, fish were acclimatized to tanks for one-week period. Thereafter, diets were randomly assigned to each of the tanks and fish were fed twice daily to apparent satiation for the 16-week period of the experiment. The 16-week period is divided into two phases: phase 1 (0-8 weeks) and phase 2 (8-16 weeks). For phase 1, fish with an average initial weight of 15.7 ± 0.41 g were randomly selected and stocked at the rate of 20 fish/tank into 12 recirculating and 12 flow-through tanks, with three replicate tanks per each of the four

dietary treatments. Recirculating culturing water temperature was maintained at 15 ºC, while flow-through culturing water temperature ranged between 14.2 and 15.5 ºC. During phase 2, an additional 12 tanks were created in both the recirculating and flow-through culturing systems, making 24 culturing tanks in both water systems. Half of the fish in each of the tanks from phase 1 were randomly selected and moved to the other water system, (i.e. from recirculating to flow-through and vice-versa), while the remaining half were maintained in the same tank and system till the end of phase 2.

2.3. Diet manufacturing

All ingredients for each of the diet were ground with an air swept pulverizer (model 18-H; Jacobson, Minneapolis, MN, USA). All the dietary treatments were pre-cooked using cooking extrusion (DNDL-44, Buhler AG, Uzwil, Switzerland) with an 18-s exposure to an average of 127 ^{o}C in the extruder barrel section. The die plate was water-cooled to an average temperature of 60 ^{o}C. Depending on the diet, pressure at the die head was varied from 15 to 30 bar. The 3-mm pellets produced were then dried in a pulse-bed drier (Buhler AG, Uzwil, Switzerland) for 25 min at 102 ºC with a 10-min cooling period. The final moisture contents in all the diets were <10%. Thereafter, oil was added to the diets by top-coating.

2.4. Digestibility determination

Standard methods (AOAC, 1995) were used for analysis of dry matter and ash of ingredients, diets and feces. Apparent digestibility coefficients (ADCs) were determined

following the method of Cho et al. [18]. Yttrium oxide was used as an indigestible marker and 1% was added to each of the dietary treatments. Fecal samples were collected from the fish in each tank at the end of each phase and freeze-dried overnight at 50 ºC. Yttrium and phosphorus were determined in diets and feces by inductively coupled plasma atomic absorption spectrophotometry (Bozeman Fish Technology Center Analytical Laboratory, Bozeman, MT).

2.5. Fish sampling

Random sampling of ten fish from the initial fish population was carried out for determination of initial whole body proximate composition. Three fish were randomly selected from each tank and were euthanized by overdose of tricane methane sulfonate (MS-222; 325 mg/L water) following the approved protocol of the USFWS. Following euthanasia and within 10 minutes, fish were aseptically processed for both initial luminal and mucosa samples. Samples were frozen rapidly in liquid nitrogen and were stored at -20 °C until further processing. During each 8-week period, fish were weighed monthly and feed intake, feed conversion ratio (FCR), and weight gain were obtained per tank. At the end of each 8-week feeding phase, three fish from each tank were randomly sampled for determination of whole body composition; three additional fish were sampled from each of the tank for determination of viscerosomatic index (VSI), hepatosomatic index (HSI) and filet ratio (FR). In addition, three fish were randomly selected from each of the tank and dissected for collection of luminal and mucosal samples as described above.

2.6. Sample amplification and sequencing

DNA was extracted from luminal and mucosal samples using MoBio PowerFecal DNA Isolation kit, while DNA extraction from water samples was carried out using the MoBio PowerWater DNA isolation kit. The manufacturer's instructions were followed except for a 3-min bead-beating step instead of a 10-min vortex for the water samples. Variable regions 3 and 4 of the 16s rRNA genes were amplified using custom primers that included indexes to identify samples after sequencing. The PCR reaction ran for 30 cycles at 94 °C for 20 s, 52 °C for 30 s, and 72 ° C for 45 s, using barcoded primers (SeqF(1-8) 5'AATGATACGGCGACCACCGAGATCTACAC (adaptor)-Index2(one of eight different 8nt codes used to distinguish among samples when pooled for sequencing)-TATGGTAATT(sequencing primer pad)-AT(linker)-CCTACGGGAGGCAGCAG(341 fprimer)-3' and SeqR(1-12) 5'- CAAGCAGAAGACGGCATACGAGAT(adaptor)-Index 1(one of twelve different 8nt codes used to distinguish among samples when pooled 67 for sequencing)-AGTCAGTCAG(sequencing primer pad)-CC(linker)-GGACTACHVGGG TWTCTAAT (806r primer-3'). Amplicons were quantified using an Agilent 2200 tape station (Agilent, Santa Clara, CA, USA) and pooled at an equimolar concentration. Pooled amplicons were purified from residual PCR reagents and nonspecific amplification products in an agarose gel using a QIAquick gel extraction kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions. Purified and pooled amplicons were subsequently quantified using a KAPA Syber quantification kit (KAPABiosystems, Wilmington, MA, USA) as per manufacturer's instructions. Purified, quantified and pooled amplicons were mixed with 10 % of an equimolar concentration of PhiX. Sequencing was

performed with an Illumina MiSeq using paired-ended 2 by 250 nucleotide (nt) dual-index sequencing. Custom primers (R1 5'-CCTACGGGAGGCAGCAG-3', R2 5'-AGTCAGTCAGCCGGACTAC-3', Index 5'-GTAGTCCGGCTGACTGACT-3') were used for sequencing and indexing.

2.7. Sequence processing and statistical analyses

Raw sequence data was filtered to remove low quality sequences (<Q30) and the remaining sequences were binned using USEARCH into operational taxonomic units (OTUs) at a threshold of over 97% sequence identity. To determine the identity or closest relative of the bacteria isolated from rainbow trout GIT, we analyzed the 16S rRNA gene from this study against the public Ribosomal Database Project ll. OTUs with a relative abundance of < 0.05% were removed. Alpha diversity of level 0.05 was performed on the OTUs and Chao 1 and Simpson indices were obtained using the RAM package [19] in R. Non-parametric analyses, including analysis of similarities (ANOSIM), permutation multivariate analysis of variance (PERMANOVA) and canonical analysis of principal (CAP) coordinates were performed using the PRIMER-E software (Plymouth, UK), and were used to explain a significant proportion of the total variation that exist in in our data. PERMANOVA was performed with 999 permutations, while CAP, a non-parametric permutation test, identified whether bacterial abundance differ among the treatment groups. P-values for the analyzed variables were corrected for false discovery rate [FDR (Q>0.05)] using R statistical software. The growth performance data were analyzed for factorial ANOVA using Proc GLM of SAS version 9.4 (SAS Institute Inc., Cary, NC,

USA). Tukey's means separations were used to determine differences within main effects. Significant effects were considered at $P<0.05$.

3. Results

3.1. Diets proximate compositions and digestibility

Proximate compositions of the diets manufactured in this study reflected the formulation targets. The results for analyzed apparent digestibility coefficients (ADCs) were 66.7, 66.2, 64.8 and 62.3% (dry matter P=0.0136); 82.1, 87.0, 83.1 and 85.7% (crude lipid P<0.001) and 86.2, 86.4, 88.4 and 90.0% (crude protein P<0.001) for balanced APD, unbalanced ADP, balanced PPD and unbalanced PPD diets, respectively.

3.2. Growth performance, body condition indexes and compositions of rainbow trout

Following the 16-week feeding trial, fish survival was within 97-100% for all dietary treatments. Average fish weight was significantly affected by diet (P=0.0008) and diet quality (P<0.0030; Table 2). Fish fed the APD diet grew larger than those on PPD diet and fish fed balanced diets were larger than those fed unbalanced diets. Feed intake was the same irrespective of dietary treatments (P=0.1986) and balancing the diets by inclusion of crystalline amino acids had no significant effect on intake (P=0.2801). In addition, balancing diets did not affect (P=0.0657) feed utilization in trout. Dietary treatments (P=0.9645) and diet quality (P=0.8910) had no significant effect on viscerosomatic index. Likewise, hepatosomatic index (P=0.6196, P=0.2043), condition index (P=0.9654, P=0.8908) and fillet ratio (P=0.9645, P=0.8910) were not significantly affected by dietary

treatments and diet quality, respectively. Analyzed whole body energy (P=0.2538, P=0.1247) and protein (P=0.0629, P=0.2849) showed no negative effect due to diet and diet quality, respectively. However, significant effects were observed in body fat (P=0.0008, P=0.0041) with diet and diet quality, respectively.

Trout raised in the recirculating system grew larger (P<0.0001) than those raised in flow-through system and switching the fish from either system to the other had no significant effect on the growth of trout. Feed intake (P=0.0008) was higher in fish raised in flow-through than those in the recirculating system. Raising trout in either recirculating or flow-through systems had no significant effect on hepatosomatic index (P=0.1247), condition index (P=0.3033) and fillet ratio (P=0.1199). Fish raised in the flow-through system stored more fat in the GIT compared to those raised in the recirculating system. Body energy (P<0.0001) and fat (P=0.0177) were significantly higher in fish raised in the flow-through water. All water physiochemical properties measured in both water systems were within the normal range. Except for nitrate which was elevated in the recirculating water system, temperature, ammonium and dissolve oxygen (DO) were not different between the recirculating and the flow-through water systems (Table 3).

3.3. Characteristics of the bacterial community compositions

In total 68,493,243 high quality sequences were obtained following quality trimming with 3,668 OTUs identified. A total of 253,425 sequences containing 15 OTUs, were shared across all samples with 95% sequence identity. Good's coverage estimates indicated that >99% of all OTUs were surveilled in this study. Overall, Chao1 diversity

indexes and measures of effective number of species (ENS) indicated greater richness and diversity in water samples than those from the fish GIT (Tables 4 and 5) and a Kruskal-Wallis test suggested significant differences between the GIT samples and the water samples (P=0.001598). Overall, bacterial richness and diversity varied between APD and PPD diets. Measures of microbial richness and diversity in the luminal and mucosal regions of the fish GIT approached significance but did not vary between APD and PPD diets (Wilcoxon rank test, P=0.07242). Flow-through water samples did not significantly differ in richness or diversity from recirculating water samples (Kruskal-Wallis P=0.1679). Greater species richness was seen in luminal GIT samples than those from the mucosa, this effect was consistent across the two diets, but significance difference was observed only in mucosal sample from APD-fed fish and luminal sample from PPD-fed fish (Wilcoxon rank test, P=0.0048).

3.4. Bacterial community composition and importance of diet type and rearing water

The five taxonomic lineages analyzed in this study are presented in Table 6. Bacteria were predominantly of the *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Spirochaetes* phyla (Fig. 1) and their relative abundances varied among water, luminal and mucosal GIT samples. Generally, the GIT samples from this study contained 31% *Firmicutes*, 31% *Proteobacteria*, 27% *Bacteroidetes*, 10% *Actinobacteria* and 2% *Acidobacteria*, while the water samples slightly differed with 38% *Firmicutes*, 34 % *Proteobacteria*, 22 % Bacteroidetes, 5 % *Actinobacteria*, and 1.1% *Acidobacteria*. CAP coordinates showed overall significance of all the OTU abundance data (R^2=0.94) in this

study. Estimations of the relative magnitude of our treatments on microbial β-diversity indicated that water alone produced a higher positive correlation (R^2=0.83) than diets (R^2=0.55), which indicated that trout GIT microbiota shared greater similarity with the rearing water. Neither water nor GIT samples varied by dietary treatment (ANOSIM: R=0.004, P=0.314, PERMANOVA: R=0.008, P=0.454). However, β-diversity differed between water samples and those obtained from trout mucosal and luminal GIT (ANOSIM: R=0.29, P=0.001), PERMANOVA: R=0.39, P=0.001) (Fig. 2). Luminal samples had higher compositional dissimilarities (ANOSIM: R=0.40, P=0.001, PERMANOVA: R=0.56, P=0.001) compared to those from the mucosal region of the GIT (ANOSIM: R=0.37, P=0.001, PERMANOVA: R=0.41, P=0.001). Pairwise comparisons of all analyzed variables revealed only significant difference between mucosa and luminal samples from unbalanced APD diet (P=0.017) as well as mucosal from unbalanced APD and luminal samples from PPD diets (Table 7). For water samples, significant differences were observed between fish raised in the flow-through and recirculating, FF-RR (P=0.001); fish raised in the flow-through system and those moved from flow-through to recirculating system, FF-RF (P=0.004); fish raised in the recirculating system and those moved from recirculating to flow-through system, RR-FR (P=0.036) and fish moved from flow-through to recirculating compare to those moved from recirculating to flow-through water systems, FR-RF (P=0.005). Bacterial diversity of the GIT was greater in trout switched from the flow-through to recirculating system compared to when trout were switched from recirculating to flow-through (Fig. 3).

The major bacterial genera composition in the diets (Fig. 4), GIT locations (Fig. 5) and rearing water (Fig. 6) were *Prevotella*, *Bacteroides*, *Arcobacter*, *Paludibacter*, *Aeromonas* and *Flavobacterium*, with enrichment of *Bacteroides* in the APD diet while *Paludibacter* was enriched in the PPD diet. Significant differences were observed in the relative abundances of 70 microbes between the mucosal and the luminal region of the GIT (Fig. 7), and each of the mucosa and luminal GIT samples and water samples (Figs. 8 and 9) ($P < 0.05$). The mucosal GIT had greater relative abundances of *Tolumonas*, *Chitinophaga, Anaerosalibacter, Coriobactineae*, *Peptoniphillus*, *Pseudobutyrivibrio propionivibrio*, *Enhydrobacter*, *Alcaligenes* and *Ethanoligenens* than observed in the GIT luminal (Fig. 7). Relative to water samples, *Arcobacter*, *Brevundimonas*, *Corynebacterineae*, *Sarcinia*, *Propionibacterineae*, *Exiguobacterium* and *Clostridium* were enriched in the trout mucosal region (Fig. 8), while *Enterobacter*, *Lactococcus*, *Paracoccus*, *Chitibacter*, *Succinispira and Gemmatimonas* were enriched in the luminal sample. (Fig. 9). *Parasporobacterium*, *Janthinobacterium*, *Flavobacterium*, *Carnobacterium*, *Acetanaerobacterium*, *Deefgea* and *Clostridium XIVa* exhibited greater relative abundances in water samples when compared to each GIT regions (Figs. 8 and 9). However, we did not observe significant separation of the bacterial composition between APD-fed fish and those fed PPD diet. But greater relative abundances of *Aeromonas* and *Arcobacter* were observed in APD-fed fish, while a greater relative abundance *Acinetobacter* was associated with PPD diet (Fig. 10), but *Flavobacterium*, *Paludibacter*, *Lactococcus*, *Propionispira*, *Enterobacter* and *Exiguobacterium* were common in the GIT of both fed APD and PPD diets (Fig. 10).

4. Discussion

In the current study, we evaluated the performance of rainbow trout fed animal- and plant-protein diets and the effect of the diets were assessed on the bacterial composition within the luminal and mucosal section of trout GIT. This study is the first to implement next-generation sequencing technology to investigate changes in the bacterial composition in rainbow trout luminal and mucosal GIT sections when the fish is switched from recirculating water system to flow-through water system and vice versa.

Animal and plant protein diets used in this study had no detrimental effect on rainbow trout survival. This observation shows that trout can survive on animal and plant protein-based diets that are devoid of fishmeal and supplemented with the essential amino acids, with improved growth. Our findings corroborate an earlier report that rainbow trout utilizes free amino acids efficiently [20]. Differences were observed in the growth performance of fish fed the animal and plant protein diets, despite supplementation with lysine, methionine and threonine. Although diets were formulated on a digestible amino acid basis, we expected no growth differences between fish fed the two diets, given similar feed intakes for both animal and plant protein diets. The reduced growth observed in trout fed the plant protein diet may indicate less-bioavailability of certain amino acids that were not supplemented in the plant diet or other reductions in bioavailability of certain nutrients due to antagonistic interactions with antinutritional factors of the plant diet. For instance, zinc bioavailability has previously been shown to be affected with soybean meal diets leading to reductions in growth of rainbow trout [21].

In addition, slight increases in body fat observed in fish fed the plant protein diet

may be as a consequence of non-starch polysaccharides in the plant ingredients affecting fat digestibility in the fish [22, 23]. These reductions in fat digestibility may have reduced the growth response from the fish. Low quality protein, especially from plant ingredients as observed from trout fed the unbalanced plant protein diet, is in accordance with what has been reported in the literature [20, 24]. Unbalanced amino acid profile of this non-supplemented plant protein diet may have resulted in the fish eating more but being unable to build protein and instead catabolizing the amino acids for energy.

In this study, trout raised in the recirculating system performed better than those raised in the flow-through system. This observation is supported by earlier findings on rainbow trout under these two water systems [25]. We hypothesize that the observed differences in microbial diversity impacted trout performance. The observation of more bacterial species richness in the recirculating system than flow-through system may have contributed significantly to the better performance of the fish that were raised in the recirculating system. Although the contribution of bacteria in rearing water to host physiology is not known in fishes, it is known that soil bacteria are important component of the world's ecosystem [26], so we assumed that more diverse microbial composition in recirculating system than flow-through system had significant impact on trout adaptation and performance in this study. Report on Indian white shrimp revealed increased activities of amylase, proteases and lipases associated with higher counts of *Bacillus* bacteria in the GIT of the treatment group when *Bacillus spp*. was added as probiotics into the water compared to the control [27]. Similarly, improved growth performance was observed in *Macrobrachium rosenbergii* when different strains of lactobacillus were fed as probiotics

compared to the control [28].

High abundances of *Proteobacteria, Firmicutes*, *Bacteroidetes* and *Actinobacteria* were observed in this study, consistent with what have previously been reported for rainbow trout [13, 15, 29-31] and Atlantic salmon [32, 33]. In particular, high abundances of the phylum *Proteobacteria* may reflect the ability of this group of bacteria to exhibit both host and environmental lifestyles [34]. Enrichment of *Bacteroides* from phylum *Bacteroidetes*, which is associated with high protein and fat diets in human studies [35, 36], observed in both the luminal, mucosal and the rearing water samples in this study especially APD diet, supports the notion that digestive physiology of trout has evolved utilizing protein and lipid for metabolic energy [37] and also possibility of close proximity between microbial ecology of trout and higher vertebrates [37]. Also, our observation agrees with earlier findings that the mucosal region of the GIT is more diverse than the luminal section of trout GIT [38]. Greater microbial diversity associated with the PPD diet may reflect a broadening of niches by the high fiber content of the plant protein-based diet. A previous study of Cichlids reported a reduction in microbial diversity among captive strains of Cichlids that were fed high protein and fat diets rather than the fiber-rich diets obtained in with low fiber diet [39].

The phylum *Acidobacteria*, generally associated with soil environments [40], observed both in the GIT and water samples, probably reflect a transient influence of the environment on the GIT. The genus *Flavobacterium* includes *F. psychrophilum*, a species that is the etiological agent of rainbow trout fry syndrome [41, 42]. *Flavobacterium* spp. were observed in luminal and mucosal samples of fish fed plant and animal protein diets

and with higher relative abundance in the water sample than samples from the luminal and mucosal GIT. This finding is consistent with an earlier suggestion that the *Flavobacterium* lineage includes non-pathogenic species or isolates [43] and rearing water have been suggested as the medium by which viable cells are transmitted into the fish [44]. Higher enrichment of cellulose-degrading bacteria: *Brevundimonas*, *Anoxybacillus and Robinsoniella* [45, 46] as well as genus *Enhydrobacter* (a member of *Vibrionaceae* family), which are known to utilize certain amino acids *viz*. L- alanine, L-serine, and L-arginine for growth and Chitinolytric bacteria, showed relative importance of exogenous and endogenous digestive enzymes associated with the digestive systems of fishes, including rainbow trout [47-49]. *Chitinophaga* has always been part of the bacteria isolated from the GIT of hatchery fed trout where they play a defense role rather than digestion [50]. We suggest further investigation of this role in rainbow trout health.

5. Conclusions

In summary, our study investigated the importance of diet types and rearing water types systems on bacterial populations in the trout GIT lumen and mucosa. Proteobacteria and Firmicutes were the two most abundant phyla in both the rainbow trout GIT and water samples. Diet is typically observed to be an important variable shaping the microbiota of the GIT, but no significant variation in the GIT microbiota was observed in our study with respect to diet. Instead water system appeared a more important factor in shaping rainbow trout microbiota. Our results suggest that rearing water enriches distinct microbiota in the GIT of rainbow trout. We suggest a more detailed study looking at the effect of specific

ingredients that could be utilized to influence the water microbial community, particularly as it relates to those bacteria associated with certain diseases in freshwater fishes.

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Table 1: Ingredients and chemical compositions of experimental diets.

Ingredient index	APD as-fed (g/100g)	Unbalanced APD as-fed (g/100g)	PPD as-fed (g/100g)	Unbalanced PPD as-fed (g/100g)
Ethanol Yeast[1]	6.52	6.52	6.52	6.52
Soybean meal[2]	6.52	6.52	6.52	6.52
Feather meal[3]	7.37	7.37	0.00	0.00
IDF Chicken 42[3]	7.29	7.29	0.00	0.00
Blood meal US[4]	4.49	4.49	0.00	0.00
Soy protein concentrate[2]	9.57	14.43	30.85	36.17
Corn Protein[4]	9.57	9.57	9.57	9.57
Wheat midds[3]	5.62	5.62	5.62	5.62
Poultry fat[3]	0.00	0.00	1.36	1.36
Wheat flour[4]	19.32	19.54	15.93	15.53
Menhaden fish oil[5]	17.60	17.51	17.50	17.50
Lecithin	1.00	1.00	1.00	1.00
Stay-C 35	0.15	0.15	0.15	0.15
Vitamin premix ARS 702	1.00	1.00	1.00	1.00
TM ARS 640	0.10	0.10	0.10	0.10
NaCl	0.28	0.28	0.28	0.28
Magnesium Oxide	0.06	0.06	0.06	0.06
Potassium chloride	0.56	0.56	0.56	0.56
Monocalcium Phosphate	3.30	3.10	3.00	2.80
Choline Cl 50%	1.00	1.00	1.00	1.00
DL-Methionine	0.76	0.00	0.75	0.00
Lysine HCl	2.94	0.00	2.86	0.00
Threonine	0.76	0.00	0.84	0.00
Tryptophan	0.00	0.00	0.00	0.00
Taurine	0.50	0.50	0.50	0.50
Biofix Plus	0.00	0.00	0.00	0.00
Yttrium oxide	0.10	0.10	0.10	0.10
Astaxanthin	0.08	0.08	0.08	0.08
Proximate composition				
Crude Protein (N*6.25)	454.2	447.2	441.3	440.8
Fat (g/kg)	140.3	167.9	132.0	135.9
Gross Energy (MJ/kg)	23.6	23.7	23.1	22.7

[1] Archer Daniels Midland (Decatur, IL, USA).
[2] Nelson & Sons Inc. (Murray, UT, USA).
[3] MGP Ingredients, Inc. (Atchison, KS, USA.
[4] Gavilon LLC, (Omaha, NE, USA).
[5]MGP Ingredients, Inc. (Atchison, KS, USA).
[6]Contributed per kg of diet: vitamin A (as retinol palmitate), 30,000 IU; vitamin D_3, 2160 IU; vitamin E (as DL-%-tocopheryl-acetate), 1590 IU; niacin, 990 mg; calcium pantothenate, 480 mg; riboflavin, 240 mg; thiamin mononitrate, 150 mg; pyridoxine hydrochloride, 135 mg; menadione sodium bisulfate, 75 mg; folacin, 39 mg; biotin, 3 mg; vitamin B_{12}, 90 µg.
[7]Contributed in mg/kg of diet: zinc, 37; manganese, 10; iodine, 5; copper, 3; selenium, 0.4.

Table 2: Growth performance and body indexes parameters of rainbow trout fed animal and plant protein diets.

Performance	Diet (D)		Diet quality (DQ)		Diet x diet quality (DDQ)				System (S)				P>F[2]				
	APD	PPD	Balanced	Un balanced	Balanced		Unbalanced		FF	FR	RR	RF	Diet	DQ	DDQ	S	D x S
					APD	PPD	APD	PPD									
Avg Fish wt	120.12	111.27	119.52	111.86	123.20	115.85	117.04	106.68	102.82	119.75	117.67	122.5	0.0008	0.0030	0.5323	<0.0001	0.2284
Feed intake (% bw/d)	2.02	1.96	1.96	2.02	1.98	1.93	2.05	1.99	2.04	2.10	1.93	1.88	0.1508	0.0991	0.9106	0.0008	0.7140
FCR (g feed/g gain)	1.03	1.03	1.01	1.05	1.00	1.01	1.04	1.06	1.08	0.98	1.00	1.04	0.5974	0.0657	0.1700	0.2972	0.3825
Visceral index (%)	11.06	11.23	11.11	11.19	11.10	11.12	11.03	11.35	12.27	10.87	9.94	11.52	0.9645	0.8910	0.4532	0.3033	0.6359
Fillet ratio (%)	26.70	26.33	26.95	26.08	27.48	26.43	25.92	26.23	25.81	27.10	26.42	26.75	0.3357	0.0271	0.0829	0.1199	0.0421
Hepatosomatic index (%)	1.38	1.48	1.30	1.57	1.29	1.30	1.48	1.67	1.27	1.32	1.28	1.86	0.6233	0.1821	0.6525	0.1247	0.9619
Condition index (%)	136.88	136.66	136.44	137.10	134.75	138.13	139.00	135.20	133.80	139.73	142.58	130.97	0.9645	0.8910	0.4532	0.3033	0.6359
Fat	11.81	11.01	11.08	11.75	11.40	12.22	10.75	11.27	11.79	10.84	11.32	11.68	0.0008	0.0041	0.4828	0.0177	0.3756
Energy	2107.77	2078.49	2073.26	2113.00	2074.47	2072.06	2141.07	2084.92	2246.85	1934.43	2194.96	1996.28	0.2538	0.1246	0.2942	<0.0001	0.0876
Protein	16.01	16.43	16.34	16.10	16.27	16.41	15.75	16.46	16.59	15.87	16.22	16.21	0.0629	0.2848	0.1974	0.1600	0.4028

FF – flow-through, FR – flow-through to recirculating, RR – recirculating, RF – recirculating to flow-through

Table 3: Physiochemical properties of recirculating and flow-through water systems.

Diet	Temperature (^{o}C)	Ammonium (mg/l)	Nitrate (mg/l)	DO
Recirculating				
APD	15.53 ± 0.06	0.12 ± 0.03	0.13 ± 0.01	6.44 ± 0.13
UnAPD	15.5 ± 0.1	0.09 ± 0.08	0.15 ± 0.01	6.55 ± 0.18
PPD	15.47 ± 0.06	0.16 ± 0.03	0.17 ± 0.02	6.32 ± 0.30
UnPPD	15.5 ± 0.1	0.13 ± 0.02	0.16 ± 0.01	6.28 ± 0.28
Flow-through				
APD	15.33 ± 0.12	0.08 ± 0.05	0.06 ± 0.01	6.75 ± 0.25
UnAPD	15.27 ± 0.15	0.10 ± 0.01	0.06 ± 0.01	7.02 ± 0.19
PPD	15.47 ± 0.06	0.09 ± 0.02	0.05 ± 0.01	6.92 ± 0.35
UnPPD	15.33 ± 0.12	0.11 ± 0.01	0.04 ± 0.01	6.96 ± 0.23

DO – dissolved oxygen

Table 4: Bacterial diversity indexes of samples from trout fed animal and plant protein diets.

GIT Samples	Balanced	Unbalanced	Luminal	Mucosal	RR	RF	FF	FR
APD-diet								
#sequence	18339	18339	18339	18339	18339	18339	18339	18339
Chao	84.9	73.5	82.8	75.3	58.9	98.7	64.7	87.2
Shannon	3.18	2.84	3.03	2.97	2.94	3.25	2.47	3.31
ENS	24	17	21	20	19	26	12	27
Evenness	0.81	0.73	0.76	0.78	0.86	0.75	0.67	0.31
Species number	84.9	73.5	108.5	52.1	58.9	86.1	66.6	64.3
Simpson	0.9	0.81	0.84	0.86	0.87	0.86	0.75	0.92
PPD-diet								
#sequence	18339	18339	18339	18339	18339	18339	18339	18339
Chao	104.6	103.9	109.5	99.5	124	157.8	61.3	87.4
Shannon	3.45	3.26	3.42	3.29	3.52	3.82	2.95	3.23
ENS	32	26	31	27	34	46	19	25
Evenness	0.8	0.79	0.76	0.83	0.77	0.25	0.77	0.81
Species number	104.6	103.9	109.5	99.5	124	157.8	61.3	87.4
Simpson	0.3	0.26	0.89	0.9	0.91	0.92	0.85	0.91

RR – recirculating, RF – recirculating to flow-through, FF – flow-through, FR – flow-through to recirculating

Table 5: Comparison of bacterial diversity indexes of water samples from trout fed animal and plant protein diets.

Water Samples	Balanced	unbalanced	RR	RF	FF	FR
APD-Diet						
#sequence	18327	18327	18327	18327	18327	18327
Chao	118.2	105.9	152.8	77.5	70.3	133.7
Shannon	3.42	2.96	3.65	2.82	2.79	3.3
ENS	31	19	39	17	16	27
Evenness	0.73	0.65	0.75	0.66	0.66	0.69
Species number	118.2	105.9	152.8	77.5	70.3	133.7
Simpson	0.92	0.85	0.93	0.87	0.87	0.87
PPD-Diet						
#sequence	18327	18327	18339	18339	18339	18339
Chao	71.7	132.4	133	74.7	99.2	70.3
Shannon	3	3.6	3.5	3.11	3.2	3
ENS	20	36	33	22	25	20
Evenness	0.73	0.77	0.17	0.73	0.73	0.72
Species number	71.7	132.4	133	74.7	99.2	70.3
Simpson	0.91	0.93	0.93	0.91	0.91	0.91

RR – recirculating, RF – recirculating to flow-through, FF – flow-through, FR – flow-through to recirculating

Table 6: Major core bacteria taxa across the five taxonomic lineages in rainbow trout fed animal and plant protein diets.

Phylum	Class	Order	Family	Genus
Firmicutes	*Actinobacteria*	*Clostridiales*	*Porphyromonadaceae*	*Paludibacter*
Proteobacteria	*Clostridia bacteroisia*	*Bacteroidales*	*Lachnospiraceae*	*Flavobacterium*
Bacteroidetes	*Gammaproteobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Prevotella*
Actinobacteria	*Alphaproteobacteria*	*Lactobacillales*	*Prevotellaceae*	*Aeromonas*
Acidobacteria	*Bacilli*	*Rhizobiales*	*Aeromonadeae*	*Parasporobacterium*
Fusobacteria	*Flavobacteria*	*Aeromonas*	*Streptococcaceae*	*Lactococcus*
	Negativicutes	*Actinobacteridae*	*Acticetalesnomy*	*Clostridium XiVa*
	Episilonproteobacteria	*Bacillales*	*Clostridiaceae*	*Sarcina*
	Fusobacteria	*Selenomonadales*	*Rhizobiaceae*	*Arcobacter*
	Acidobacteria	*Campylobacterales*	*Campylobacteraceae*	*Bacteroides*
		Pseudomonadales	*Bacteroidaceae*	*Clostridium sensu*
		Xanthomonadales	*Moraxellaceae*	*Streptococcus*
		Sphingomonadales	*Sphingomonadaceae*	*Sphingomonas*
		Fusobacteriales	*Pseudomonadaceae*	*Steroidobacter*
		Gp4	*Acidaminococccaceae*	
			Planococcaceae	
			Bacillaceae	
			Sinobacteraceae	

Table 7: Results of PEMANOVA analysis for the different diets, GIT locations, and water samples.

PERMANOVA	P-value	FDR
Diet	0.141	0.187
Diet quality	0.308	0.32
Water	0.003	0.011
Location	0.001	0.005

PERMANOVA pair wise test	P-value	FDR	PERMANOVA pair wise test	P-value	FDR
GIT locations			Water samples		
$UnAPD_M$ – PPD_L	0.001	0.006	FR, FF	0.043	0.065
$UnAPD_M$ – $UnAPD_L$	0.006	0.018	FR, RR	0.296	0.32
APD_M – PPD_L	0.061	0.0732	FR, RF	0.004	0.013
PPD_L – PPD_M	0.088	0.088	FF, RR	0.001	0.005
APD_M – $UnAPD_L$	0.022	0.044	FF, RF	0.016	0.034
PPD_M – $UnAPD_L$	0.037	0.0555	RR, RF	0.155	0.191

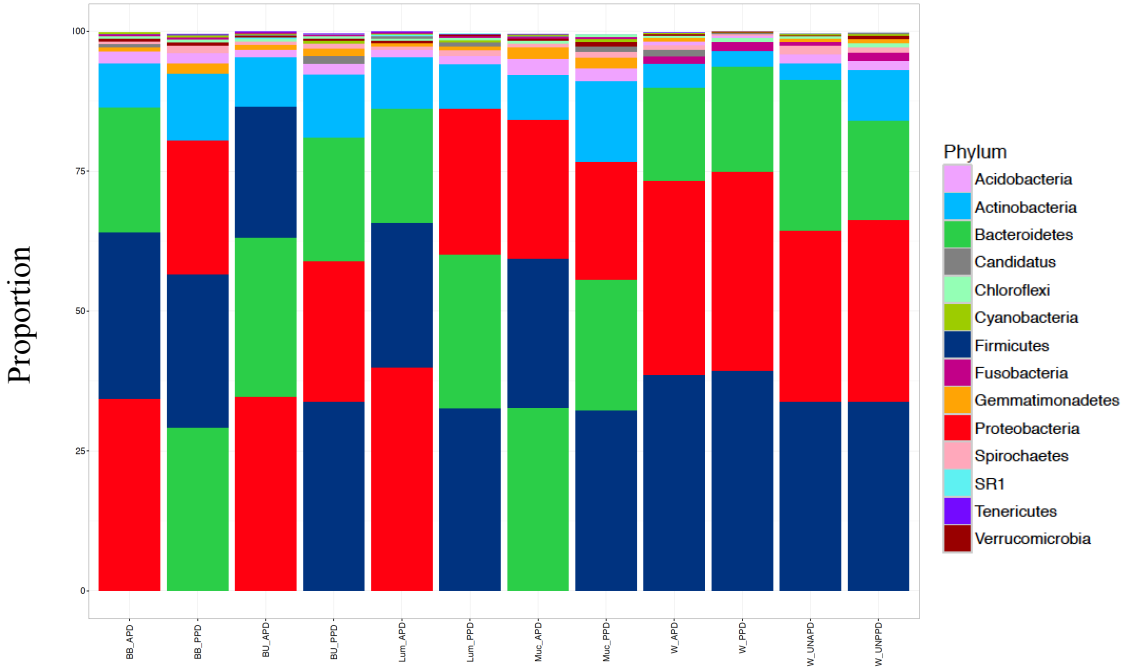

Fig. 1. Bacterial composition at phylum level of rainbow trout fed animal and plant protein diets. Digesta, and water samples are grouped by dietary treatments. Dietary treatments: Balanced Animal protein diet (A_APDdiet), Unbalancedanimal protein diet (A_UnAPPDdiet), Plant protein diet (A_PPDdiet),Unbalanced plant protein diet) A_UnPPDdiet), Initial fish sample (A_Fish), Digesta samples: Fish fed Balanced APD diet (BB_APD), Fish fed Balanced PPD diet (BB_PPD), Fish fed Unbalanced APD diet (BU_APD), Fish fed Unbalanced PPD diet (BU_PPD), Luminal samples by Balanced APD diet (Lum_APD), Luminal samples by Balanced PPD diet (Lum_PPD), Mucosal samples by Balanced APD diet (Muc_APD), Mucosal samples by Balanced PPD diet Muc_PPD); and Water samples: Samples from APD diet (W_APD), Samples from PPD diet (W_PPD), Samples from Unbalanced APD diet (W_UNAPD), Samples from Unbalanced PPD diet (W_UNPPD).

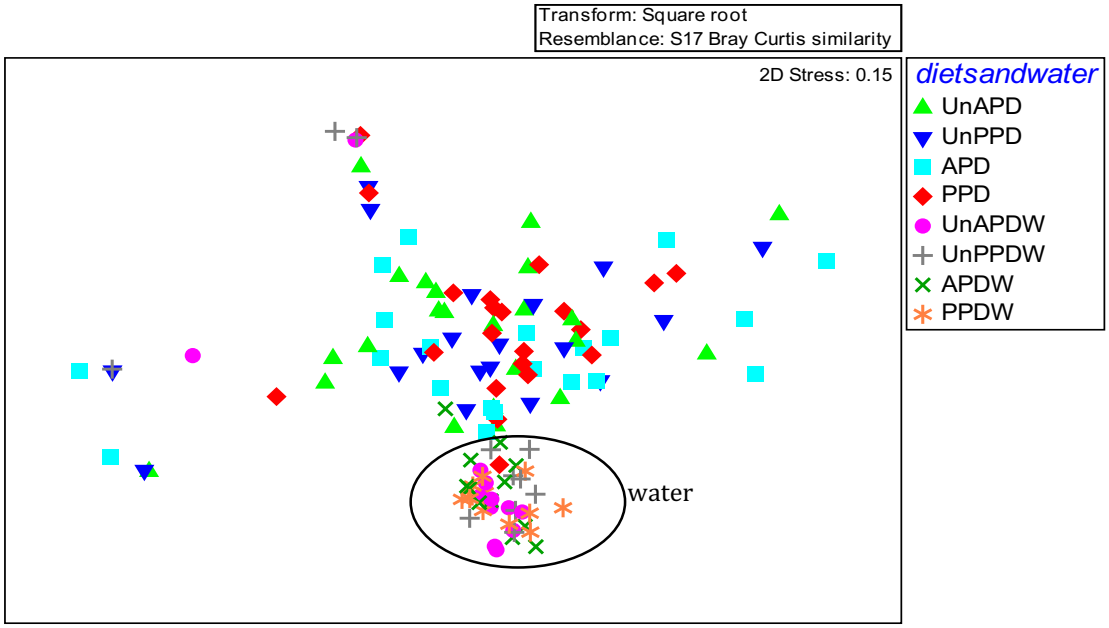

Fig. 2. Non-metric multidimensional scaling plot based on Bray-curtis similarity of bacterial communities from fish GIT and water samples. Digesta samples: Balanced animal protein diet (APD), Balanced plant protein diet (PPD), Unbalanced animal protein diet (UnAPD), Unbalanced plant protein diet (UnPPD); water samples: Balanced animal protein diet (APDW), Balanced plant protein diet (PPDW), Unbalanced animal protein diet (UnAPDW), and Unbalanced plant protein diet (UnPPDW).

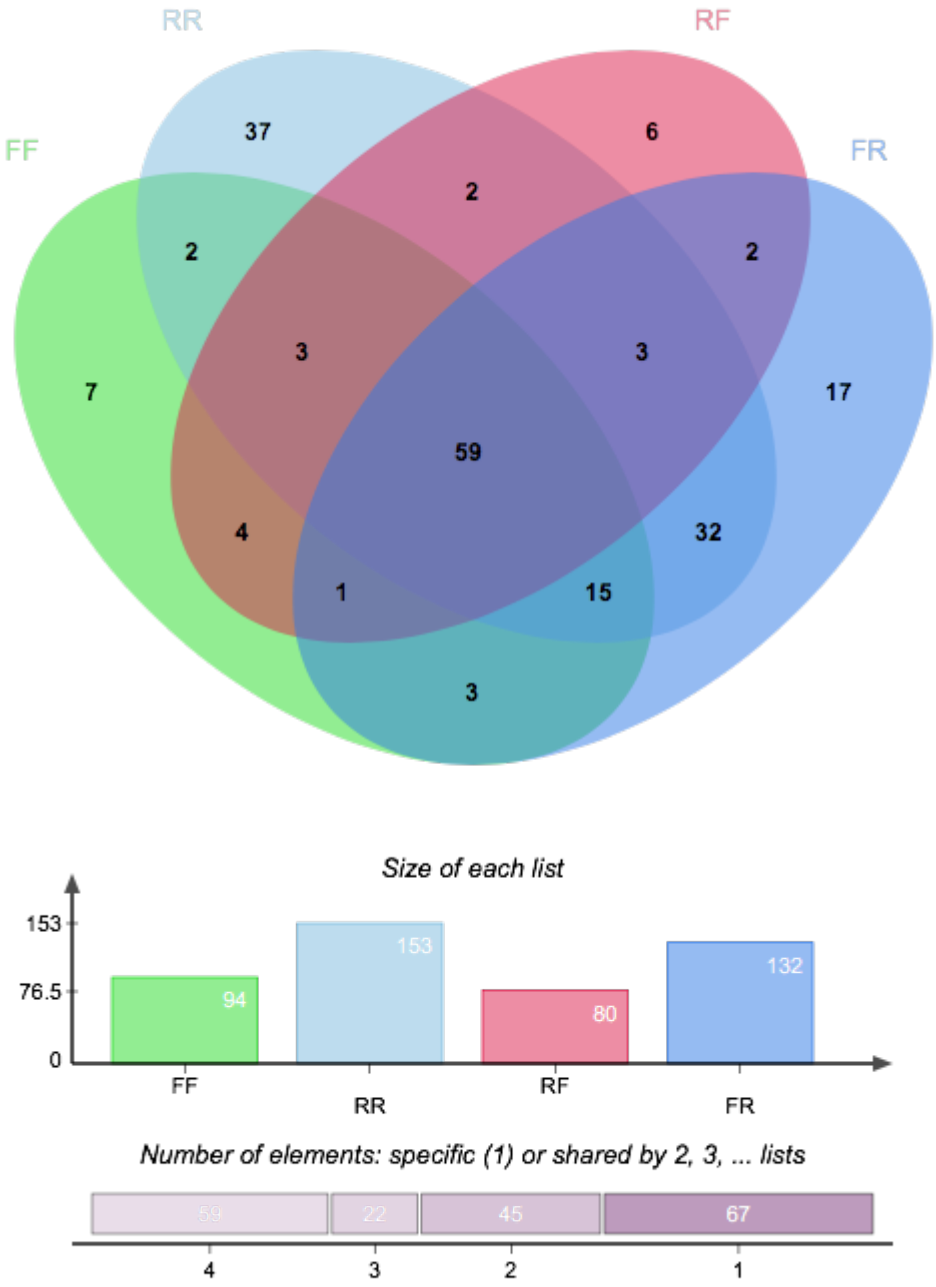

Fig. 3. Venn diagram showing bacterial richness of trout GIT when reared in recirculating and flow-through water systems. RR: recirculating, FF: flow-through, FR: flow-through-recirculating, and RF: recirculating-flow-through.

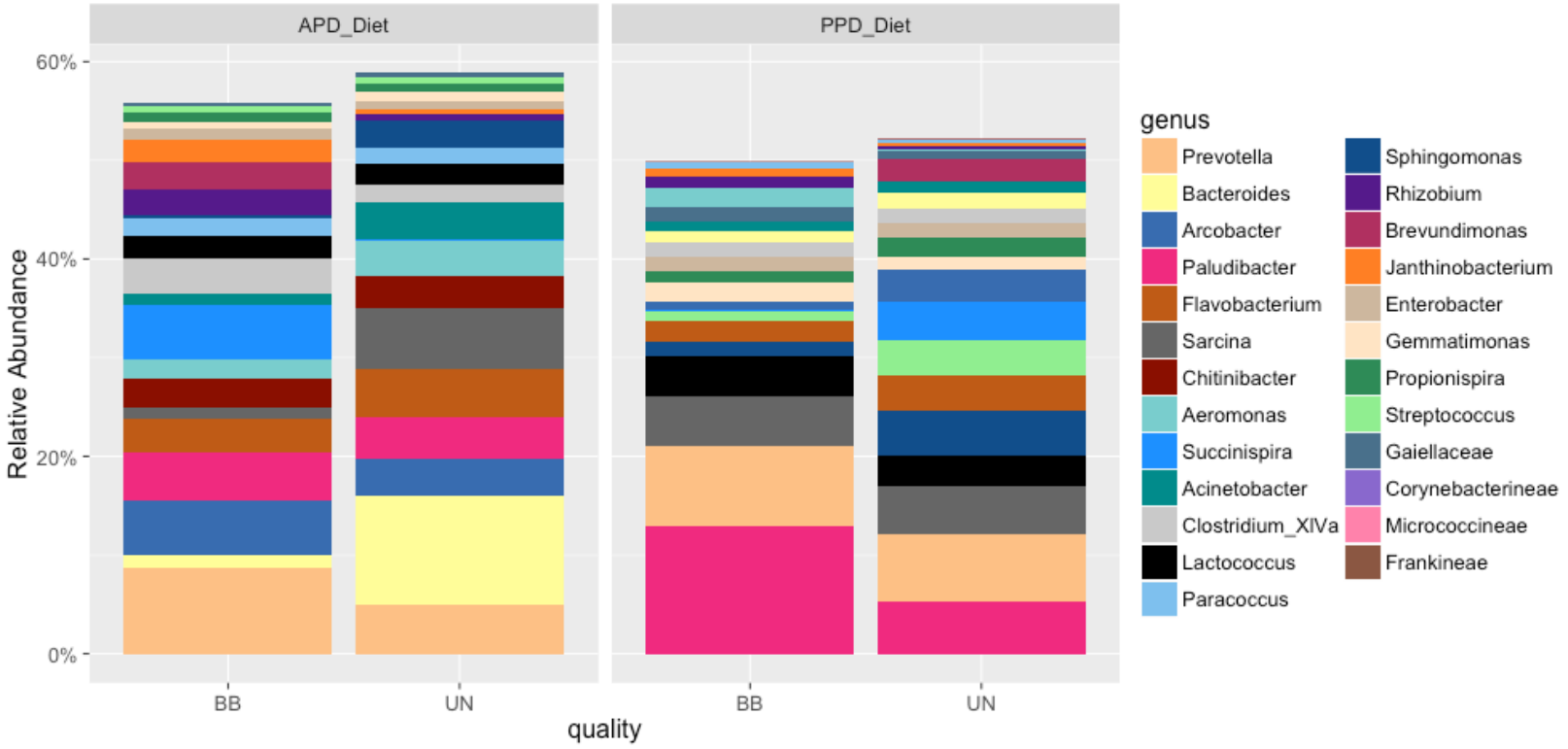

Fig. 4. Bar chart of the relative abundance of bacterial community compositions at genus level at dietary treatments level, with (BB) or without (UN) substitution with crystalline amino acids.

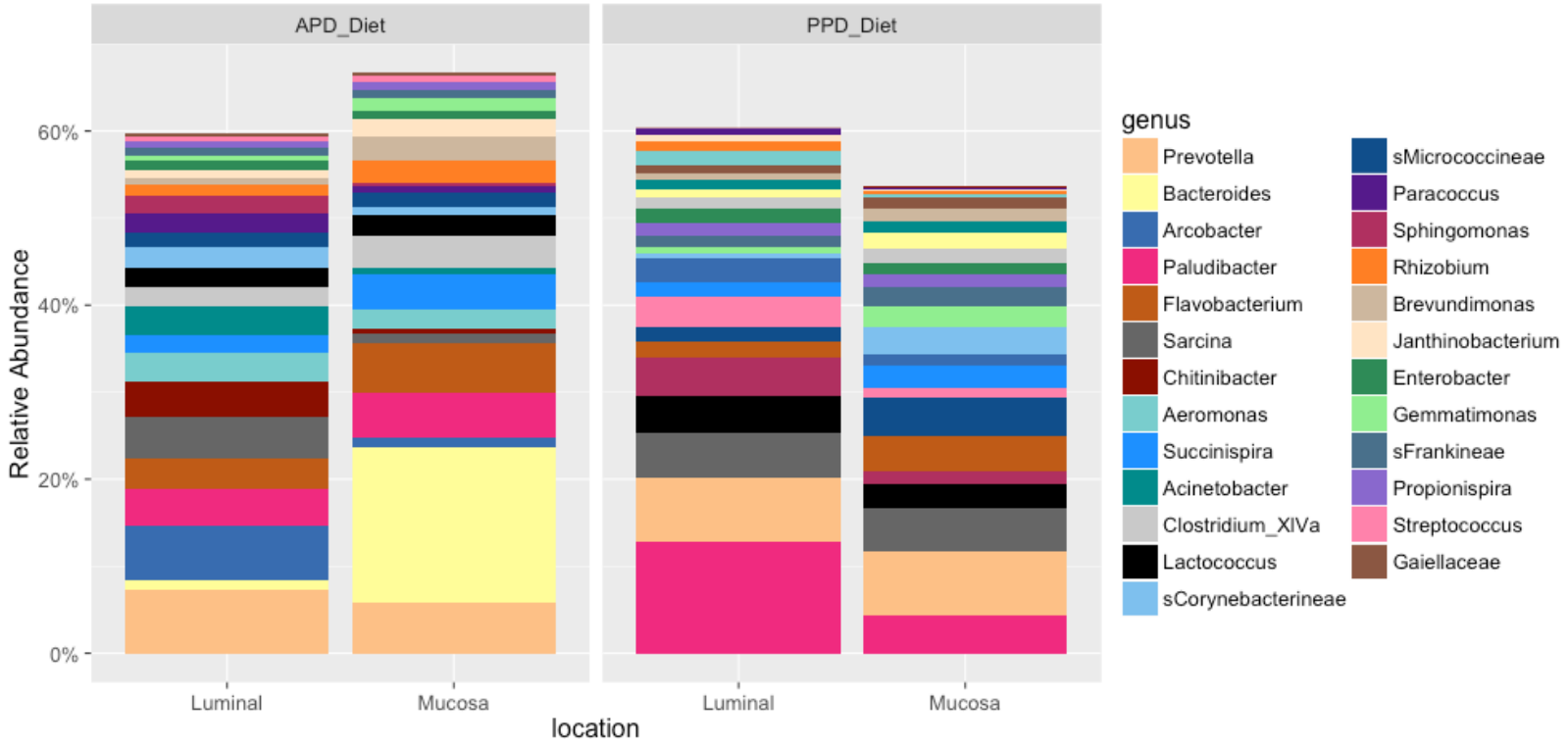

Fig. 5. Bar chart of the relative abundance of bacterial community compositions at genus level at GIT locations.

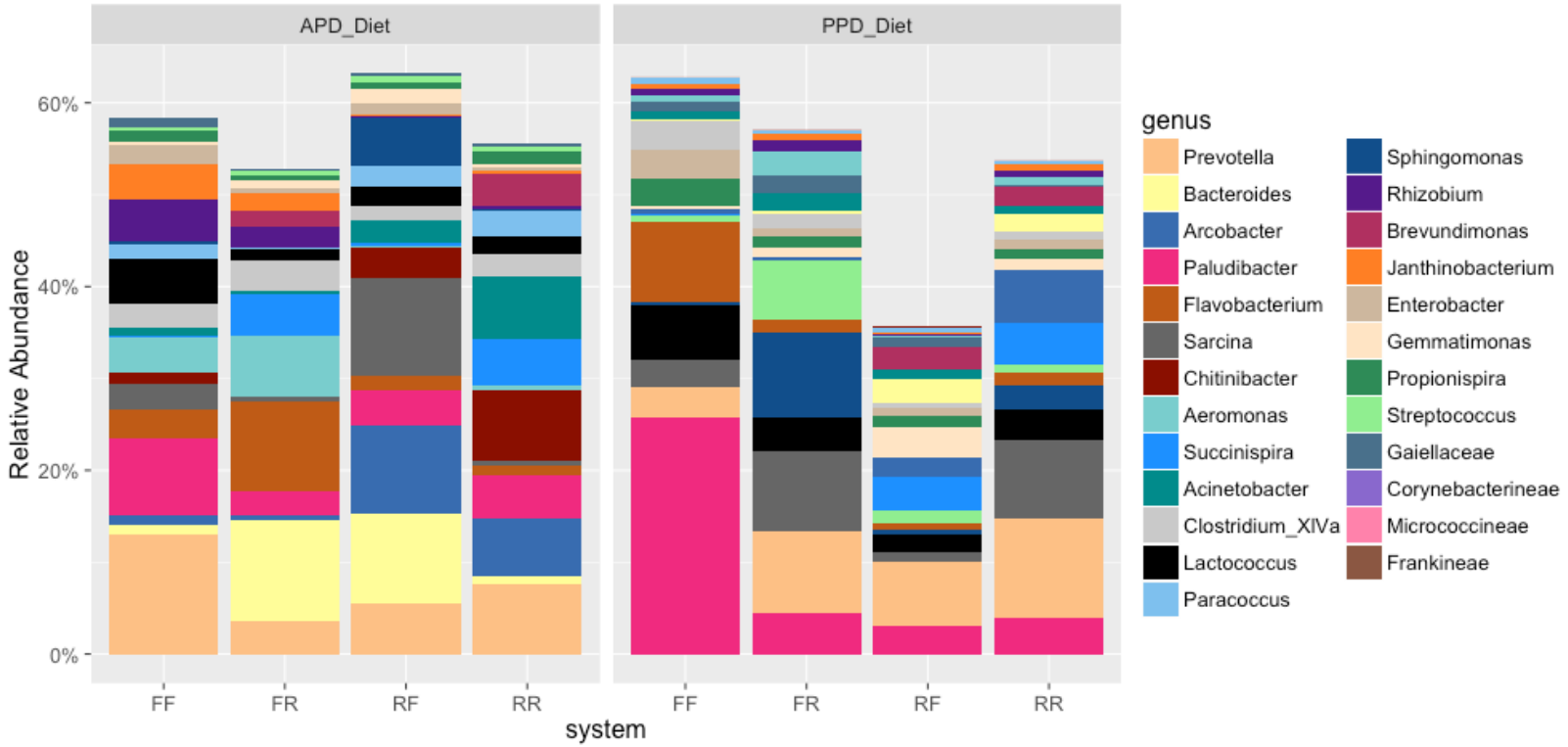

Fig. 6. Bar chart of the relative abundance of bacterial community compositions at genus level at rearing water levels. Flow-through (FF), moved from flow-through to recirculating (FR), recirculating (RR) and moved from recirculating to flow-through (RF).

Fig. 7. Bacterial composition that are significantly different at the genus level between luminal (L) and mucosal (M) GIT sections.

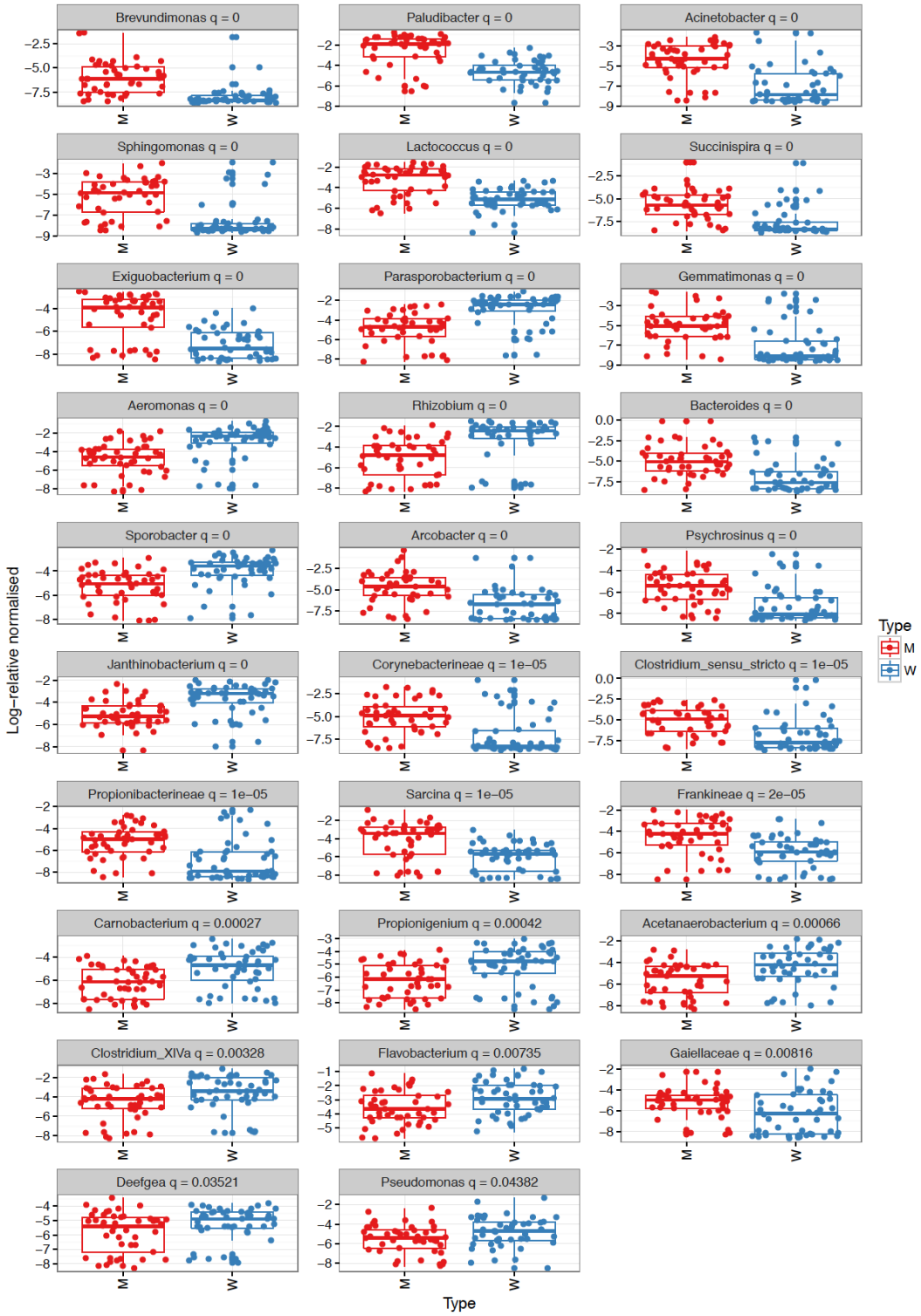

Fig. 8. Bacterial composition that are significantly different at the genus level between mucosal (M) and water (W) samples.

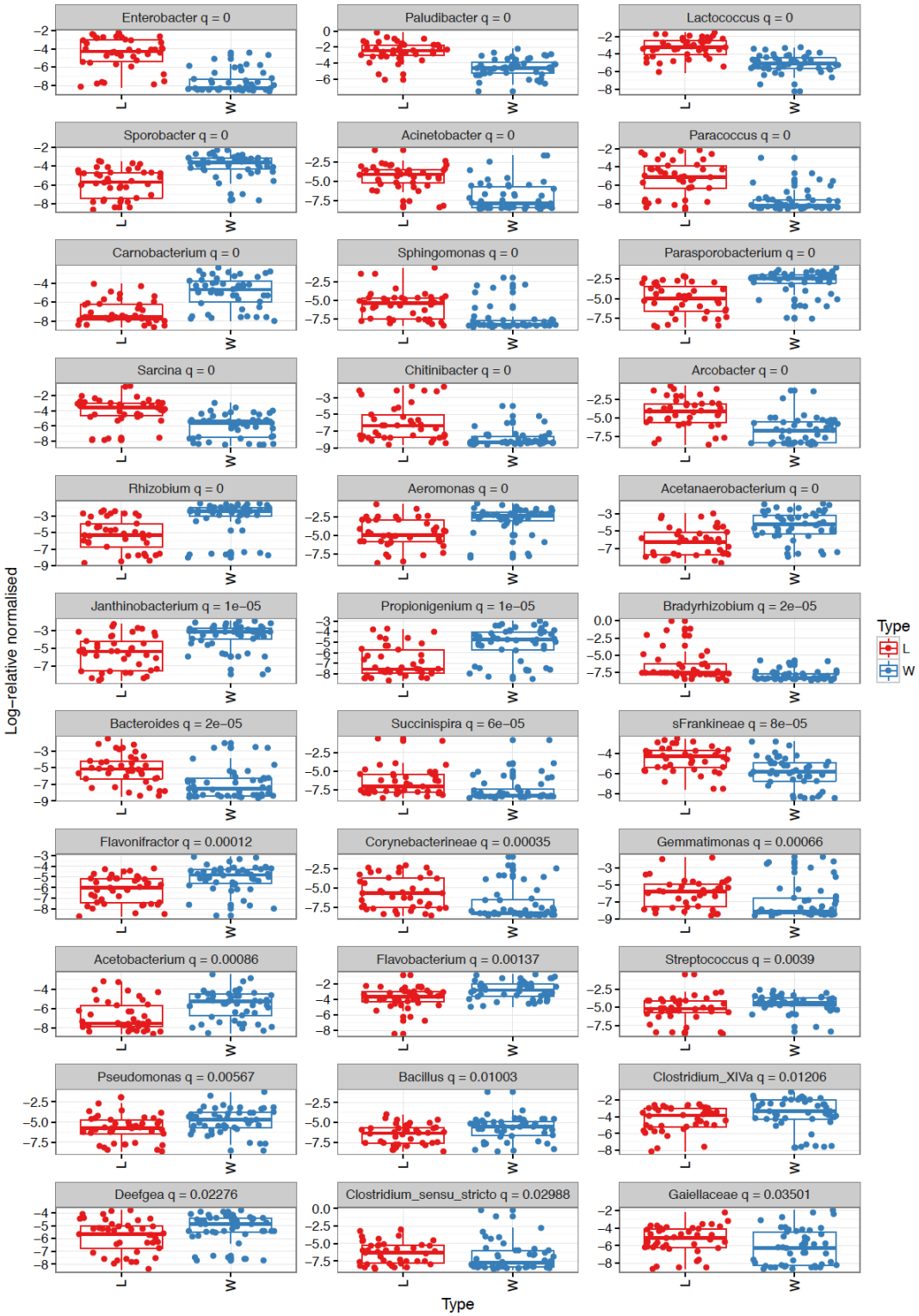

Fig. 9. Bacterial composition that are significantly different at the genus level between luminal (L) and water (W) samples.

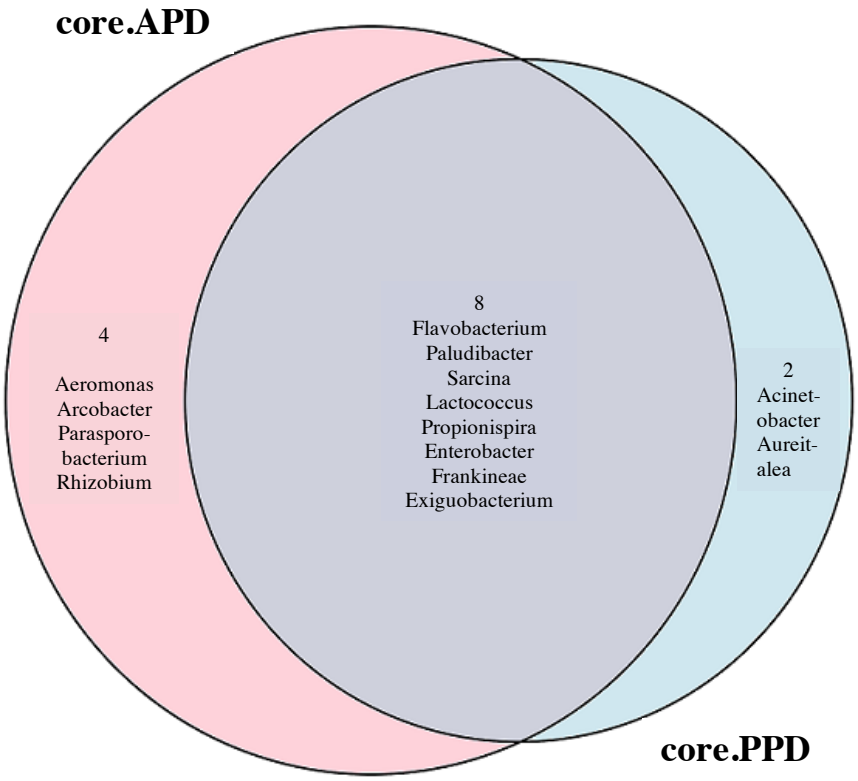

Fig. 10. Venn diagram showing core bacteria associated with animal protein diet (APD) and plant protein diet (PPD).

CHAPTER SEVEN

DISSERTATION CONCLUSION

For centuries, fishmeal has been utilized as the primary protein source for aquaculture as it provides all nutrients required by fish for growth and development. However, static or declining availability of fishmeal from capture fisheries necessitates alternative protein sources to meet the growing demands of the aquaculture industry and ensure its sustainability. Consequently, in recent years, nutritional research has focused on alternative protein sources (Gomes et al., 1995; De Francesco et al., 2004; Burr et al., 2012; Hauptman, 2012) leading to substantial progress. In particular, the use of crystalline amino acids to balance amino acid profiles of plant and animal diets to the meet fish's nutritional requirements have proved successful in overcoming production deficiencies (Davies and Morris, 1997; Furuya et al., 2004; Gaylord and Barrows, 2009; Gaylord et al., 2009). However, suboptimal growth and feed utilization are commonly observed when complete replacement of fishmeal has been attempted (Barrows et al., 2007).

Carnivorous fishes like rainbow trout are an important aquaculture species in North America that requires a quality protein diet to meet their metabolic demands. Hence, development of alternative diets, that are sustainable economically, and in terms of availability, and suitability to replace conventional fishmeal diet is critically needed to ensure the continued success of the aquaculture industry.

The focus of the current study was to identify alternative ingredient combinations that can efficiently replace the conventional fishmeal diet for commercial production of

rainbow trout and moreover, to investigate changes in microbial compositions and functions that are related to nutrient utilization in trout when fed alternative protein diets. In addition, to determine if MOS and GroBiotic-A prebiotics inclusion in GDDY diet could improve nutrient utilization and how this impacts GIT microbiota and their functions. Finally, to determine the influence of diet and rearing water, as environmental factors, on 16S ribosomal RNA gene bacterial composition in trout GIT. Outcomes from these studies indicated that reducing protein level from 45% CP to 38% digestible protein (40% CP) with amino acids supplementation improved growth performance of rainbow trout. Microbiota composition in the trout GIT were primarily bacterial, mainly from *Firmicutes*, *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes*. However, significant population from Eukaryotes and Archaea were also observed. The rainbow trout GIT microbiome appeared to be partitioned into midgut and hindgut specific populations who were focused on clearly defined nutrient metabolisms. GIT microbiota of rainbow trout demonstrated distinctive metabolic functions along the GIT, with active metabolism of sulphur and aromatic amino acids in the midgut region of fishmeal fed trout while microbial genes related to carbohydrate metabolism were observed in the hindgut section of trout fed the plant protein-based diet. While diet appeared to modulate the hindgut microbiome in our first study, the water environment appeared a greater influence than diet in modulating the microbial community composition in the GIT.

Results from the second feeding trial indicated that 75% of GDDY could replace fishmeal in trout diet when lysine, methionine and threonine are balanced based on the amino acids profile in trout muscle and this improvement in growth was not associated to

prebiotics inclusion. However, the high level of GDDY increased feed intake and feed conversion ratio. Insignificant marked inflammation, increased thickness of connective tissue and lamina propria were observed in same diet, but did not affect acetic, propionic, and butyrate acids production compared to the fishmeal-based diet. Microbial gene functions suggested greater potential for amino acid catabolism through glutamate dehydrogenase pathway due to high GDDY inclusion levels.

Third feeding trial suggested that rearing water has a greater influence on the composition of rainbow trout GIT than diet with substantial enrichment of *Aeromonas* and *Acinetobacter* genera in the rearing water samples. These results also confirmed the importance of host physiology influencing microbial population in the GIT sections of trout, with more bacterial population in the mucosal region of the intestine and substantial populations of these bacteria were associated with metabolisms of certain amino acids and carbohydrate.

This work is the first to provide information on the GIT microbial community of rainbow trout and their potential functions. In addition, information on the bacterial population which colonize recirculating and flow-through rearing environments and how they impact GIT bacterial population in cultured rainbow trout has been lacking. From the results obtained from this research, I suggest further nutrition studies to determine how specific ingredients alter the microbial gene functions for possible elimination of anti-nutritional factors that are present in alternative ingredients to improve their utilization in aquafeed industry for rainbow trout production.

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