Investigating the response of the gut bacterial community and enzyme activity during the challenge of diet manipulation in the herbivorous fish *Campostoma anomalum* (Central stoneroller) and the carnivorous fish *Etheostoma caeruleum* (Rainbow darter)

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Abstract:

Enzymes are biologically important as they are involved in metabolic processes including catabolizing macromolecules for cellular fuel production and maintaining homeostasis. The combined influence of the GIT (gastrointestinal tract) bacterial community and altered diets on the activity of enzymes has been previously postulated but not studied in depth. Therefore, a proper understanding of the contribution of the GIT bacterial community and diet towards the enzyme activity in the GIT and other tissues was required. Hence, in this thesis, I characterized the GIT bacterial communities and enzyme activates in the herbivorous *C.anomalum* (central stoneroller) and the carnivorous *E.caeruleum* (rainbow darter) for the first time. Through this thesis, I deduced that the GIT, GIT bacterial community, and GIT enzymes of both fish species each responded distinctly against the challenge of an altered diet and positively benefitted their host in maintaining an overall pinnacle digestive physiology. This response appears confined to the GIT.

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ADP- Adenosine diphosphate
AKG- Alpha-ketoglutarate
ATP- Adenosine triphosphate
BAPNA- Nα-benzoyl-L-arginine-p-nitroanilide hydrochloride
BBE- Brush border enzyme
CMC- Carboxymethylcellulose
CS- Citrate synthase enzyme
DNS- Dinitrosalicylic Acid
DTNB- 5, 5'-dithiobis-(2-nitrobenzoic acid)
DTT- Dithiothreitol
EDTA- Ethylenediaminetetraacetic acid
FAO- Food and agriculture organization of the United Nations
FiaF- Fasting induced-adipose factor
GALT- Gut-associated lymphoid tissue
GDH- Glutamate dehydrogenase enzyme
gDNA- Genomic deoxyribonucleic acid
GIT – Gastrointestinal tract
HEPES- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
KEGG- Kyoto encyclopedia of genes and genomes
LDH- Lactate dehydrogenase enzyme
MID- Molecular identifier
MOL- Map of life
MS-222- Tricaine mesylate
NAD- Nicotinamide adenine dinucleotide
NADH- Nicotinamide adenine dinucleotide (reduced Hydrogen)
NKA- Na^+/K^+ ATPase enzyme
OAA- Oxaloacetate
OTU- Operational taxonomic units
PBS- Phosphate-buffer saline
PCR- Polymerase chain reaction
PEP- Phosphoenolpyruvate
PK- Pyruvate kinase enzyme
P-PAR- Peroxisome-proliferation activated receptor
RDA- Redundancy analysis
rRNA- Ribosomal ribonucleic acid
SCFA- Short-chained fatty acids
UPGMA- Unweighted Pair Group Method with Arithmetic mean

Chapter 1: General Background

1.1. An overview of the gastrointestinal tract (GIT):

The GIT is the mediator for food intake, energy/nutrient absorption, and fuel provision for cellular metabolism in all higher organisms (Karasov and Douglas 2015). These vital roles of the GIT were traditionally viewed as dependent on endogenous digestive and metabolic processes supplemented by enterocyte enzyme complements and nutrient transporters. However, the role of the bacterial inhabitants in these processes has rarely been studied. Enzymes are important biological catalysts involved in metabolic processes, such as catabolizing macromolecules, and in other physiological functions, such as the maintenance of homeostasis. In the GIT, enzymes are particularly important in the conversion of ingested food to cellular fuel. For example, enzymes of the Krebs cycle like Citrate Synthase (CS) are used by the host GIT to convert energy derived from food nutrients like carbohydrates, fats, and proteins to fuel for cellular metabolism and maintenance of homeostasis (Mommsen et al. 2003; Willmott et al. 2005). The traditional paradigm of the piscine GIT attributes enzyme activity patterns to both feeding patterns and the food consumed (German et al. 2003). In particular, most studies in fish have focused on the impact of diet manipulation on the activity of the brush border (the microvilli-covered surface of intestinal epithelial cells) enzymes (BBE) and have uncovered the importance of dietary composition in many GIT enzyme activities across diverse species (e.g. Chakrabarti et al. 1995; Al Hafedh 1999; Krogdahl et al. 1999; Furne et al. 2005). However, the GIT is also home to millions of individuals from the domains of Archaea, Eukaryota, and Bacteria, forming a diverse microbiome community residing within the digestive tract (Luczkovich and Stellwag 1993; Klein et al. 1998; Saha and Ray 1998; Hakim et al. 2006;

German and Bittong 2009; Ghanbari et al. 2015). This community may also contribute to the vital roles of the GIT in obtaining energy from ingested food through exogenously produced enzymes which bolster the overall digestive capability of the intestinal tract (Liu et al. 2016). This area is currently under researched, and my thesis will contribute to this novel understanding of gut physiology. In particular, I will provide insight into the interplay between diet manipulation, bacterial species, and enzyme activity across a suit of enzymes involved in energy metabolism. Associations that I discover could be used to maximize energy metabolism, and hence growth of species, a particularly important aspect of aquaculture.

1.2. The GIT Microbiome:

Bacteria, prokaryotic microorganisms inhabiting most environments on the planet, can live as parasites or as symbionts within animal GITs. In humans, the GIT resident bacteria are so abundant that they outnumber the somatic and germ cells at a ratio of 10:1 (Shanahan 2002; Hara and Shanahan 2006). As a result, the bacterial community and its genome are present well in excess of those of the host, leading some researchers to consider the bacterial community as a separate organ within the GIT (Shanahan 2002; Hara and Shanahan 2006). Two distinct groups of bacteria form the primary GIT populations. The first are autochthonous, or adherent, which consistently reside in the intestinal tract (Yoshimizu et al. 1976; Savage 1989; Ringo and Olsen 1999; Nayak 2010). The second are allochthonous, or transient bacteria, that may be ingested with a meal, but are subsequently expelled from the GIT along with the digesta (Yoshimizu et al. 1976; Savage 1989; Ringo and Olsen 1999; Nayak 2010).

While bacteria form a large and diverse domain, the fish microbiome itself is home to a much narrower diversity of autochthonous bacteria. At the phylum level, Firmicutes, Bacteroidetes, and Proteobacteria are abundant in the GIT, forming the core bacterial community

present across fish species (Bolnick et al. 2014; Ghanbari et al. 2015). An increase in the abundance of one GIT bacterial phylum generally occurs at the expense of the other two, and these changes have been correlated with changes in the host's habitat and diet (Ghanbari et al. 2015). The GIT of the teleost fish is an open system and comes in constant contact with surrounding water of varying contents, and the surrounding environment plays a significant role in forming the teleost's GIT bacteria community (Hansen et al. 1992; Perez et al. 2010). For example, members of genera *Carnobacterium* and *Vibrio* are frequently found in marine fish GIT bacterial communities, while the genera *Clostridium* and *Fusobacterium* are found in freshwater fish bacterial communities (Cahill 1990; Hansen et al. 1992; Austin 2006; Gomez and Balcazar 2008; Perez et al. 2010). Additionally, the influence of diet upon the fish GIT bacterial community can be dependent on the level of variety in that diet -i.e. fish consuming a homogenous diet have less GIT bacterial community richness than fish feeding on varying diets (Bolnick et al. 2014). However, certain dietary ingredients are often associated with particular phyla, such as Firmicutes with lipid rich diets (Turnbaugh et al. 2006; Parfrey et al. 2011; Angelakis et al. 2012; Carmody and Turnbaugh 2012; Givens 2012; Semova et al. 2012), Bacteriodetes with cellulose-rich diets (Ley et al. 2006; Turnbaugh et al. 2006; Ni et al. 2014; Miyake et al. 2015), and Proteobacteria with protein-rich diets (Bairagi et al. 2002; Ghosh et al. 2002, Ray et al. 2012; Banarjee et al. 2016; Andrade et al. 2017). Therefore, habitat and dietary differences can be the determinants of the GIT bacterial community composition.

1.2.1 Technological advancement in the study of the GIT bacterial community:

99% of all known bacterial species, including those that inhabit the GIT, are poorly culturable or currently unculturable (Hopkins et al. 2002; Baker et al. 2003; Hara and Shanahan 2006; Welch and Huse 2011; Ghanbari et al. 2015). As a result, identification of bacteria species

within the GIT was limited to only those that could be grown on agar plates and characterized. Research relying upon plate culture techniques was only able to tell part of the story of how bacterial symbionts function within the intestinal tracts of fish and other vertebrates. However, with the advent of next generation sequencing, unculturable bacterial species have been identified and quantified, providing valuable insight into the understanding of the GIT bacterial community structure (Hopkins et al. 2002; Baker et al. 2003; Hara and Shanahan 2006). This advancement has resulted in an explosion of research, characterizing the GIT microbiome across animal species.

In essence, next generation sequencing techniques rely on the 16S rRNA gene. This gene has several hypervariable regions surrounded by conserved regions. Little has changed in the conserved portions of the gene sequence over millions of years of evolution and among bacterial species, allowing researchers to amplify all species with a common set of primers targeting these regions and PCR. However, the hypervariable regions are specific to each species, and sequencing of each region can be used to identify individuals. Ultimately, amplifying all species present and then using next generation sequencing to identify individual species, provides a basis for estimating bacterial phylogenetic diversity and generating taxonomic inventories of the bacterial community populations. Indeed, through the identification of the evolutionary distances between orthologous sequences or similarities to database entries in a BLAST or FASTA classifier, bacterial communities can succinctly be identified into operational taxonomic units (OTUs) which corresponds to the class, the genera, or the species of the bacteria (Li et al. 2014).

1.3. Mutualistic relationship between the host and the GIT bacterial community:

Mutualism between the host GIT (providing a favorable environment) and the bacterial community (digesting, assimilating, and sometimes transporting essential nutrients) is an

essential relationship for optimal digestive performance (Hooper and Jeffrey 2001; Sachs et al. 2011; Sullam et al. 2012; Tremaroli and Bäckhed 2012). Recent studies have shown that host-bacteria interactions are essential to many aspects of normal physiology that include metabolic activity and immune system homeostasis (Rawls et al. 2004; Perez et al. 2010). For example, the GIT bacterial community has been shown to enhance nutrient transport by modulating the GIT enterocytes, and by enhancing proliferation of existing blood vessels through angiogenesis, or the creation of new blood vessels (Rawls et al. 2004; De et al. 2015; Wu et al. 2015). Furthermore, the GIT bacterial community can influence its host's ability to efficiently process a meal, through manipulation of the bacteria-associated catabolic (Xu et al. 2003; Sonnenburg et al. 2005; Ray et al. 2012) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways (Kanehisa et al. 2000; Mardanov et al. 2013; Ni et al. 2014; Kanehisa et al. 2016; Kanehisa et al. 2017). For example, various cellulolytic and amylolytic *Bacillus* strains were introduced into a strict carnivore, the Asian seabass (*Lates calcalifer*), that was then fed a plant protein diet for 30 days (De et al. 2015). Following the introduction of these *Bacillus* strains, the seabass could effectively digest the plant proteins, providing the host with the increased ability to absorb nutrients from this otherwise non-native diet. This resulted in a higher survival and protein efficiency ratio in comparison to the wild control group (Dhage 1968; Bairagi et al. 2002; De et al. 2015). By manipulating the shift in the GIT bacterial composition to favor digestion of a plant-based diet, this carnivorous fish was then able to digest plant-based proteins.

However, the effects of an altered diet and the GIT bacterial community composition on the activity of many enzymes are not fully understood. For example, lactate dehydrogenase (LDH) is a metabolic enzyme catalyzing the reversible conversion of pyruvate to lactate, and this enzyme plays a significant role in connecting aerobic and anaerobic metabolism. LDH-encoding

gene clusters (lutABC) have been identified in bacterial species commonly found in the human GIT, such as *Escherichia coli, Saccharomyces cerevisiae, Bacillus subtilis, Campylobacter jejuni,* and *Shewanella onedensis* (Gao and Gupta 2005; Chai et al. 2009; Thomas et al. 2011). The survival of these bacterial species depends on the expression of the lutABC genes for exogenous LDH production in response to a low-fat diet fed to the human host (Marshall et al. 1976). This correlation between the GIT LDH enzyme activity, GIT bacterial community, and the diet has only been studied in mammals and studies are still in the preliminary phase in fish. Another glycolytic enzyme, pyruvate kinase (PK) is an allosteric enzyme that links glycolysis (primary energy metabolism) to cellular metabolism. It catalyzes the conversion of phosphoenolpyruvate and adenosine diphosphate (ADP) to pyruvate and adenosine triphosphate (ATP) which then goes on to be used in the citric acid cycle (Thillart and Smit 1984; Seeto et al. 1996; Mommsen et al. 2003; Veith et al. 2013). Lactic acid bacteria, especially *Streptococcus pyogenes*, *Enterococcus faecalis,* and *Lactobacillus plantarum* rely on glycolysis to integrate PK into their metabolic processes (Thillart and Smit 1984; Seeto et al. 1996; Veith et al. 2013). Previous studies have shown that the above-mentioned bacteria are also present in the GIT of humans and other terrestrial vertebrates, where they induce PK production in response to increased dietary carbohydrate influx (Veith et al. 2013). However, this correlation between the GIT PK enzyme activity, the GIT bacterial community, and the diet is only understood in case of the terrestrial vertebrates and our understanding of this correlation in fish is unknown. Furthermore, the possibility that any changes in the activities or gene expression of these glycolysis enzymes influencing the activity of other downstream enzymes in glycolysis or the citric acid cycle requires further contemplation. For example, citrate synthase (CS) is a mitochondrial enzyme of the citric acid cycle that catalyzes the condensation of acetyl-CoA

(obtained from the glycolysis pathway) and oxaloacetic acid to form citrate. This initiates the first reaction of the aerobic metabolic process known as the citric acid cycle for energy production in the form of ATP (Mommsen et al. 2003; Willmott et al. 2005). Although some previous studies have demonstrated a decrease in CS activity among several strains of the bacteria *Corynebacterium glutamicum* (Ooyen et al. 2012), the influence of the diet, possible GIT bacterial community abundance change, and the possible effects of a modified glycolysis pathway on the activity of this enzyme in the fish GIT is not yet studied.

1.3.1. Contribution of the GIT bacterial community towards digestive physiology in herbivores:

Herbivory, or the ingestion of plant material, is a trait often found in terrestrial animals that have developed digestive chambers like the colon and fermentation chambers like the rumen (Sellers et al. 1982; Sutherland 1988; Sakaguchi 2003; Flint et al. 2008). These chambers are used to house a bacterial population necessary for the digestion of ingested food for which the organism lacks the required endogenous mechanisms i.e. cellulose (Sellers et al. 1982; Sutherland 1988; Flint et al. 2008). Aquatic herbivores also take advantage of bacterial enzymes to obtain nutrients (Luczkovich and Stellwag 1993; Saha and Ray 1998), often in the absence of these dedicated chambers. Herbivory in the aquatic environment presents a unique challenge, since aquatic plants contain only a fraction of the amino acid and lipid concentration found in their terrestrial counterparts (Rimmer and Wiebe 1987; Clements et al. 1989; Angert et al. 1993; Mountfort et al. 2002). To compensate for this, the host must rely on its GIT bacterial community to extract and process these precious nutrients. For example, in the herbivorous grass carp, amino acid requirements are fulfilled by the metabolic and catabolic function of the GIT Bacteroidetes community (German and Bittong 2009, German 2009; Ni et al. 2014). Moreover, Ni et al. (2014) demonstrated that the phylum Bacteroidetes is critical in the biosynthesis of at

least 12 amino acids in herbivorous fish. Furthermore, it has been proposed that Bacteroidetes activate the metabolic/catabolic pathway by expressing the Fasting induced-adipose factor (FiaF) which activates the Peroxisome-proliferation activated receptor (P-PAR), increasing the metabolic activity of the cellular peroxisomes in the host's GIT (Kihara and Sakata 2001; Ni et al. 2014; Konishi et al. 2015).

1.3.2. Contribution of the GIT bacterial community towards digestive physiology in carnivores:

Unlike herbivores, most of the diet of carnivores is readily digestible. However, a carnivorous diet may also contain some nutritionally rich material that cannot be processed by the host. For example, glycoprotein-rich matter like bone, tendon, and hair also compose a carnivore's diet, yet these components are difficult to break down through the host's endogenous resources and pathways. They do, however, provide a potential substrate for the GIT bacterial community (Depauw et al. 2013), and the resident GIT bacterial communities have demonstrated to aid in the digestion and absorption of these glycans (Sonnenburg et al. 2005). Like their terrestrial counterparts, aquatic carnivores feed on a diet rich in animal protein, and they depend on their GIT bacteria for the complete digestion and absorption of the dietary amino acids. Although not yet identified in fish; 9 bacteria-induced amino-acid degradation pathways have been previously identified in mice models fed commercial protein diets (Ni et al. 2014). Furthermore, it has previously been shown that saccharides resistant to digestion by endogenous enzymes are fermented by the GIT bacterial community, by way of their exogenous enzymes, into short-chain fatty acids (SCFA), such as acetic, propanoic, and n-butyric acids (Furne et al. 2005). Additionally, increases in the levels of fatty acid deposition in hosts that feed on a commercial diet has been attributed to the GIT bacterial phylum Firmicutes (Semova et al. 2012), and increases in the dietary amino-acid degradation through the production of serine

protease enzyme like trypsin in hosts that generally feed on a protein diet has been attributed to the GIT bacterial phylum Proteobacteria (Krogdahl et al. 1994; Bairagi et al. 2002; Ghosh et al. 2002; Ray et al. 2012; Banerjee and Ray 2016; Andrade et al. 2017).

1.4. The objective of this project:

The GIT is a remarkable organ made of multiple contributing units including the endogenous enzymes, the endogenous nutrient transport pathways, the microbiome, the exogenous enzymes, and the exogenous microbiome mediated digestion/energy transport pathways. Therefore, understanding the adjustments made by the individual components of the GIT system is key to understanding the overall response of the GIT and the entire body of the host when faced with an external challenge, such as that of an altered diet. Furthermore, identifying responses of pathways involved in energy metabolism (such as CS) and energy use (such as NKA) is useful in predicting if these responses are either beneficial or harmful for the host's growth rate in response to diet manipulation. Therefore, the primary objective of this thesis was to understand the response of the GIT bacterial community and the corresponding digestive and metabolic enzyme activity adjustments following dietary alterations in the herbivorous central stoneroller (*Campstoma anomalum*) and the carnivorous rainbow darter (*Etheostoma caeruleum*). In the second chapter of this thesis, I will elaborate on the response of both the GIT bacterial community and the host digestive and metabolic enzyme activities toward a commercial fish feed diet in the central stoneroller. In the third chapter of this thesis, I will elaborate on the response of both the GIT bacterial community and the host digestive and metabolic enzyme activities towards a commercial fish feed diet compared to a protein diet in the rainbow darter. Finally, in the fourth chapter of this thesis, I will draw a conclusion based upon

my findings about the response of the GIT bacterial community and the digestive and metabolic enzyme activities of the two fish species in response to an altered diet.

Chapter 2: The response of the GIT bacterial community and the digestive/metabolic enzyme activity in response to an altered diet in the herbivorous fish Campostoma anomalum

Introduction:

2.1. The diet, GIT bacterial community, and digestive enzymes in an herbivorous fish:

The diet of herbivorous fish consists primarily of algae material rich in cellulose and hemicellulose (Ojeda and Caceres 1995; Jurick II et al. 2012; Anand et al. 2014; De et al. 2015). However, the endogenous production of cellulase to degrade this dietary cellulose is not well understood. Cellulose is the primary structural material of a plant cell wall, and the main source of glucose and fiber for herbivorous fish. To digest algae and plant material, the cell walls are lysed in the acidic stomach (PH 1.6 – 2; Ojeda and Caceres 1995) or in the anterior intestine of fish that lack a stomach (Lobel 1981). The cellulose is then degraded into β-glucose by cellulase, but the production of the cellulase enzyme is poorly understood. It was initially thought that herbivorous fish possessed endogenous cellulose, which would facilitate the breakdown of cellulose (Ojeda and Caceres 1995). However, it was later determined through the isolation of *Trichoderma*, *Aspergillus*, *Bacillus*, *Aeromonas*, and *Clostridium* that these GIT bacteria were capable of producing cellulase exogenously in the GIT of herbivorous fish (Luczkovich and Stellwag 1993; Saha and Ray 1998). While this suggests that exogenous bacterial enzymes also play a key role in digestion alongside endogenous enzymes, it is still unknown whether these cellulase enzyme producing bacteria are part of the allochthonous or the autochthonous bacterial communities within many herbivorous fish.

However, it is clear that bacterial symbionts are involved in digestion and fermentation of the ingested algae material (Mountfort et al. 2002; Givens 2012). The colonization of the herbivorous fish GIT by different bacterial phyla, genera, or species is influenced by the endogenous factors such as the genetics and the immune response of the host (Fuller 1989). Therefore, the survival of the GIT bacterial community depends on the capacity of the bacterial species to overcome these internal challenges posed by the host. In addition, exogenous stressors, particularly diet, also pose a challenge to the survival and proper functioning of the GIT bacterial community. For example, some herbivorous fish such as the pinfish (*Lagodon rhomboids*) undergo dietary transitions that accompany development. Interestingly, this dietary transition correlates with a shift in the organization of the GIT bacterial community which favors symbionts that can hydrolyze the ingested plant materials of the adult's diet (Luczkovich and Stellwag 1993; Benavides et al. 1994; Ojeda and Caceres 1995; Ringo et al. 1995; Givens 2012; Ringo et al. 2016).

The herbivorous piscine diet, is primarily composed of carbohydrates such as glucose, but lacks adequate protein content. Interestingly, herbivorous fish have a well-developed GIT bacterial community, maximizing yield from the protein-deficient diet (German et al. 2014). The endogenous trypsinogen production in the GIT (Divakaran et al. 1999; Pujante et al. 2017) and exogenous trypsin production by the resident GIT bacterial community (Sumathi et al. 2012; Andrade et al. 2017; *Escherichia coli*) may provide herbivores with an increased protein assimilation, that would otherwise be limited through endogenous mechanisms alone (Sumathi et al. 2012; German et al. 2014). Additionally, herbivorous fish demonstrate low endogenous lipase activity (Tengjaroenkul et al. 2000; Tilapia) compared to carnivorous or omnivorous fish. As with proteins, it has been proposed that lipid digestion in the herbivorous fish is supported by the

exogenous lipase produced by resident GIT bacteria such as *Acinetobacter venetianus*, *Aeromonas* sp, *Bacillus* sp, and *Citrobacter braakii (*Dutta et al. 2015; *Catla*, Dey et al. 2016; Catfish, Chen et al. 2016; Turbot). This suggests that herbivore hosts process, extract, and absorb lipids from a nutrient-deficient diet via support from the microbiome (Tengjaroenkul et al. 2000; Bairagi et al. 2002; Dutta et al. 2015; Koca et al. 2015; Chen et al. 2016; Dey et al. 2016). However, like the other digestive enzymes mentioned above, the subsequent influence of the challenge of an altered diet and the effect of the restructuring of the GIT bacterial community on lipase activity has not yet been established in either terrestrial or aquatic herbivores.

2.2. Effects of an altered diet on the GIT metabolic enzyme activity of an herbivorous fish:

The combined effects of an altered diet and the GIT bacterial community on the activity of the electrogenic transmembrane ATPase enzyme is not well understood. Na^+/K^+ ATPase (NKA) is a transmembrane enzyme that maintains the ionic/electrochemical gradient in a cell through the active transport of sodium and potassium ions across the cell membrane using ATP as an energy source (Skou 1989). The ionic gradient created by NKA is critical in regulating Na^+-coupled transport of nutrients and amino acids across the cells (Hakim et al. 2006). Diet is a major exogenous stressor faced by the enterocytes and the NKA (Skulachev 1978, Dibrova et al. 2015). Most previous studies however have focused only on the endogenous NKA activity in the kidney of terrestrial vertebrates and the gill, kidney, and liver of fish, especially on widely-used model omnivorous fish such as *Danio rerio* (zebrafish) and carnivorous fish, like *Oncorhynchus mykiss* (rainbow trout). Unfortunately, whether the composition of the GIT bacterial community benefits or hinders GIT NKA activity is not yet known for herbivorous fish. Therefore, understanding the challenge of an altered diet on the GIT bacterial community and the GIT NKA

activity in an herbivorous fish is an initial step to further understand the interplay between the diet, the resident bacterial community, and the enzyme.

The effect of an altered diet and the GIT bacterial community influencing the ability of the herbivorous fish intestinal tract to detoxify ammonia from its system also requires further investigation. For example, glutamate dehydrogenase (GDH) is an enzyme that catalyzes the conversion of α-ketoglutarate (glutamic acid) into glutamate (amino acid) by removing ammonium. Previous studies indicate the forward reaction is mediated by the GIT bacterial community; especially by the Firmicutes (Nazarczuk et al. 1981; Sanchez-Muros et al. 1998). For example, GDH activity in the intestinal tract of the wood-eating termite *Nasutitermes exitiosus* decreased by 75 % when the anaerobic bacteria *Spirochaetes* was eliminated using an oxygen treatment (Nazarczuk et al. 1981). Although it is postulated that the GIT bacterial community would play a similar mediatory role in herbivorous fish, this is currently unknown. Hence, it is critical to understand the overall influence of an altered diet and the possible GIT bacterial community restructuring as a result of dietary alteration on the GDH activity in the GIT and on the ability of the herbivorous fish GIT to detoxify ammonia.

2.3. Objectives and Hypothesis:

C. anomalum is a benthic species, whose diet consists of algae, such as *Spirogyra*, *Rhizoclonium*, and *Chlorella* (Power 1983). This fibrous, cellulose-rich plant material is nutritionally devoid (Power 1983). We explored the GIT of this species, both in terms of the microbiome but also the enzymatic activities. Furthermore, we wanted to understand how this species would react to an increase in dietary fat and protein. In this study, the native bacterial community of the central stoneroller was characterized for the first time. This is also the first-time enzymes have been measured in this fish. We hypothesized that the composition of the GIT

bacterial community will transition in abundance from the phylum Bacteroidetes to the phylum Firmicutes in response to a shift in diet from that of a natural algal diet to that of a commercial fish feed (high-lipid) diet. Secondly, as observed but not completely understood in previous studies, we predicted that the GIT bacterial community abundance transition from the phylum Bacteroidetes to that of phylum Firmicutes will influence the GIT enzyme activities in response to the above dietary change. Finally, to understand the response of the full-body enzymatic physiology in response to an altered diet, an altered GIT bacterial community abundance, and an overall change in the digestive and metabolic enzyme activity in the GIT, we hypothesized that the enzyme activity in reference tissues (white muscle, liver, kidney, and gill) will be altered.

Overall, the main purpose of this novel study was to understand how the GIT bacterial community, the digestive and metabolic GIT enzyme activities, and full-body enzyme activities respond to transitioning from a natural algal diet to a commercial fish feed diet in herbivorous fish, using the central stoneroller as a model organism.

Materials and Methods:

3.1. Model fish species collection:

The sampling location for fish collection was initially identified using the Map of Life species distribution database (MOL, Yale University). We collected stonerollers from a single sampling site (Irvine Creek; 43°46’12” N,80°21’29” W), a tributary of the Grand River, adjacent to the town of Bellwood, Ontario. Fish were collected using dip nets, float nets, and minnow traps. The collected fish were then placed in a 20 L cooler chest filled with source water and transported back to the lab. Precautionary measures were taken to prevent the stress of over-heating, ammonia toxicity, and hypoxia during the transportation process. Finally, 10 individuals (average mass= 8 ± 3 gram, average length= 7 ± 2 cm) were sampled the same day aseptically as described below in 3.4. The remaining fish were held under laboratory conditions.

3.2. General fish care:

Individuals were kept in a large (50 L) solid plastic tank supplied with flow-through dechlorinated City of Toronto water, and aerated with an individual air flow at 11° C. Water temperature and fish health was monitored daily, and water quality, including ammonia, nitrate, and chlorine levels were monitored weekly.

3.3 Diet treatments:

After 7-days of laboratory acclimation during which the animals were unfed, the fish were separated into two tanks containing 20 and 25 individuals respectively. Individuals in tank 1 were fed a UV-sterilized algae diet (Table 2) and individuals in tank 2 were fed a UV-sterilized

commercial fish feed diet (Table 2). 10 individuals from each tank were then aseptically sampled 1-month and then 3-month following the first meal.

3.4. Aseptic sampling for tissue extraction and tissue storage:

All dissections were performed under aseptic conditions to prevent external agents, such as lab bacteria from contaminating the samples. The bench space was first sterilized using 70 % ethanol, and the forceps and scissors were sterilized under UV-light for 10 minutes before being placed into fresh ethanol for an additional 20 minutes of sterilization. Separate, sterile falcon tubes containing fresh ethanol were also set up and the dissection tools were transferred from one falcon tube to the next after each fish was sampled for an additional layer of protection against contamination.

Fish were euthanized with an overdose of buffered tricaine mesylate (MS-222; Siga-Aldrich, Oakville, ON, CA), and subsequently measured for mass and length. A lateral incision was then made along the ventral side of the fish to expose the internal organs. Two equal demarcations were made, and the posterior intestine was then removed, placed inside a sterile 2 ml bullet tube, snap-frozen on dry ice, and stored at -80°C for future extraction of the bacterial gDNA. The anterior intestine was freeze-clamped on dry ice and stored at -80°C for future enzyme activity assays. The gill basket was then removed and filaments were excised from the supporting cartilage. The liver and kidney were then collected, followed by white muscle tissue from the posterior area of the fish. Each of these tissues were placed in individual aluminum packets, snap-frozen on dry ice using an aluminum press, and stored at – 80°C for future enzyme activity analysis.

3.5. Genomic bacterial DNA extraction from the GIT:

The bacterial gDNA from the GIT was extracted and purified using three separate kits: Qiagen Stool kit, Qiagen DNeasy kit, and Qiagen Microbiome kit (QIAGEN, Toronto, ON) using the manufacturer's protocol. Contents of the kit, as well as pipettes and pipette tips were initially UV sterilized for 20 minutes in a flow through fume hood prior to the extraction procedure. The extracted gDNA was then quantified spectrophotometrically with a BioTek Synergy HT spectrophotometer (BioTek Instruments Inc, Winooski, VT, USA) and analyzed at 260 and 280 nm using a 260/280 ratio > 2 to confirm sufficient quality of the gDNA. With each extraction, a blank (no intestinal tract tissue present) was also tested for contamination.

3.6. Universal primer selection, design, and PCR to test for contamination:

Universal primers were designed based on the V3 and V6 hypervariable regions of the 16S rRNA (Watanabe et al. 2001; Baker et al. 2003; Welch and Huse 2011). Universal bacterial primers designed for this purpose were 338/341F – 785R for the V3 region and 967F-1177R for the V6 region (Watanabe et al. 2001; Baker et al. 2003; Welch and Huse 2011; Table 3). The primers (Sigma-Aldrich Co, The Woodlands, Texas, USA) were resuspended in RNAase and DNAase free molecular water at a concentration of 100 µM and stored at -20°C.

To test for contamination in the extraction buffers, and to confirm successful extraction of the tissue samples, PCR was performed on all samples in a Bio-Rad MyCycler (Bio-Rad Laboratories Ltd, Mississauga, Ontario, Canada) to amplify the V3 and V6 regions (Table 3). The PCR reaction consisted of 6.25 µl Dreamtaq PCR mastermix (Thermo-Fisher Scientific, Waltham, MA, USA), 1.25 µl each of forward and reverse primers, 2 µl of gDNA, and 1.75 µl of water to a total volume of 12.5 µl. The PCR conditions were as follows: an initial one-time

denaturation step at 95°C for 3 minutes, followed by a repeated cycle of 30 seconds of 95°C denaturation step, a 45 second annealing step at 55-65°C (depending on the primer being used), and a 1-minute elongation step at 72°C, followed by a final elongation step at 72°C for 5 minutes and holding at 4°C for the required amount of time. After PCR amplification, the reaction products were run on a 1.5 % agarose gel in a Tris-HCl-Acetic acid-EDTA buffer containing 0.5µg/ml of ethidium bromide. The UV image of the gel was acquired using a MiniBis Pro Imager (FroggaBio Scientific Solutions, Toronto, ON, Canada). Any indication of a band in the negative controls (extraction buffers without tissue) resulted in re-extraction of those samples with a new kit.

3.7. Sample pooling and packaging:

The extracted bacterial gDNA was pooled together within each treatment and then quantified spectrophotometrically at 260 and 280 nm using a 260/280 ratio > 2 to indicate sufficient quality of the DNA at a total concentration of 600-3200 ng/µl and was sent to Molecular Research Lab (Shallowater, Texas, USA) for Illumina sequencing.

3.8. Bacterial gDNA Illumina sequencing:

The bacterial gDNA from the pooled samples was sequenced using an Illumina MiSeq sequencer (Illumina, San Diego, CA) by the Molecular Research lab (MrDNA; Shallowater, Texas) following the manufacturer's standard protocols. Briefly, a fragment of the bacterial 16S rRNA gene, spanning the V1 – V2 hypervariable region was amplified using universal bacterial primers modified by adding ligation adaptors and/or molecular identifier (MID) barcodes, which function as sample identification sequences to the 5' ends. Then PCR was performed using a high-fidelity polymerase (HotStarTaq Plus, Qiagen, Valencia, CA) with the following

conditions: 94ºC for 3 minutes, followed by 28 cycles of 94ºC for 30 seconds; 53ºC for 40 seconds, and 72ºC for 1 minute after which a final elongation step at 72ºC for 5 minutes was performed. PCR products were examined (in 2% agarose gel) to determine the success of amplification, the relative intensity of the bands, and the quality of the DNA. Amplicons (DNA obtained from the PCR replication process) were then quantified, pooled, and purified using Agencourt AMPure beads (Agencourt Bioscience Corporation, MA, USA) before sequencing.

3.9. Sequencing data analysis:

A fasta sequence file, a qual quality file, and a mapping file was obtained from Mr DNA lab after the completion of the Illumina sequencing process. The sequences were then filtered using Qiime software for; low quality reads (quality score< 25 bp), short and long sequences (< 200 nt; >1000 nt), zero ambiguous base calls, gaps, zero primer sequence mismatch, and sequences with homopolymer runs exceeding 6 nucleotides. The filtered sequences were then checked for chimeras for removal (Edgar et al. 2011, uchime as part of usearch v5.2.236). The remaining sequences were then sorted into operational taxonomic units (OTUs) based on 97% sequence similarity using usearch (Edgar 2010) against the curated GreenGenes database through the Qiime software pipeline using a closed-reference OTU picking protocol (Caporaso et al. 2010; Caporaso et al. 2011; Kuczynski et al. 2012). All unassigned bacterial species (not identified to the kingdom level) were removed from the data set before further analysis. After the quality control process, each of the treatments were then rarefied to the lowest number of sequences detected in an individual sample before conducting diversity analyses to eliminate uneven sampling depth impacts on observed differences.

Qiime software was then used to generate rarefaction curves for the Observed species (count of unique species), Chao1 (estimate of rare species), Phylogenetic distance (phylogenetic

distance between species), and Shannon Index (estimate of richness and evenness or distribution of species) metrics for each sample for the alpha diversity (within community diversity) analysis. Beta diversity (between sample diversity) was then examined by creating a weighted (species abundance based) and unweighted (species identity based) Unifrac metric (based on normalized abundance data; Lozupone et al. 2010). These metrics were then used to perform a jackknifed beta diversity analysis (Caporaso et al. 2010; Caporaso et al. 2011) to compare the GIT bacterial community abundance and the richness composition of each treatment. A bootstrap consensus tree was then constructed from the jackknifed analysis using UPGMA (Unweighted Pair Group Method with Arithmetic mean) clustering. GIT bacterial OTU's were then transformed as a presence/absence data of the individual OTUs, averaged across the samples, and then the software package Krona (Ondov et al. 2011) was used to create a non-metric multidimensional scaling visualization. Finally, the GIT bacterial community OTU was analyzed using a multivariate analysis called a redundancy analysis (RDA) which is a method to extract and summarize the variation in a set of response variables (bacterial species) that can be explained by a set of explanatory variables (treatment groups). A Hellinger transformation was then performed to remove species with low counts to determine the impact of abundance on the observed correlations. The multivariate RDA analysis was performed on the biome table using the package vegan (Oksanen et al. 2016) in R. The scripts used in QIIME and R to process and analyze the bacterial gDNA sequences and generate the multivariate RDA is described more in depth in the APPENDIX.

3.10. Tissue preparation for the enzyme activity analysis:

GIT, white muscle, liver, kidney, and gill tissues were homogenized using a glass homogenizer in ice-cold, enzyme-specific homogenization buffer described individually below.

3.11. Cellulase enzyme activity assay:

GIT tissue was initially homogenized in a 0.1 M PBS buffer at a pH of 6.8 using a glass homogenizer pre-chilled in ice before being centrifuged at 12000 g for 20 minutes. 125 µl of the homogenate was mixed with 125 µl of 1% weight/volume carboxymethylcellulose (CMC) substrate and incubated at 37°C for 1 hour. The reaction was then stopped by adding 750 µl of Dinitrosalicylic Acid (DNS) reagent (0.2 M DNS, 2M NaOH in dH_2O) and the mixture was then incubated at 90°C for 10 minutes. Finally, absorbance was measured at 574 nm along with a glucose standard curve and the cellulase activity was calculated as the final difference between enzyme activity with CMC and enzyme activity without CMC and expressed as mg of glucose min^{-1} mg^{-1} protein (Miller 1959; Anand et al. 2014; Liu et al. 2016).

3.12. Trypsin enzyme activity assay:

GIT tissue was initially homogenized in a 0.05 M Tris-HCl buffer at a pH of 7.5 using a glass homogenizer pre-chilled in ice, followed by centrifugation at 12000 g for 15 minutes. 10 µl of homogenate was mixed with 190 µl of reaction buffer (100 mM Tris-HCl at pH 7.5) either with or without 2 mM Nα-benzoyl-L-arginine-*p*-nitroanilide hydrochloride (BAPNA) substrate. Dissociation of the BAPNA into 4-nitroaniline was measured at 410 nm for 10 minutes at 37°C, and the trypsin activity was calculated as the final difference in trypsin activity between the samples with BAPNA and those without BAPNA. Activities were expressed as µmol min^{-1} g^{-1} protein.

3.13. Lipase enzyme activity assay:

The lipase enzyme activity assay was performed on the GIT tissue using the Abcam lipase activity assay kit (Abcam, Toronto, Canada) according to the manufacturer's protocol, and was expressed as nmol of triglyceride converted to glycerol min^{-1} at 37°C.

3.14. NKA enzyme activity assay:

The NKA activity assay was performed based on previously published techniques (McCormick 1993). The homogenization buffer was prepared with SEI (150 mM Sucrose, 10 mM EDTA, and 50 mM imidazole) at a pH of 7.5. Around 20-30 mg of the designated tissue was placed into a 2-ml bullet tube containing 150 µl of the SEI homogenization buffer plus 50 µl of SEID buffer (SEI buffer plus 5g/L deoxycholic acid) at a pH of 7.5 to lyse the cells and to solubilize the cellular and membrane components of the cell. The tissue was then homogenized in the SEI-SEID buffer mixture using a glass homogenizer pre-chilled in ice. The homogenate was then centrifuged at 10000 rpm for 1 minute at 4° C to pellet the solid tissue. The supernatant was then used to analyze the NKA enzyme activity.

The reaction buffer for the NKA enzyme activity assay was prepared in two solutions: Solution A (4U/ µl LDH, 5U/ µl of PK, 2.8 mM of Na^{+}- PEP, 0.7 mM of ATP, 0.22 mM of NADH, and 50 mM of imidazole in dH_2O) and Solution B (Solution A with 0.5 mM ouabain octahydrate). Both solutions were prepared fresh daily. NADH was added to the concentrated solutions only 5 minutes before the assay, and was remade every 4 hours. Solution B containers were wrapped with aluminum foil always to protect the light-sensitive ouabain from degradation. The pH of the solution was also measured and maintained at 7.5. A separate salt solution (50 mM NaCl, 10.5 mM $MgCl_2$, 42 mM KCl, and 50 mM imidazole in dH_2O) was also prepared and

maintained at a pH of 7.5. The salt solution was then mixed with solutions A and B, respectively at a ratio of 4:1.

The assay was performed on a pre-chilled 96-well microplate. 10 µl of the homogenate was then pipetted in triplicate. The plate was then immediately placed in the spectrophotometer. The temperature for the assay was maintained at 26°C and the change in absorbance was then measured at 340 nm for 12 minutes following a 2-minute wait time in a spectrophotometer (BioTek Instruments Inc, Winooski, VT, USA). The rate of NADH oxidation was calculated from the slope/ max V or linear rate between 2-12 minutes after initiation of the reaction. The final NKA activity was calculated as the difference between ouabain-sensitive and insensitive ATP hydrolysis and expressed as µmol ADP mg^{-1} protein h^{-1}.

3.15. Homogenization buffer for the lactate dehydrogenase (LDH), pyruvate kinase (PK), and glutamate dehydrogenase (GDH) enzyme activity assay:

The homogenization buffer for the LDH, PK, and GDH enzyme activity assay was prepared as a 500-ml stock solution and kept at 4° C (75 mM Tris-HCl, 3mM $MgCl_2$, and 5mM EDTA in dH_2O). 1mM Dithiothreitol (DTT) was added fresh daily to stabilize the enzyme activity and the mixture was maintained at a pH of 7.5.

3.15.1. LDH enzyme activity assay:

Tissue was homogenized in the ice-cold homogenization buffer mentioned above in 3.15 using a glass homogenizer pre-chilled on ice. The reaction buffer (0.15 mM NADH, 0.2 mM Pyruvate-Na, and 50 mM imidazole in dH_2O) was prepared fresh daily. The pH was maintained at 7 to favor pyruvate reduction. All buffer preparations and dilutions were kept ice-cold. The assay was performed on a pre-chilled 96-well microplate. 10 µl of the homogenate was pipetted

into the microplate in triplicate. 200 µl of ice-cold reaction buffer was added to the wells, and the plate was then immediately placed in the spectrophotometer. The temperature for the assay was maintained at 26º C and the change in absorbance was measured at 340 nm for 6 minutes in a spectrophotometer (BioTek Instruments Inc, Winooski, VT, USA). The rate of NADH oxidation was calculated from the slope/ max V or linear rate between 1-6 minutes after initiation of the reaction and the LDH enzyme activity was calculated as the catalyzation of the pyruvate to lactate with oxidation of NADH to NAD and expressed as µmol min^{-1} g^{-1} protein.

3.15.2. PK enzyme activity assay:

Tissue was homogenized in a pre-chilled glass homogenizer with the ice-cold homogenization buffer mentioned above in 3.15. The reaction buffer (5mM ADP,0.15 mM NADH, 5-10 U LDH and 50 mM imidazole in dH_2O) was prepared fresh daily. The reaction mixture was split into two equal volumes, with 5mM of phosphoenolpyruvate (PEP) substrate added only to the experimental buffer. Buffer preparation and dilutions were maintained at ice-cold temperatures, and the pH was maintained at 7.4 to favor PEP reduction. The assay was performed on a pre-chilled 96-well microplate. 10 µl of the homogenate was pipetted into the microplate in triplicate. 200 µl of the ice-cold reaction buffer with PEP was added to the experimental wells and 200 µl of the reaction buffer without PEP was added to control wells. The temperature for the assay was maintained at 26º C and the change in absorbance was then measured at 340 nm for 6 minutes in a BioTek Synergy HT spectrophotometer (BioTek Instruments Inc, Winooski, VT, USA). The rate of NADH oxidation was calculated from the slope/ max V or linear rate between 1-6 minutes after initiation of the reaction and the PK enzyme activity was calculated as the final difference between the reactions with PEP vs without PEP and expressed as µmol min^{-1} g^{-1} protein.

3.15.3. GDH enzyme activity assay:

Tissue was homogenized in a pre-chilled glass homogenizer with the ice-cold homogenization buffer mentioned above in 3.15. The reaction buffer (0.12 mM NADH, 1mM ADP, and 50mM imidazole in dH_2O) was prepared fresh daily. The reaction mix was then split into two equal volumes and 14 mM of alpha-ketoglutarate (AKG) substrate was added only to the experimental reaction buffer. Buffer preparation and dilutions were maintained at ice-cold temperatures, and the pH was maintained at 7.4 to favor alpha ketoglutarate reduction. The assay was performed on a pre-chilled 96-well microplate. 10 µl of the homogenate was pipetted into the microplate in triplicate. 200 µl of the ice-cold reaction buffer with AKG was added to the experimental wells and 200 µl of the reaction buffer without AKG was added to control wells. The temperature for the assay was maintained at 26° C and the change in absorbance was then measured at 340 nm for 10 minutes in a BioTek Synergy HT spectrophotometer (BioTek Instruments Inc, Winooski, VT, USA). The rate of NADH oxidation was calculated from the slope/ max V or linear rate between 1-10 minutes after initiation of the reaction and the GDH enzyme activity was calculated as the oxidation of NADH to NAD with the final difference in reactions between with-AKG vs without-AKG and expressed as µmol min^{-1} g^{-1} protein.

3.16. Citrate Synthase (CS) enzyme activity assay:

A section of the designated tissue was homogenized in a Triton-X homogenization buffer (20 mM HEPES, 1 mM EDTA, and 0.1 % Triton-X-100 in dH_2O at pH of 8) in a glass homogenizer pre-chilled in ice and was kept at -20° C freezer overnight. Reaction buffer (0.1 mM DTNB, 0.3 mM Acetyl-CoA) was prepared fresh daily and maintained at a pH of 8.0 and Acetyl-CoA was added only 5-10 minutes prior to the start of the reaction. The solution was then split into two

equal volumes and 0.5 mM Oxaloacetate (OAA) substrate was added only to the experimental reaction buffer. Buffer preparation and dilutions were maintained at ice-cold temperatures and the reaction buffer was always covered with aluminum foil to prevent DTNB degradation. The assay was performed on a pre-chilled 96-well microplate. 10 µl of the homogenate was pipetted into the microplate in triplicate. 200 µl of the ice-cold reaction buffer with OAA was added to the experimental wells and 200 µl of the reaction buffer without OAA was added to control wells. The temperature for the assay was maintained at 26° C and the change in absorbance was measured at 412 nm in a BioTek Synergy HT spectrophotometer (BioTek Instruments Inc, Winooski, VT, USA). The rate of Acetyl-CoA reduction was calculated from the slope/ max V or linear rate between 1-6 minutes after initiation of the reaction. The CS enzyme activity was calculated as the reduction of Acetyl-CoA to CoA-DTNB with the final difference in reactions between with-OAA vs without-OAA minus the blank and expressed as µmol min^{-1} g^{-1} protein.

For all the above enzymes (except lipase), protein concentration in the homogenates was measured using the Bradford assay protocol (Bradford 1976). All enzyme assays were first optimized to determine optimal reaction conditions.

Table 1: Summary of *C.anomalum* collected from the sampling site of Irvine Creek:

Date of collection	Number of individuals collected	Temperature of source water (C°)	Temperature of lab water (C°)
October 4th, 2015	25	14 ± 2	11 ± 2
October 12th, 2015	30	16± 2	16 ± 2

Table 2: Summary of nutritional values in the *C. anomalum* diet treatments:

Algae	Commercial fish feed
Protein = 33 % Fat = 4 % Fiber = 3 % Moisture = 10 % P = 0.8 % Ash = 17 %	Protein = 42 % Fat = 15 % Fiber = 2 % Moisture= 6.5 % P = 1.1 %

Table 3: Universal bacterial primers extrapolated or designed to perform contamination test on the extracted gDNA samples:

Bacterial gene hypervariable region	Primer sequence	From
V3	Forward: 338 = ACTCCTACGGGAGGCAGC 341= CCTACGGGAGGCAGCAGTG Reverse: 785 = CTACCAGGGTATCTAATCC	Baker et al. 2003 Welch and Huse 2011
V6	Forward :967 = CAACGCGAAGAACCTTACCT Reverse: 1177 = GACGTCATCCCCACCTTCCT	Watanabe et al. 2001

Results:

4.1. GIT bacterial abundance and richness in response to dietary alterations:

238,304 bacterial sequences were detected and a total of 2246 bacterial OTUs were identified after the quality control procedure. Sequences and OTU's removed during the quality control procedure totaled only to be less than 1 % of the entire data set. The identified 2246 OTUs belonged to the phyla Bacteroidetes, Proteobacteria, and Firmicutes. In wild (control) animals, Bacteroidetes, Proteobacteria, and Firmicutes made up the GIT bacterial community at 56%, 6%, and 34 % total abundance respectively (Figure 1). However, by the end of the 1-month algae diet treatment, the bacterial phylum Proteobacteria dominated the GIT with abundance at 97.5% (an increase of 16-fold), followed by Bacteroidetes at 0.57%, and Firmicutes at 0.53% (Figure 1). Bacterial phylum Proteobacteria remained the highest proportional representation at the end of the 3-month of the algae diet treatment (67% abundance), however, recoveries in the proportions of Bacteroidetes (21.6%) and Firmicutes (9.5%) were also observed (Figure 1).

The 1-month fish feed diet treatment initially demonstrated a bacterial phylum composition similar to the 1-month algae diet treatment, with Proteobacteria abundance at 79%, followed by Firmicutes at 6%, and Bacteroidetes at 8% (Figure 1). However, after 3-month of the fish feed diet treatment, the proportion of the Firmicutes in the GIT increased by 12-fold (to 72%), while the proportion of Proteobacteria decreased by 3-fold to 24% and proportion of Bacteroidetes decreased by 4-fold to 2% compared to the treatments fed the algae and the fish feed diet for 1 month (Figure 1).

The rarefied observed species curve showed a high count of species (OTUs) in the wild treatment (total OTUs = 1755) at 19,372 sequences (Figure 2.A) followed by the 3-month algae diet treatment (total OTUs = 475), 1-month fish feed diet treatment (total OTUs= 424), and 3-

month fish feed diet treatment (total OTUs = 377; Figure 2.A). The lowest GIT OTU richness was observed in the 1-month algae diet treatment (total OTUs = 305; Figure 2.A). Similarly, the number of rare species (Chao1), phylogenetic distance between the species (PD whole tree), and distribution of the species (Shannon Index) was also the highest in the wild treatment compared to the other dietary treatments (Figure 2.B, 2.C, and 2. D). Interestingly, when we compared the 1-month fish feed versus the 3-month algae diet treatment, the observed OTU's, rarer species, and the phylogenetic distance between the species was higher in the 3-month algae diet treatment (Figure 2.A, Figure 2.B and Figure 2.C). However, they had a similar Shannon Index which means that the distribution of the bacterial species was similar in these two treatments (Figure 2.D).

Unweighted beta-analysis (similarity between the treatments based on species identity) showed that the wild samples clustered separately from the diet treatments (Figure 3.B). Furthermore, it also showed that the 1-month fish feed and the 3-month algae diet treatments clustered together possibly because of the similarity between the treatments based on species identity (Figure 3.B). We also found that the clustering of the 3-month fish feed diet treatment was weakly supported (< 50 %) in comparison to the other treatments (Figure 3.B). This clustering of the diet treatments was further supported by Bonferroni corrected p-values at $p \leq 0.01$ and a bootstrapped tree at a distance of 0.1 (Figure 3.B).

On the other hand, the weighted beta-analysis (similarity between the treatments based on species abundance) showed that the 1- and 3-month algae diet treatments clustered together (Figure 3.A), possibly due the abundance of similar bacterial species. Interestingly, the 3-month fish feed diet treatment clustered separately possibly due to a different abundance of bacterial species compared to the other treatments (Figure 3.A). The wild treatment was again clustered

separately compared to the diet treatments in the weighted analysis (Figure 3.A). Animals subjected to the diet treatments demonstrated altered bacterial OTU communities, as compared to the wild treatments, which formed their own cluster, apart from the lab-reared samples. This clustering of the diet treatments in the weighted beta-analysis was also further supported by Bonferroni correction ($p \leq 0.01$) and a bootstrapped tree at a distance of 0.1 (Figure 3.A).

Identification of the bacteria species correlating with the diet treatments was achieved through the multivariate redundancy analysis (RDA). The axis that displayed the most variation was RDA 1 (Eigenvalue of 0.2168, with 50.18% of the variation explained) followed by RDA 2 (Eigenvalue of 0.1566, 36.24% of the variation explained) (Figure 4). Together, the two axes explained 86.42% of the total variation observed in the bacterial species abundance in the diet treatments at an adjusted R^2 of 0.894. Furthermore, a comparison of the Hellinger-transformed data (Figure 4.B) to the non-transformed data (Figure 4.A) showed that filtering out the bacterial species with low weight and abundance did not alter the redundancy analysis results, therefore establishing that the treatments were distinguished by the identity of a few highly abundant bacterial species, rather than total species richness (Figure 4).

Most bacterial species were shared among the diet treatment groups (the clustering in the center of the graph in Figure 4.A and Figure 4.B), however there were a few species correlated specifically with the treatments. Bacterial species of the genus *Bacteroidia* and *Lachnospiraceae* (phylum Bacteroidetes) had a strong positive correlation to the wild samples (Figure 4.B). Bacterial species of the genus *Proterobacteria* (phylum Proteobacteria) positively defined (increased in abundance) the 1- and 3-month algae diet treatments, but were negetively correlated to the 3-month fish feed diet (Figure 4.B) . Finally, bacterial species of the genus *Leuconostocaceae* and *Bacilli* (phylum Firmicutes) increased in abundance with the 3-month fish

feed diet treatment, but decreased in abundance with both the 1- and 3-month algae diet treatments (Figure 4.B).

4.2. Enzyme activities

4.2.1. Cellulase enzyme activity in the GIT:

Cellulase enzyme activity was highest in the wild samples (0.945 ± 0.287 mg of glucose min^{-1} mg^{-1} protein). The dietary treatments (3-month algae, 1-month commercial fish feed, and 3-month commercial fish feed) significantly lowered the enzyme rates ($p < 0.0022$, n= 7; Figure 5).

4.2.2. Trypsin enzyme activity in the GIT:

The highest trypsin enzyme activity was again observed in the wild samples at 1225 ± 204.233 µmol min^{-1} g^{-1} protein (Figure 6). As with cellulose, trypsin activity decreased with diet treatments, with significantly lower activity ($p < 0.05$, n= 7) in the 3-month commercial fish feed treatment (101.899 ± 19.80 µmol min^{-1} g^{-1} protein; Figure 6).

4.2.3. Lipase enzyme activity in the GIT:

Lipase activity was significantly higher ($p < 0.05$, n= 5) in the GIT of animals fed the commercial fish feed diet for either 1- or 3-month (Figure 7). Lipase activity was not affected by the 1-month and 3-month algae diet treatments compared to the wild animals (Figure 7).

4.2.4.1. Na^+/K^+ ATPase enzyme activity in the GIT :

NKA activity in the GIT of the wild individuals was initially 1.27 ± 0.26 µmol ADP mg^{-1} protein h^{-1} and was not altered by feeding either the algae or commercial fish feed diets for 1-month (Figure 8). However, the activity increased by 6-fold (7.77±2.89 µmol ADP mg^{-1} protein

h^{-1}) and 2-fold (2.69 ± 0.76 µmol ADP mg^{-1} protein h^{-1}) respectively with the continued feeding of the above-mentioned diets for 3-month ($p < 0.05$, n=8; Figure 8).

4.2.4.2. Na^+/K^+ ATPase enzyme activity in the white muscles, liver, kidney, and gill:

NKA activity was not impacted by diet treatments in the white muscle (Figure 9.A), liver (Figure 9.B), and gill (Figure 9.C). In contrast, NKA activity in the kidney increased significantly with the introduction and continuation of the algae diet for 1- and 3-month and the fish feed diet for 1-month compared to the wild samples ($p < 0.05$, n=8) (Figure 9.D).

4.2.5.1. LDH enzyme activity in the GIT:

Diet did not impact LDH enzyme activity in the GIT compared to the wild animals (Figure 10). However, there was a significant difference within the algae treatment. LDH activity was highest in the GIT of the 3-month algae diet treatment (1601.05 ± 395.65 µmol min^{-1} g^{-1} protein) compared to the 1-month algae diet treatment (n=6, p=0.03; Figure 10).

4.2.5.2. LDH enzyme activity in the white muscle, liver, kidney, and gill:

A significant increase in the white muscle LDH activity (Figure 11.A) was detected in the 3-month algae diet treatment (compared to all treatments; p = 0.000169, n= 6). LDH activity in the liver decreased significantly (Figure 11.B) with the introduction of the 1- month algae diet treatment compared to the wild samples (p= 0.01, n= 6). No significant effect of diet treatment was detected in the gill (Figure 11.C) or the kidney (Figure 11.D).

4.2.6.1. PK enzyme activity in the GIT:

The PK enzyme activity in the wild samples was 1790 ± 864.55 µmol min^{-1} g^{-1} protein and was unaffected by the algae diet treatments, and the 1-month fish feed diet treatment (Figure

12). Interestingly, the PK activity increased significantly ($p= 0.005$, $n=6$) following the 3-month fish feed diet treatment (7705 ± 832.75 µmol min^{-1} g^{-1} protein; Figure 12).

4.2.6.2. PK enzyme activity in the white muscle, liver, kidney, and gill:

PK activity was not impacted by the diet treatments in the white muscle (Figure 13.A), liver (Figure 13.B), and kidney (Figure 13.D). PK activity in the gill increased significantly with the highest activity observed in the 1-month fish feed diet treatment ($n= 6$, $p = 0.0131$) compared to the wild and the 1-month algae diet treatment (Figure 13.C).

4.2.7.1. CS enzyme activity in the GIT:

CS enzyme activity was initially 126.78 ± 13.78 µmol min^{-1} g^{-1} protein in the wild samples which was significantly higher ($p<0.05$, $n=5$) compared to the 1- and 3-month fish feed, and 3-month algae diet treatments (Figure 14).

4.2.7.2. CS enzyme activity in the white muscle, liver, kidney, and gill:

CS enzyme activity in the white muscle (Figure 15.A), gill (Figure 15.C), and kidney (Figure 15.D) was not impacted by the diet treatments. Activity in the liver was significantly lower in the 3-month fish feed diet treatment (2.344±0.48 µmol min^{-1} g^{-1} protein) compared to the other diet treatments ($p<0.05$, $n= 5$) (Figure 15.B).

4.2.8.1. GDH enzyme activity in the GIT:

GDH enzyme activity in the GIT was not impacted by the introduction of either the algae or fish feed diet treatments for 1-month (Figure 16). After the continuation of the algae diet treatment for 3- months, GDH activity increased significantly to 1532.8±134.34 µmol min^{-1} g^{-1} protein ($p<0.05$, $n=6$; Figure 16). GDH activity also increased with the continuation of the fish

feed diet for 3 months (2768.44±962.530 µmol min^{-1} g^{-1} protein) which was significantly higher compared to the other treatments ($p<0.05$, n=6; Figure 16).

4.2.8.2. GDH enzyme activity in the white muscle, liver, kidney, and gill:

GDH enzyme activity was not impacted by the diet treatments in the white muscle (Figure 17.A). Activity in the liver was significantly higher ($p< 0.05$, n=6) in the 1-month fish feed diet treatment compared to the other treatments (4327±918.35 µmol min^{-1} g^{-1} protein). Activity in the gill increased significantly ($p< 0.05$, n=6) with the continuation of the algae diet (1153.7±88.65 µmol min^{-1} g^{-1} protein) and the fish feed diet (1341.63±610.88 µmol min^{-1} g^{-1} protein) for 3-month (Figure 17.C). Activity in the kidney was not impacted by both the diet treatments for 1-month, however, it increased significantly with the continuation of both the diet treatments for 3-month ($p<0.05$, n=6; Figure 17.D).

4.3. Multivariate association of the GIT bacterial abundance and the GIT enzyme activity in response to an altered diet:

Several prominent correlations between the distinct diet treatments, the GIT bacterial species abundance, and the GIT enzyme activity were observed when a multivariate analysis was performed (Figure 18). The cellulase and the trypsin enzyme activities correlated with the abundance of the *Lachnospiraceae* and *Bacteroidia* species (phylum Bacteroidetes) in the wild samples (Figure 18). LDH and CS enzyme activities correlated with the abundance of *Proterobacteria* (phylum Proteobacteria) in the 1- and 3-month algae diet treatments, and the 1-month fish feed diet treatment (Figure 18). Finally, the lipase, PK, GDH, and NKA enzyme activities were strongly correlated with the abundance of *Bacilli* and *Leuconostocaceae* species (phylum Firmicutes) in the 3-month fish feed diet treatments (Figure 18).

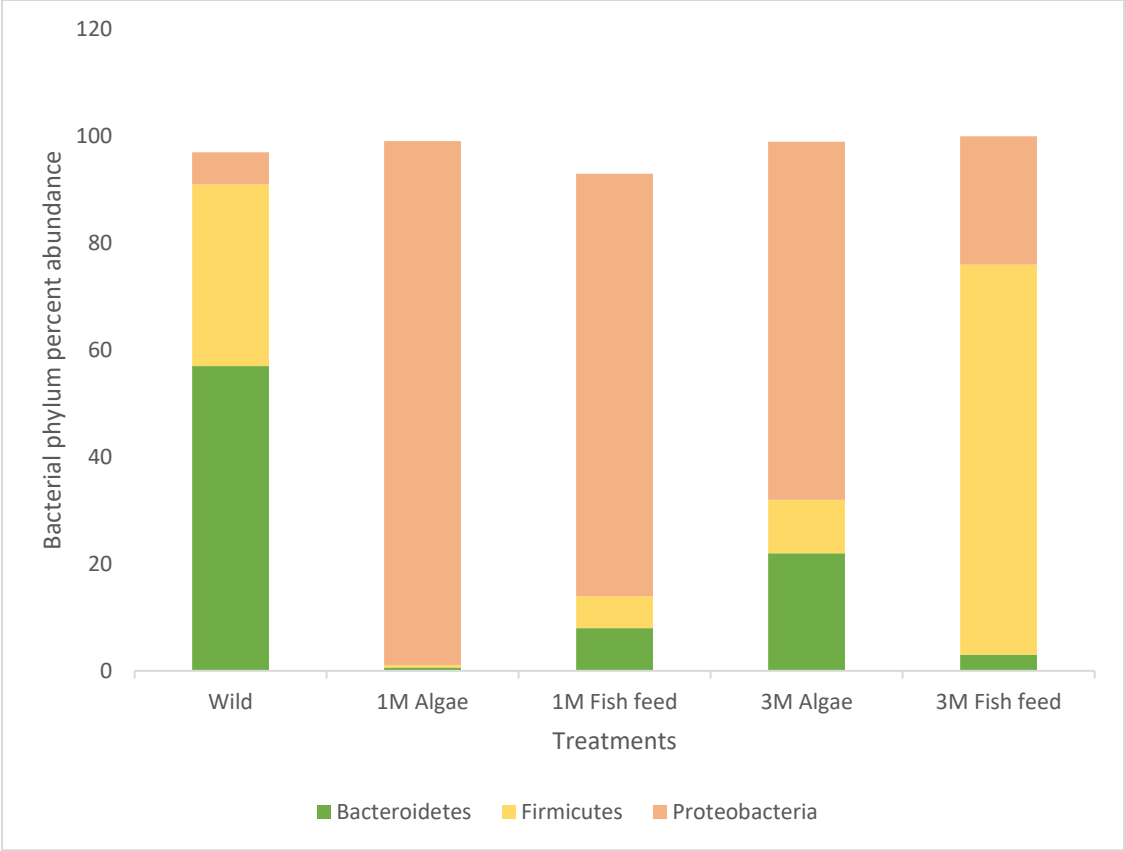

Figure 1: Percent GIT-bacterial community phylum abundance distribution in samples with unique diet treatment across a time-frame of 3 months. GIT bacterial phyla Bacteroidetes (green), Firmicutes (yellow), and Proteobacteria (maroon) dominated the GIT bacterial abundance, however, one phylum dominated in expense of the other two based on the properties of the diet composition.
(Wild = control animals, 1M Algae = animals fed algae diet for 1 month, 3M Algae = animals fed algae diet for 3 months, 1M Fish feed = animals fed fish feed diet for 1 month, and 3M Fish feed = animals fed fish feed diet for 3 months).

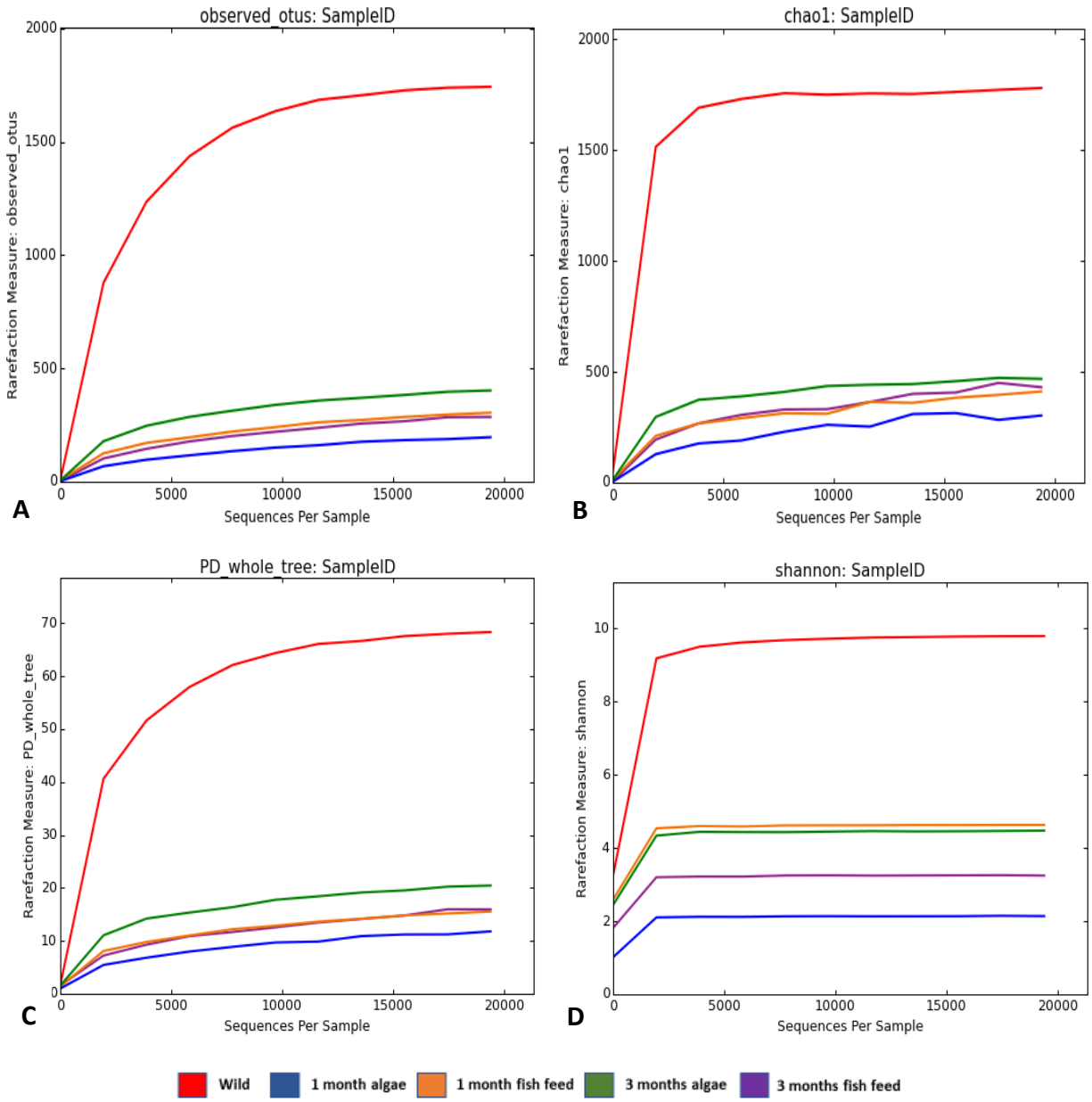

Figure 2: The observed alpha-rarefaction plot (**A.** Observed Species, **B.** Chao1, **C.** PD whole tree, and **D.** Shannon Index) of the distinct treatments at an increment sequence depth of 5000 sequences per sample. Rarefaction was performed on 19,372 random sequences from each sample.
(Wild = control animals, 1-month algae = animals fed algae diet for 1 month, 3-month algae = animals fed algae diet for 3 months, 1-month fish feed = animals fed fish feed diet for 1 month, 3-month fish feed = animals fed fish feed diet for 3 months).

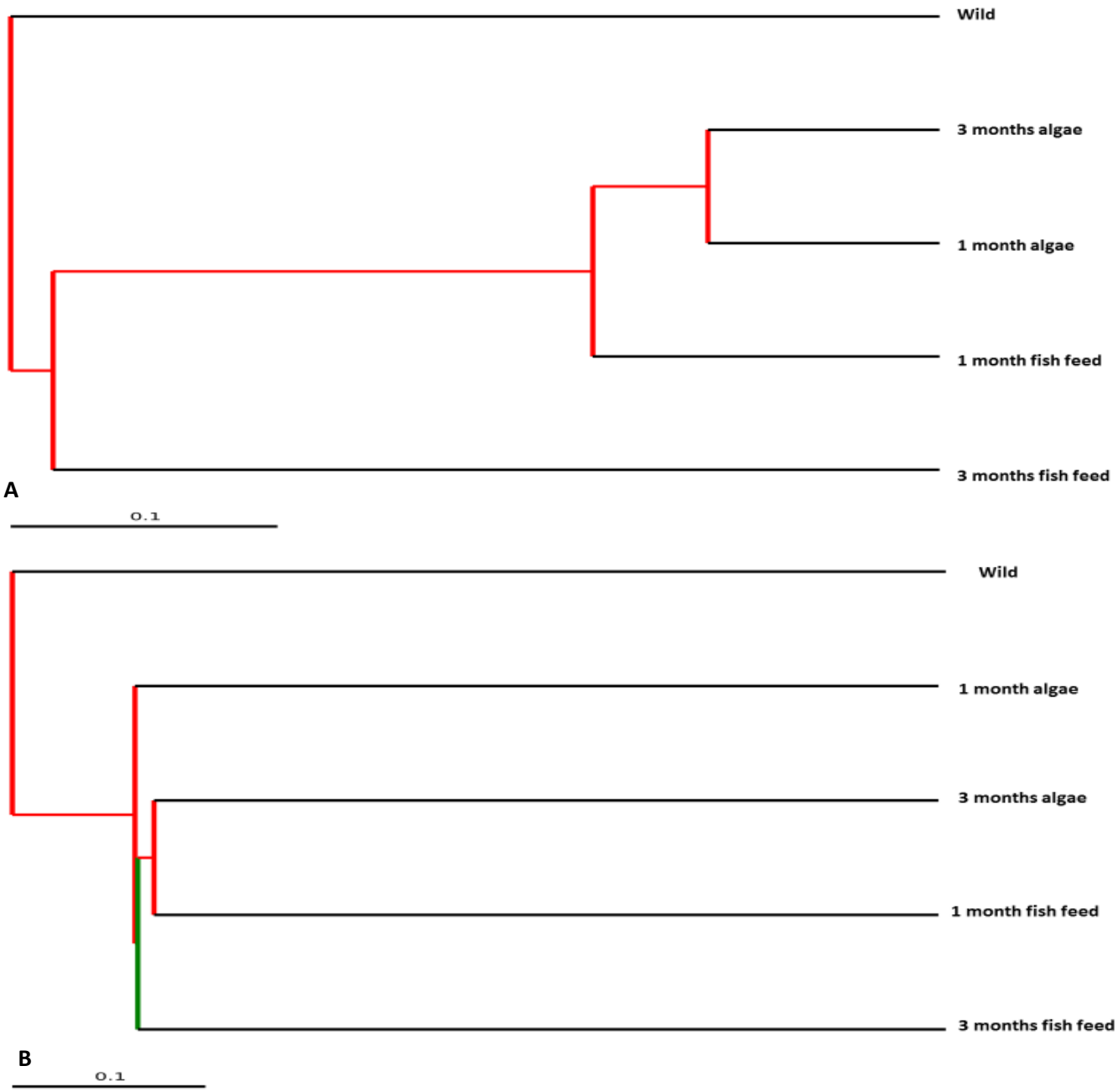

Figure 3: Jackknife weighted (**A**) and unweighted (**B**) bootstrap trees at a distance of 0.1. The red line resembles 75% or more support and the green line resembles 25-50 % support. Jackknife beta analysis was performed using 90 % of the random sequences as a baseline adopted from the sample containing the least number of OTUs.
(Wild = control animals, 1-month algae = animals fed algae diet for 1 month, 3-month algae = animals fed algae diet for 3 months, 1-month fish feed = animals fed fish feed diet for 1 month, 3-month fish feed = animals fed fish feed diet for 3 months).

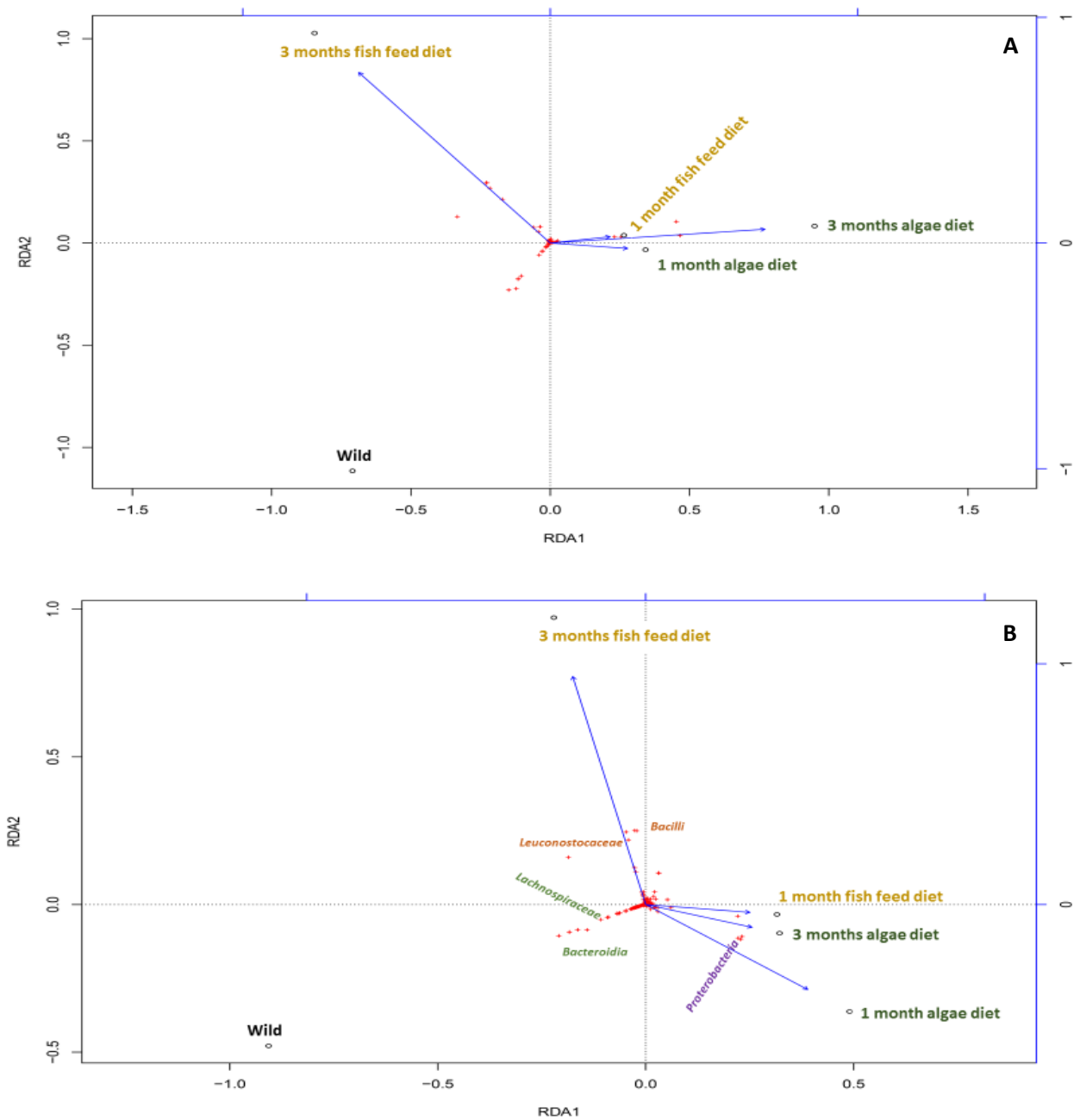

Figure 4: Multivariate RDA analysis showing the treatments seperated by the function of the abundance of the bacterial species present with **A** (RDA analysis without hellinger transformation) and **B** (RDA analysis with hellinger transformation). Bacterial species *Lachnospiraceae* and *Bacteroidia* = phylum Bacteroidetes, bacterial species *Proterobacteria* = phylum Proteobacteria, and bacterial species *Bacilli* and *Leuconostocaceae* = phylum Firmicutes. (Wild = control animals, 1-month algae = animals fed algae diet for 1 month, 3-month algae = animals fed algae diet for 3 months, 1-month fish feed = animals fed fish feed diet for 1 month, 3-month fish feed = animals fed fish feed diet for 3 months).

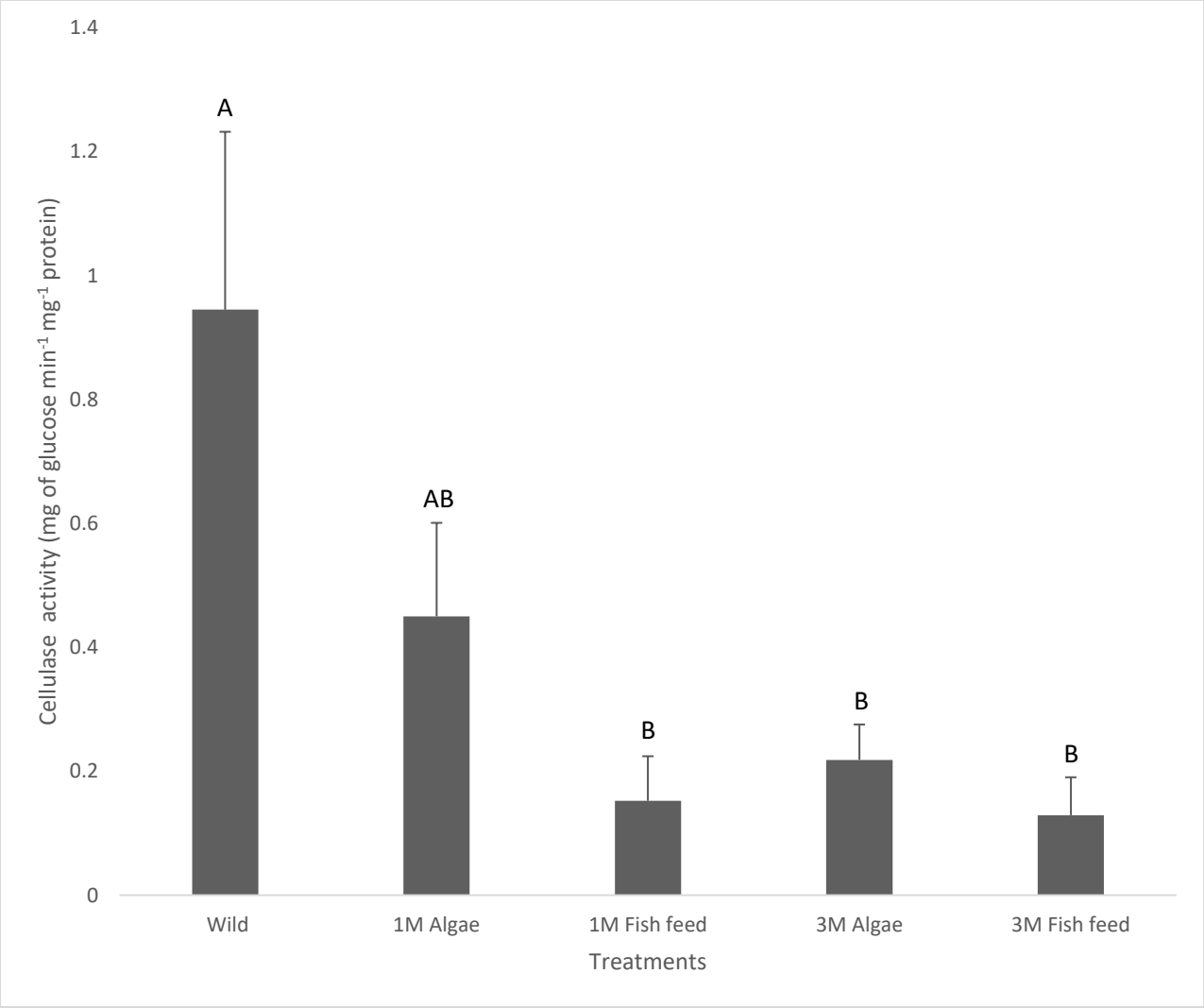

Figure 5: Cellulase enzyme activity (mg of glucose min^{-1} mg^{-1} protein) in the GIT with the introduction and the continuation of the distinct diet treatments. Bars that share the same letter are not significantly different (one-way ANOVA, $p < 0.05$, n= 7).
(Wild = control animals, 1M Algae = animals fed algae diet for 1 month, 3M Algae = animals fed algae diet for 3 months, 1M Fish feed = animals fed fish feed diet for 1 month, and 3M Fish feed = animals fed fish feed diet for 3 months).

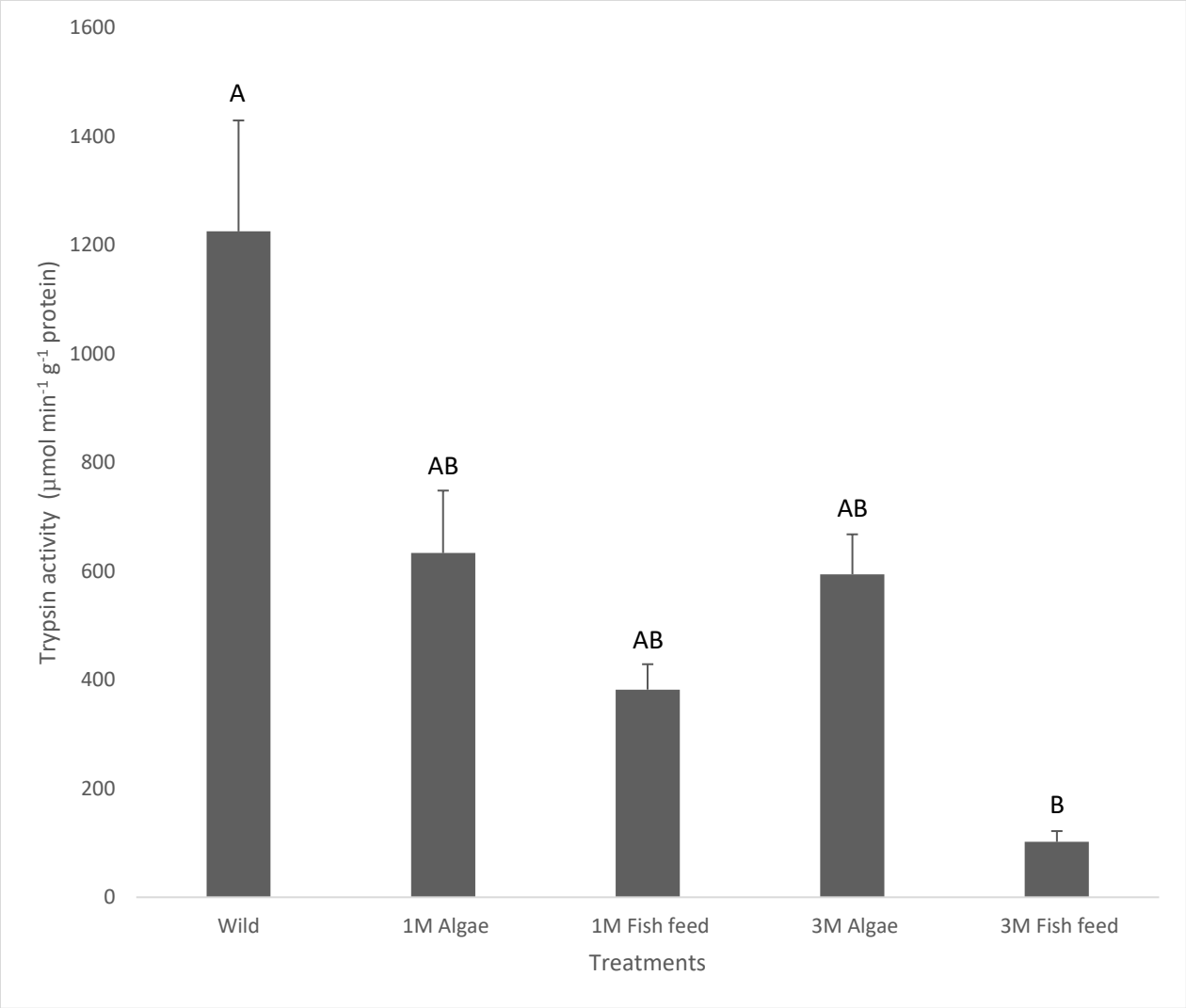

Figure 6: Trypsin enzyme activity (µmol min^{-1} g^{-1} protein) in the GIT with the introduction and the continuation of the distinct diet treatments. Bars that share the same letter are not significantly different (one-way ANOVA, $p < 0.05$, n= 7).
(Wild = control animals, 1M Algae = animals fed algae diet for 1 month, 3M Algae = animals fed algae diet for 3 months, 1M Fish feed = animals fed fish feed diet for 1 month, and 3M Fish feed = animals fed fish feed diet for 3 months).

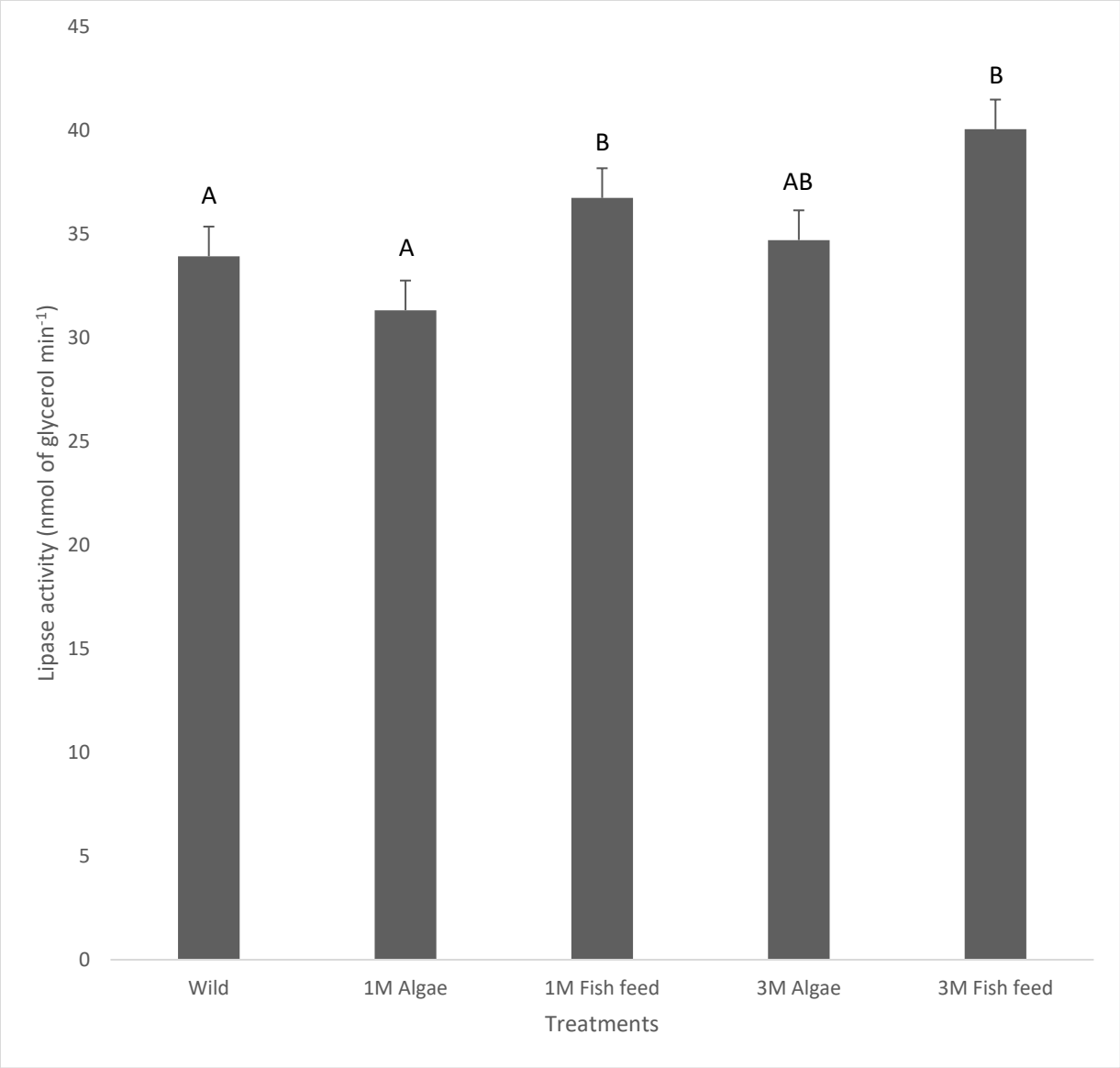

Figure 7: Lipase enzyme activity (nmol of glycerol min^{-1}) in the GIT with the introduction and the continuation of the distinct diet treatments. Bars that share the same letter are not significantly different (one-way ANOVA, $p < 0.05$, n= 5).
(Wild = control animals, 1M Algae = animals fed algae diet for 1 month, 3M Algae = animals fed algae diet for 3 months, 1M Fish feed = animals fed fish feed diet for 1 month, and 3M Fish feed = animals fed fish feed diet for 3 months).

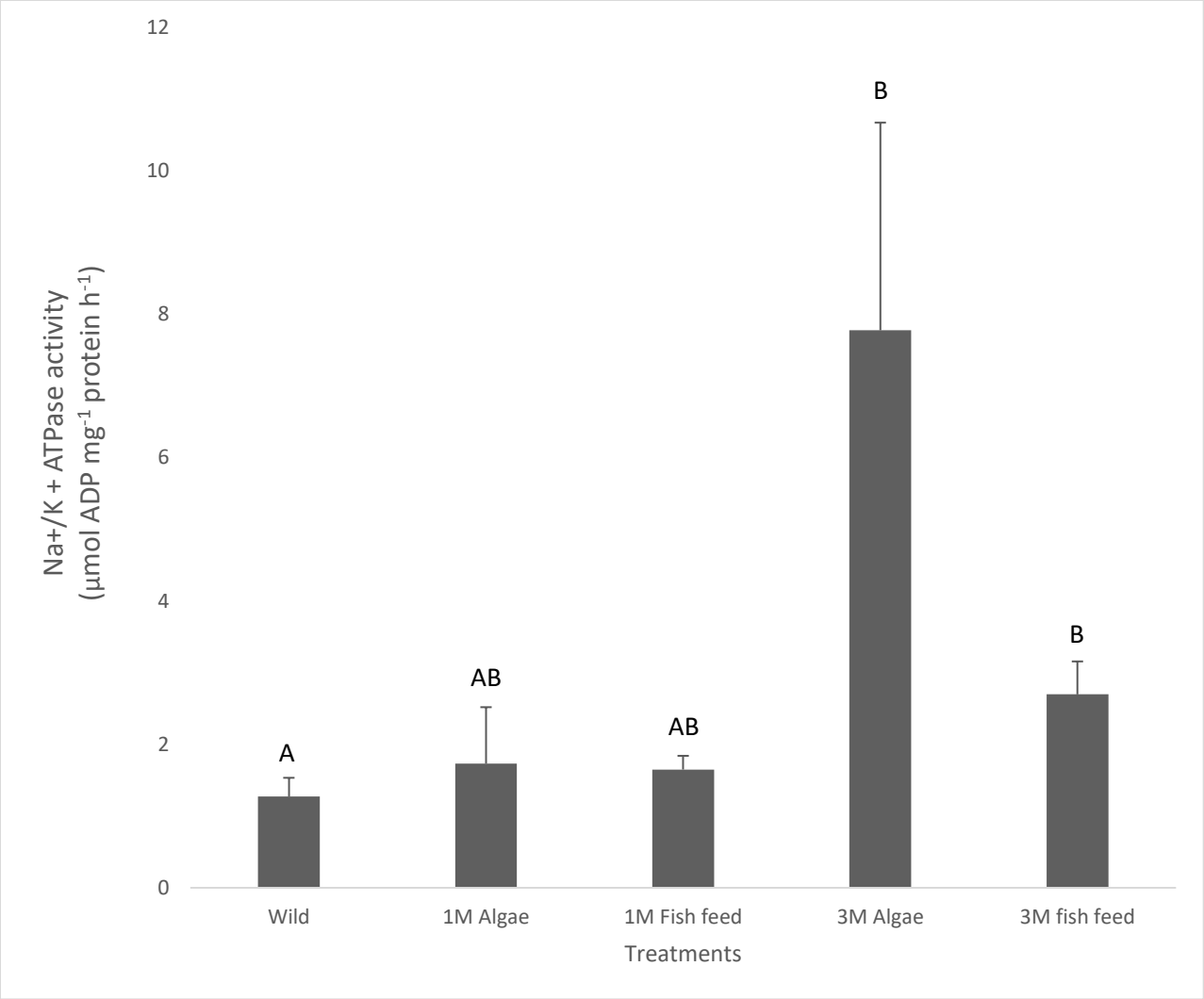

Figure 8: Na^+/K^+ ATPase enzyme activity (µmol ADP mg^{-1} protein h^{-1}) in the GIT with the introduction and continuation of distinct diet treatments. Bars that share the same letter are not significantly different (1-way ANOVA, $p < 0.05$, n=8).
(Wild = control animals, 1M Algae = animals fed algae diet for 1 month, 3M Algae = animals fed algae diet for 3 months, 1M Fish feed = animals fed fish feed diet for 1 month, and 3M Fish feed = animals fed fish feed diet for 3 months).

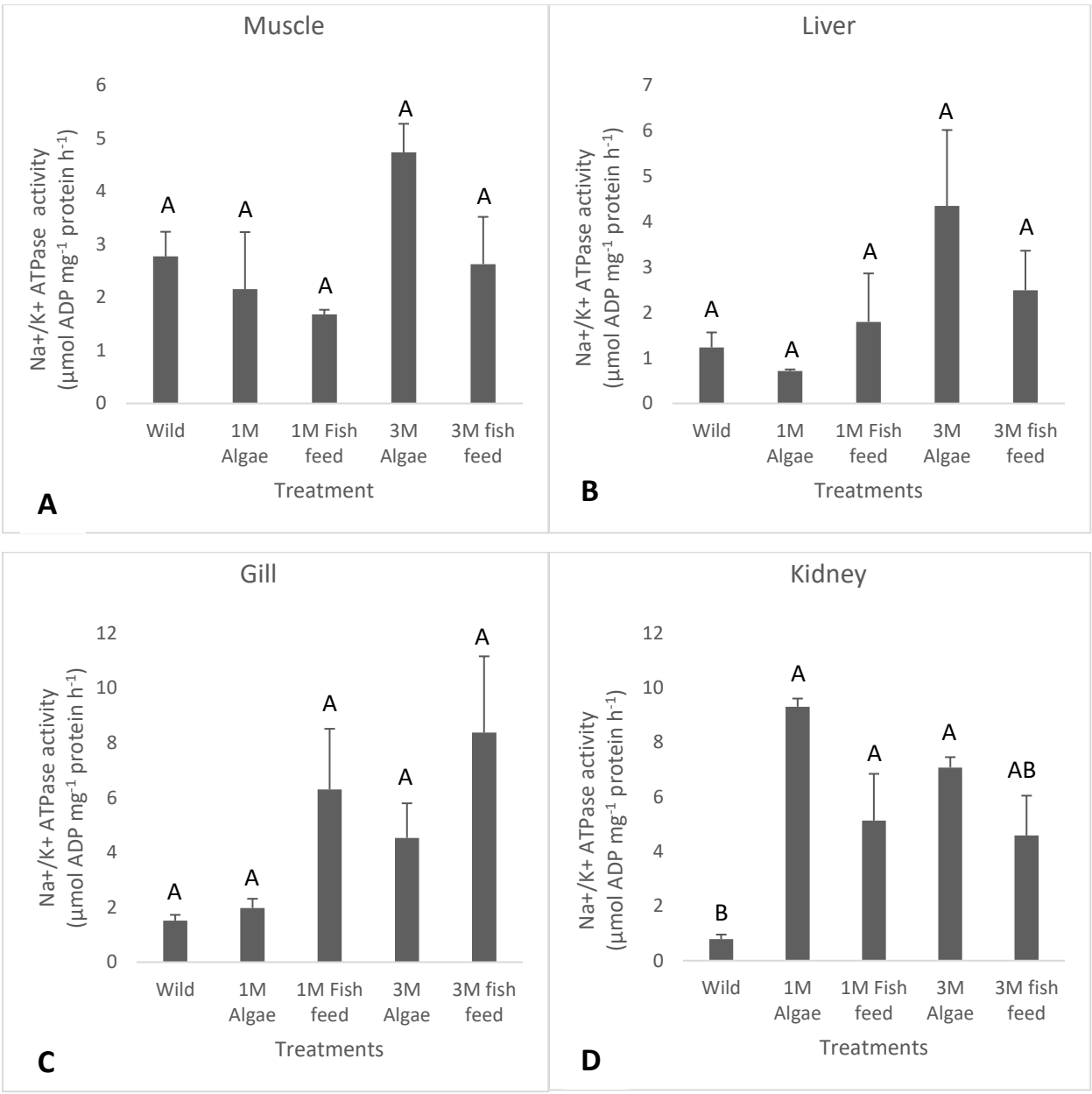

Figure 9: Na^+/K^+ ATPase enzyme activity (µmol ADP mg^{-1} protein h^{-1}) in the **A.** white muscle, **B.** liver, **C.** gill, and **D.** kidney with the introduction and continuation of the distinct diet treatments. Bars that share the same letter are not significantly different (one-way ANOVA, $p < 0.05$, n=8). (Wild = control animals, 1M Algae = animals fed algae diet for 1 month, 3M Algae = animals fed algae diet for 3 months, 1M Fish feed = animals fed fish feed diet for 1 month, and 3M Fish feed = animals fed fish feed diet for 3 months).

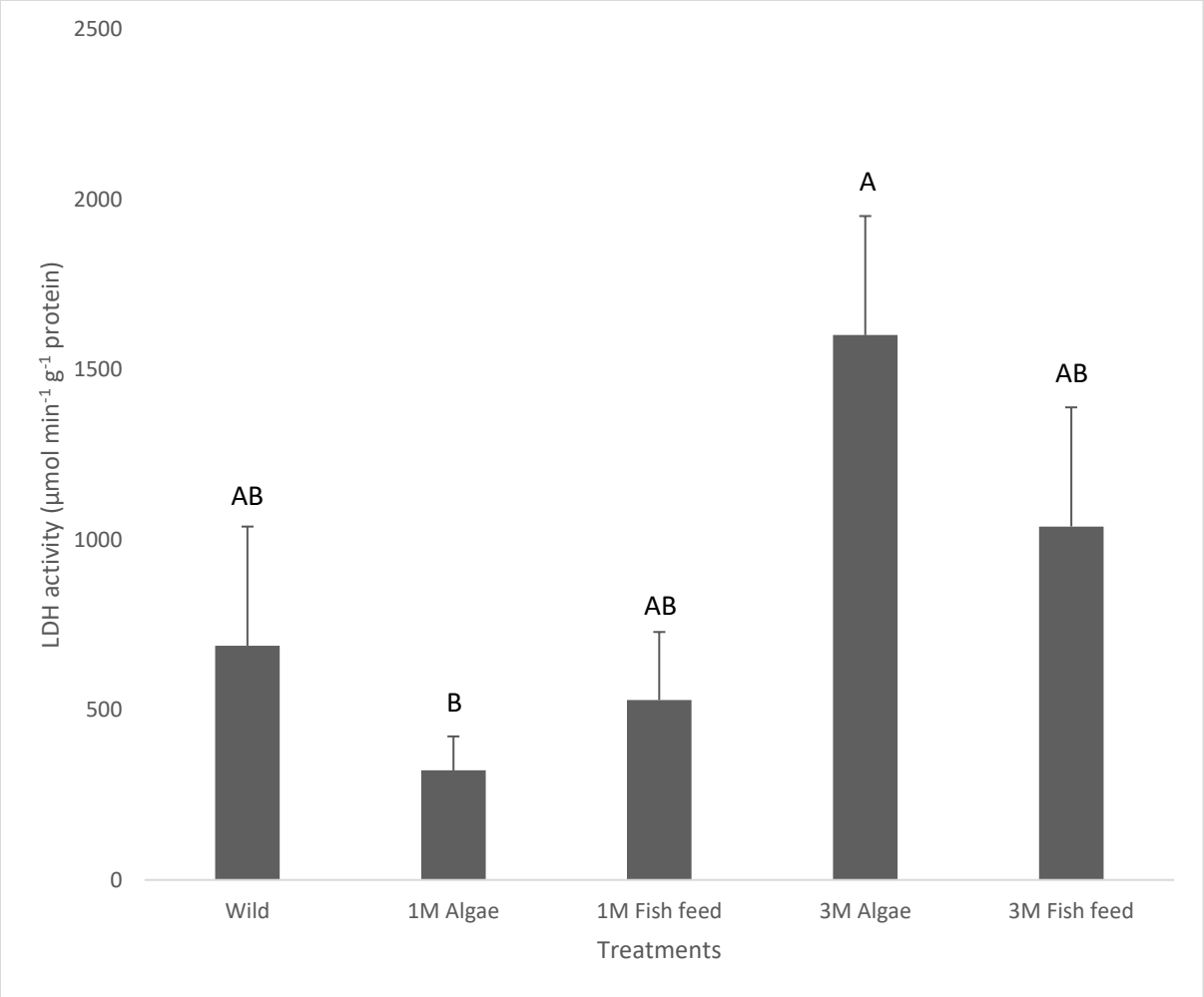

Figure 10: LDH enzyme activity (µmol min^{-1} g^{-1} protein) in the GIT with the introduction and continuation of the distinct diet treatments. Bars that share the same letter are not significantly different (one-way ANOVA, $p < 0.05$, n= 6).
(Wild = control animals, 1M Algae = animals fed algae diet for 1 month, 3M Algae = animals fed algae diet for 3 months, 1M Fish feed = animals fed fish feed diet for 1 month, and 3M Fish feed = animals fed fish feed diet for 3 months).

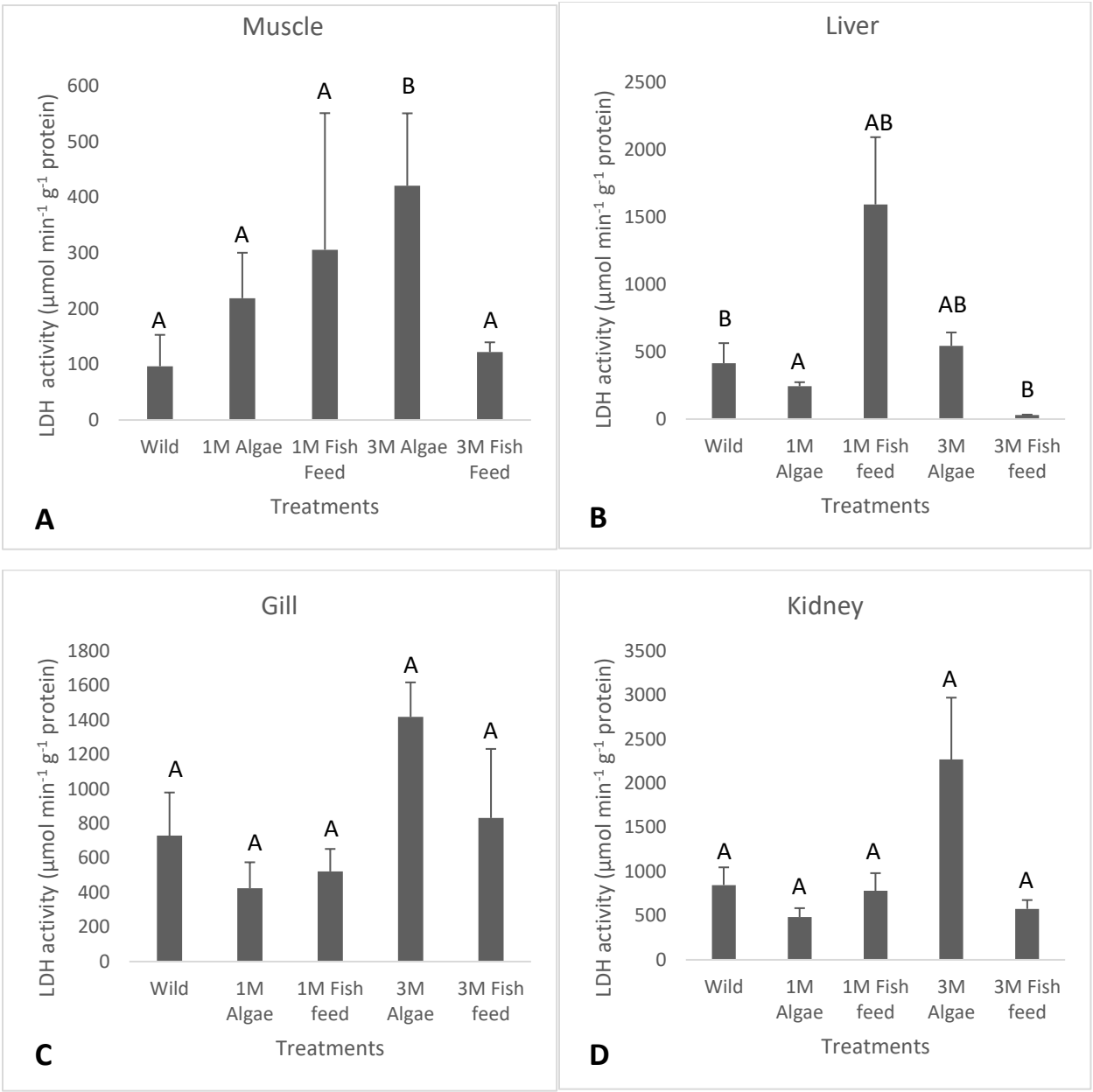

Figure 11: LDH enzyme activity (µmol min^{-1} g^{-1} protein) in the **A.** white muscle, **B.** liver, **C.** gill, and **D.** kidney with the introduction and continuation of distinct diet treatments. Bars that share the same letter are not significantly different (one-way ANOVA, $p < 0.05$, n= 6). (Wild = control animals, 1M Algae = animals fed algae diet for 1 month, 3M Algae = animals fed algae diet for 3 months, 1M Fish feed = animals fed fish feed diet for 1 month, and 3M Fish feed = animals fed fish feed diet for 3 months).

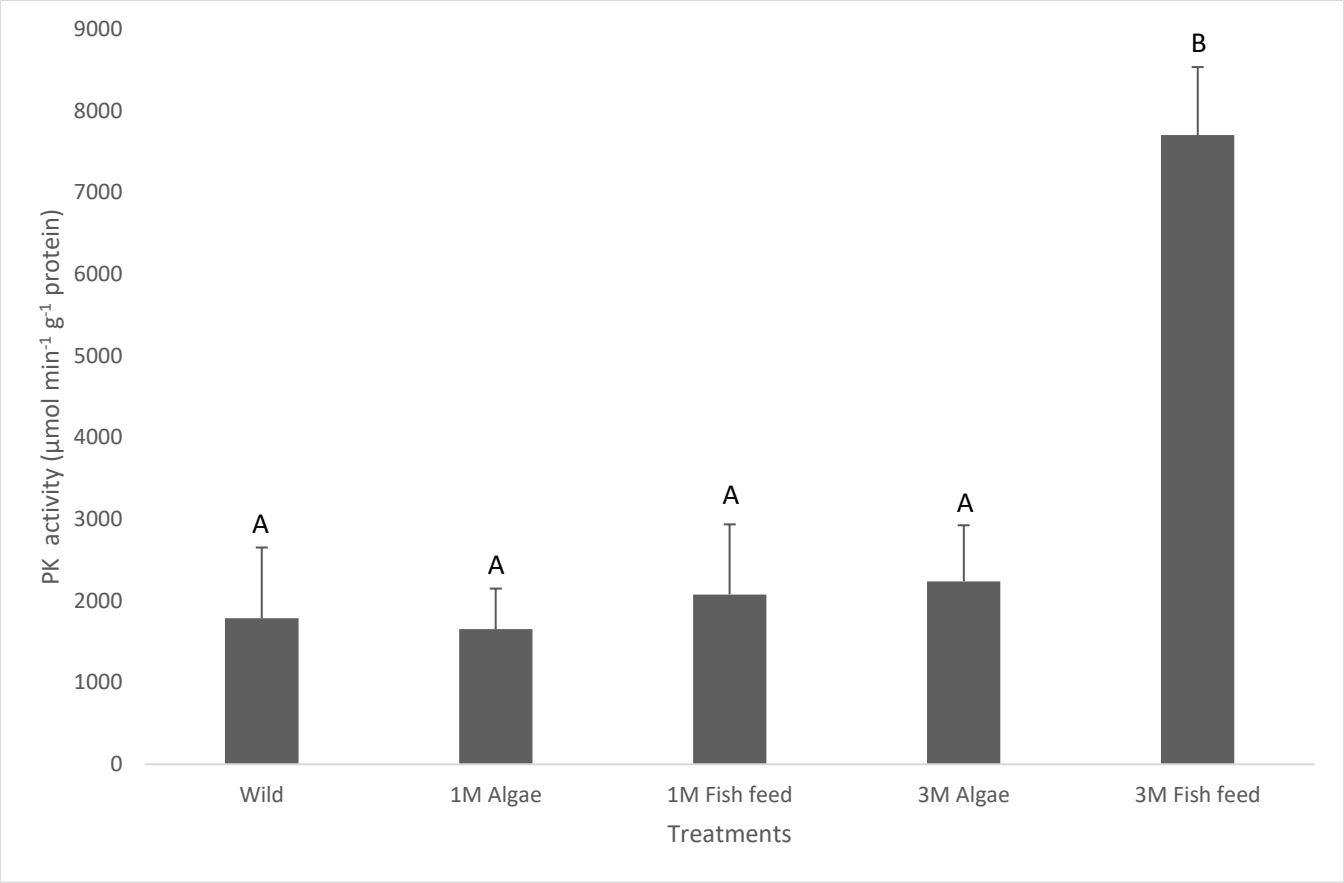

Figure 12: PK enzyme activity (µmol min^{-1} g^{-1} protein) in the GIT with the introduction and continuation of distinct diet treatments. Bars that share the same letter are not significantly different (one-way ANOVA, $p < 0.05$, n= 6).
(Wild = control animals, 1M Algae = animals fed algae diet for 1 month, 3M Algae = animals fed algae diet for 3 months, 1M Fish feed = animals fed fish feed diet for 1 month, and 3M Fish feed = animals fed fish feed diet for 3 months).

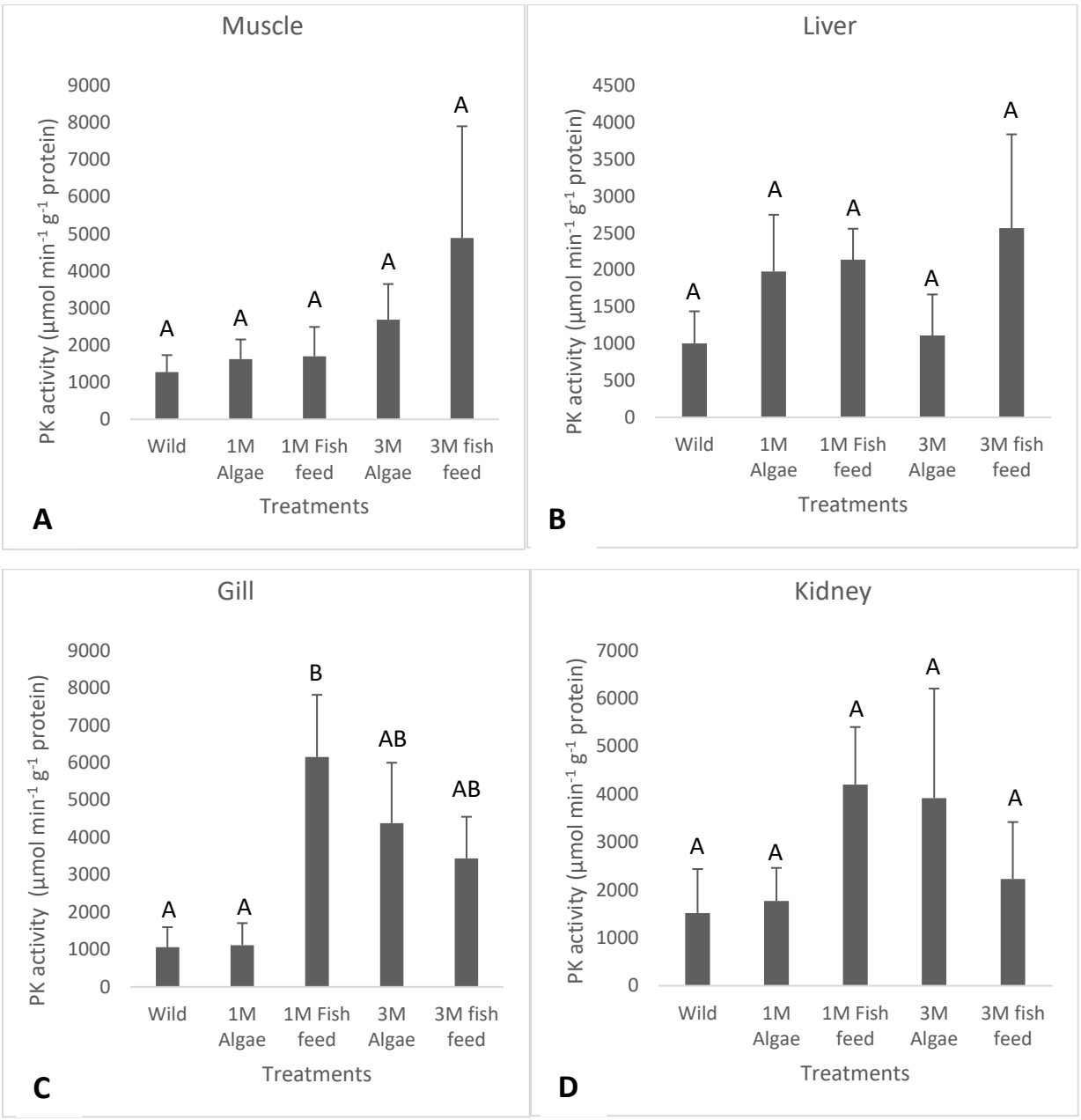

Figure 13: PK enzyme activity (µmol min^{-1} g^{-1} protein) in the **A.** white muscle, **B.** liver, **C.** gill, and **D.** kidney with the introduction and continuation of distinct diet treatments. Bars that share the same letter are not significantly different (one-way ANOVA, $p < 0.05$, n= 6).
(Wild = control animals, 1M Algae = animals fed algae diet for 1 month, 3M Algae = animals fed algae diet for 3 months, 1M Fish feed = animals fed fish feed diet for 1 month, and 3M Fish feed = animals fed fish feed diet for 3 months).

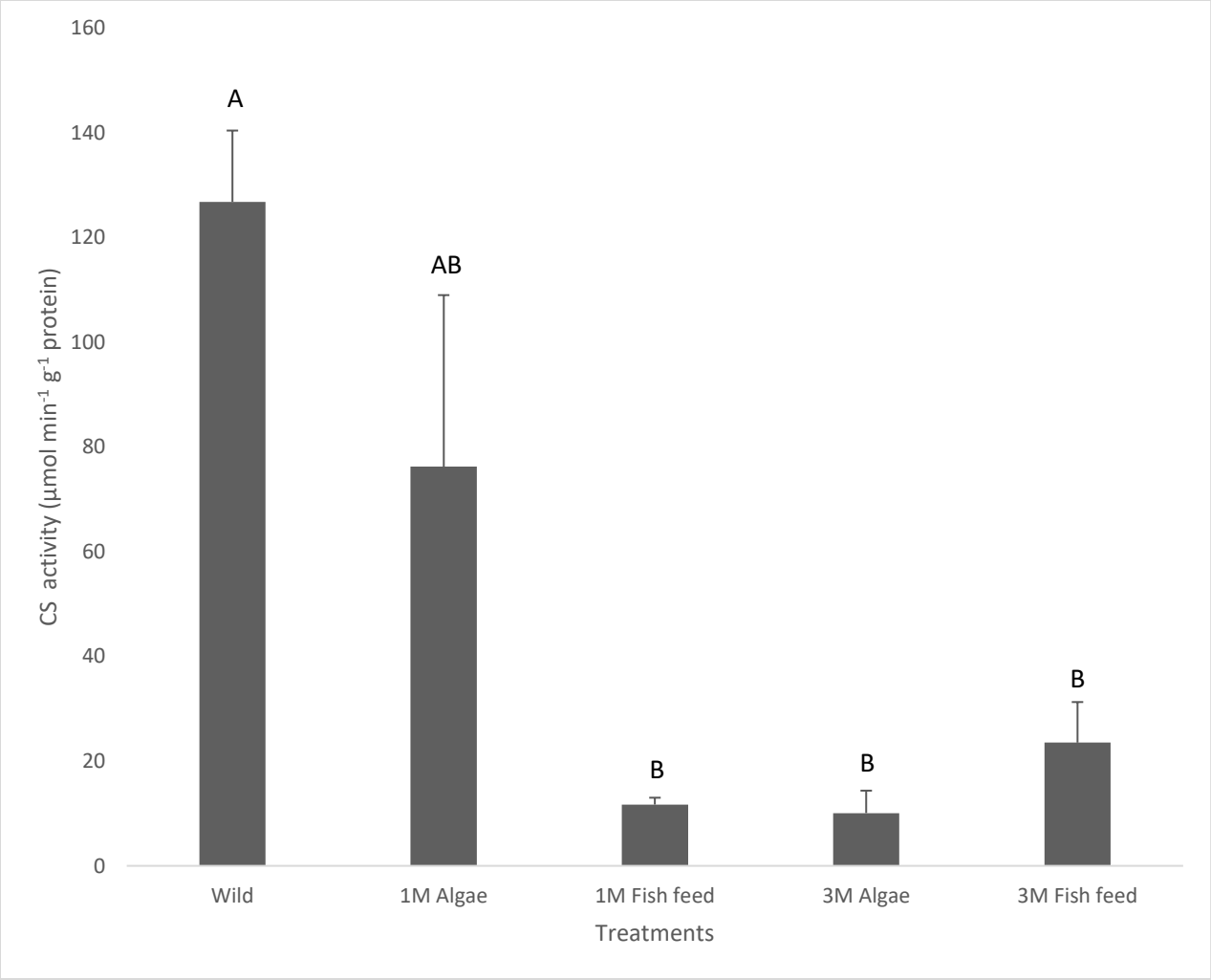

Figure 14: CS enzyme activity (µmol min^{-1} g^{-1} protein) in the GIT with the introduction and the continuation of the distinct diet treatments. Bars that share the same letter are not significantly different (one-way ANOVA, $p < 0.05$, n= 5).
(Wild = control animals, 1M Algae = animals fed algae diet for 1 month, 3M Algae = animals fed algae diet for 3 months, 1M Fish feed = animals fed fish feed diet for 1 month, and 3M Fish feed = animals fed fish feed diet for 3 months).

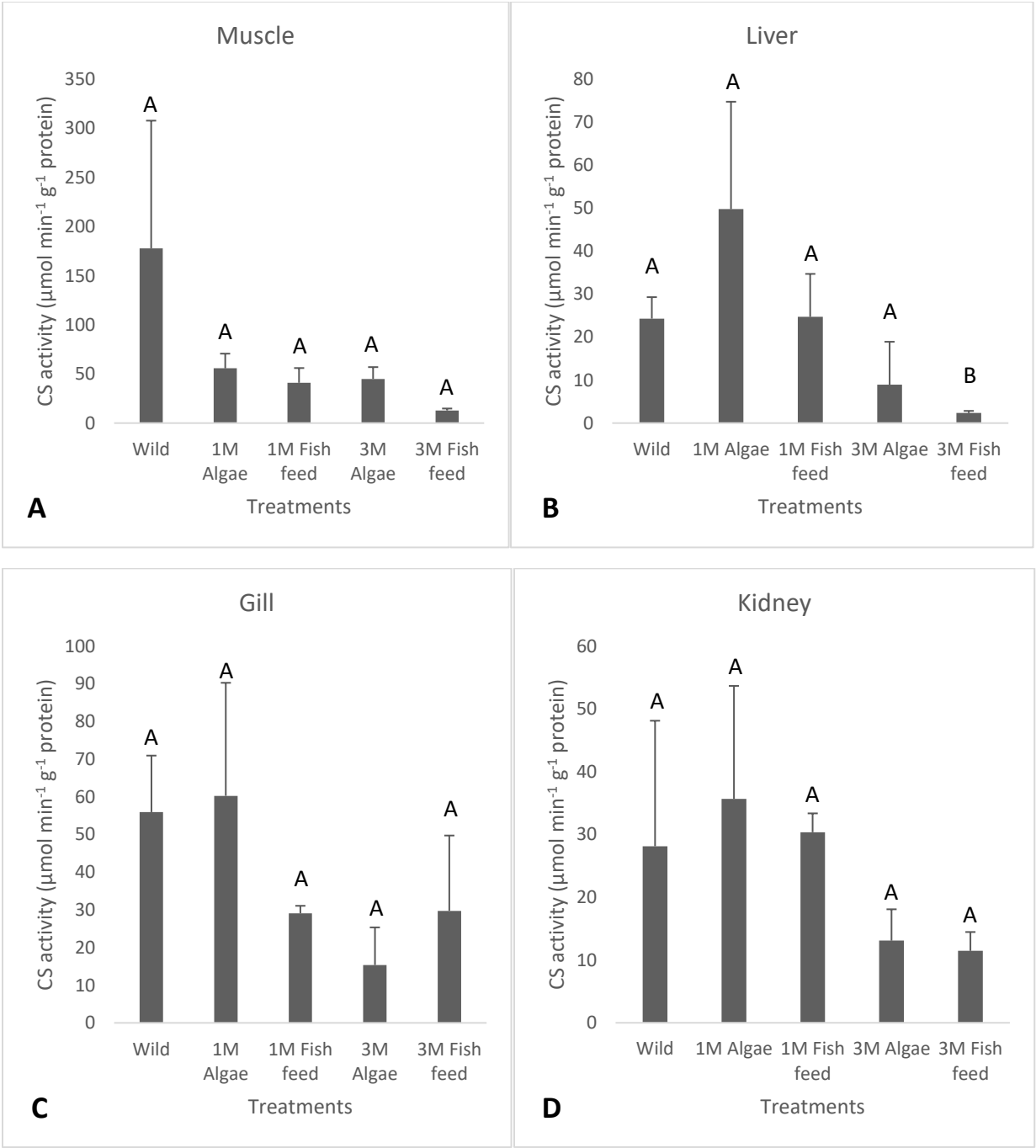

Figure 15: CS enzyme activity (µmol min^{-1} g^{-1} protein) in the **A.** white muscle, **B.** liver, **C.** gill, and **D.** kidney with the introduction and continuation of distinct diet treatments. Bars that share the same letter are not significantly different (one-way ANOVA, $p < 0.05$, n= 5).
(Wild = control animals, 1M Algae = animals fed algae diet for 1 month, 3M Algae = animals fed algae diet for 3 months, 1M Fish feed = animals fed fish feed diet for 1 month, and 3M Fish feed = animals fed fish feed diet for 3 months).

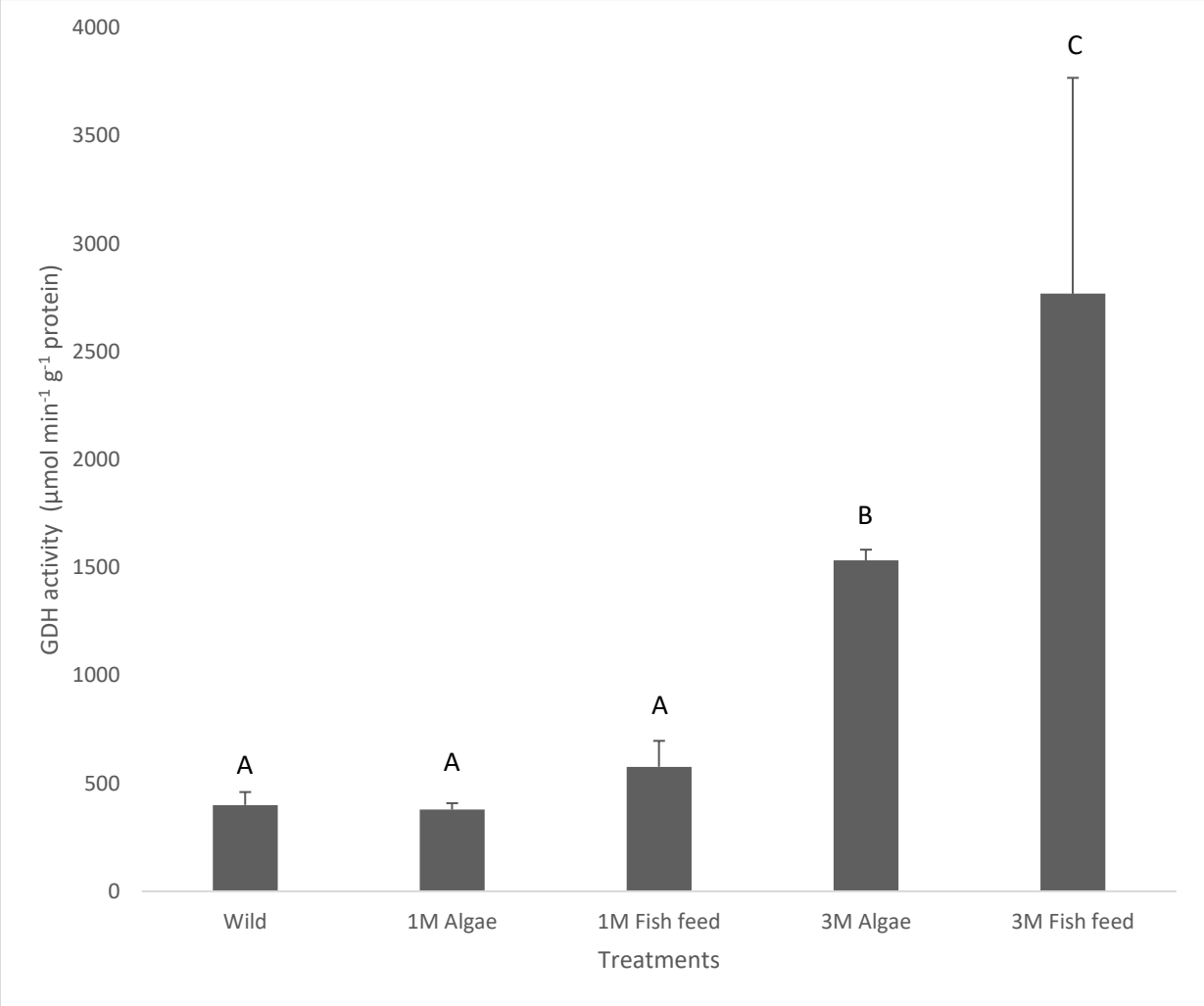

Figure 16: GDH enzyme activity (µmol min^{-1} g^{-1} protein) in the GIT with the introduction and continuation of distinct diet treatments. Bars that share the same letter are not significantly different (one-way ANOVA, $p < 0.05$, n= 6).
(Wild = control animals, 1M Algae = animals fed algae diet for 1 month, 3M Algae = animals fed algae diet for 3 months, 1M Fish feed = animals fed fish feed diet for 1 month, and 3M Fish feed = animals fed fish feed diet for 3 months).

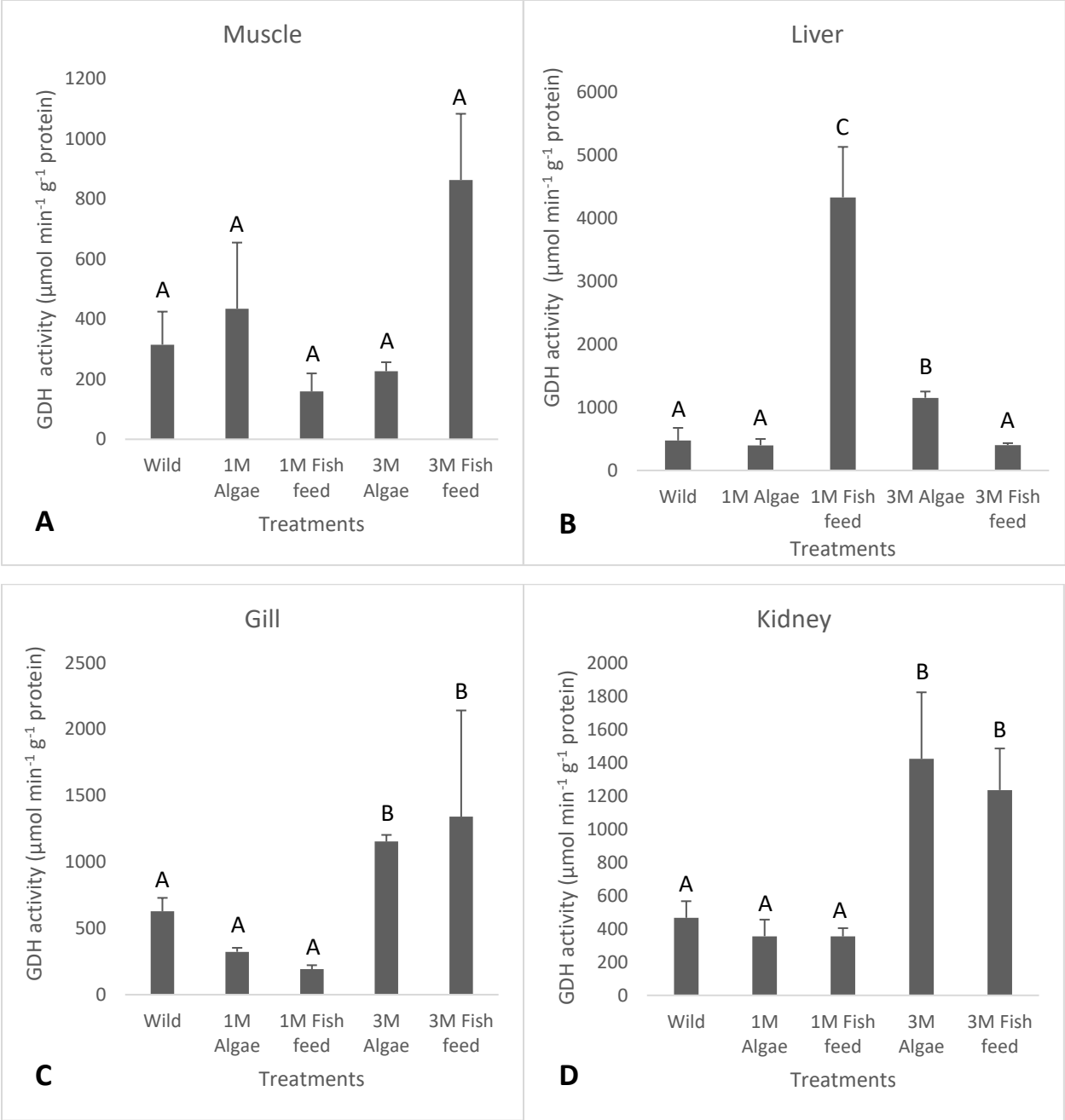

Figure 17: GDH enzyme activity (µmol min^{-1} g^{-1} protein) in the **A.** white muscle, **B.** liver, **C.** gills, and **D.** kidney with the introduction and continuation of distinct diet treatments. Bars that share the same letter are not significantly different (one-way ANOVA, $p < 0.05$, n= 6). (Wild = control animals, 1M Algae = animals fed algae diet for 1 month, 3M Algae = animals fed algae diet for 3 months, 1M Fish feed = animals fed fish feed diet for 1 month, and 3M Fish feed = animals fed fish feed diet for 3 months).

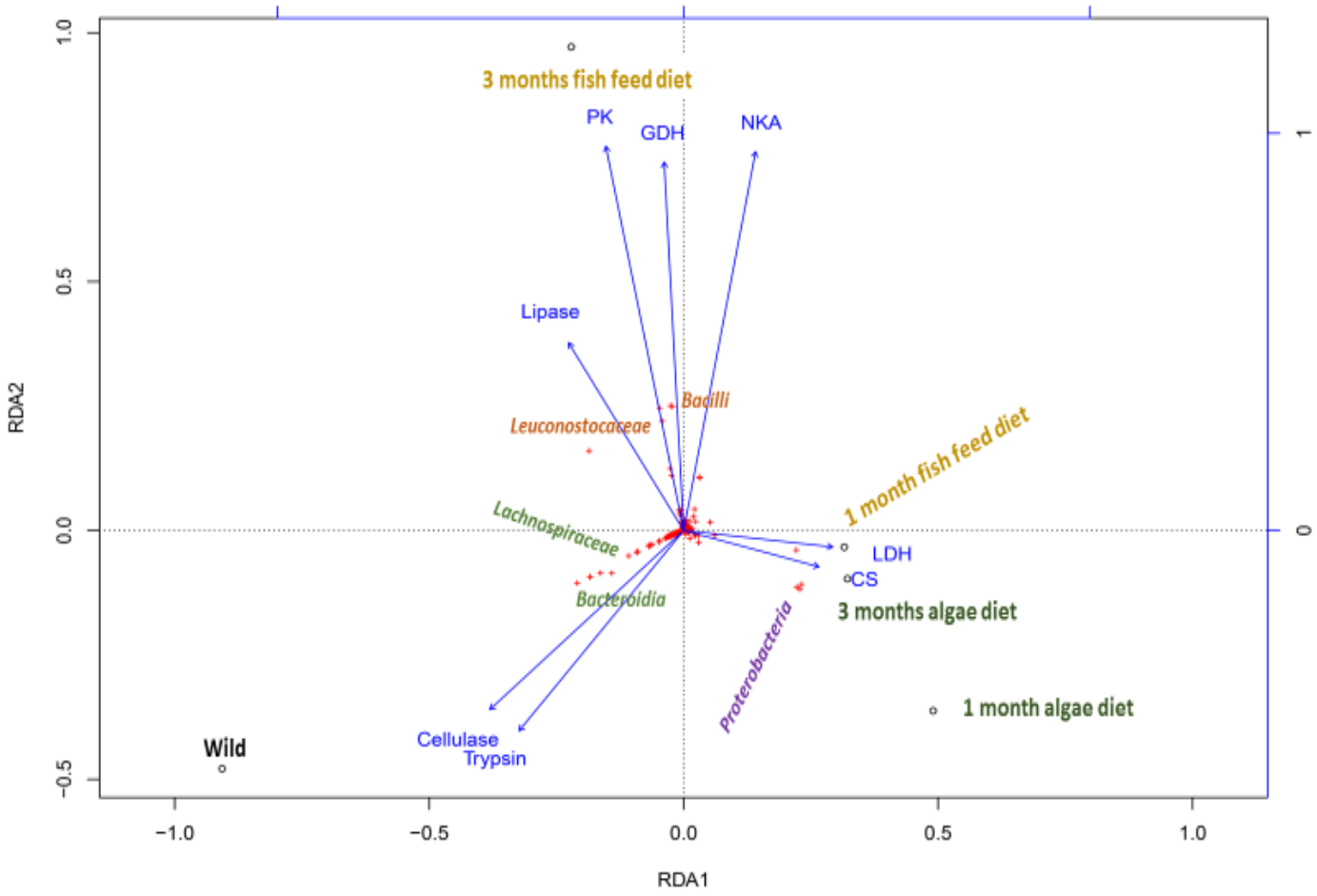

Figure 18: Multivariate RDA analysis showing the distinct separation and the correlation of the diet treatments with the abundance of the bacterial species and the enzyme activities. Enzymes are shown in blue. Diet treatments are in green and yellow. Bacterial species *Lachnospiraceae* and *Bacteroidia* = phylum Bacteroidetes, bacterial species *Proterobacteria* = phylum Proteobacteria, and bacterial species *Bacilli* and *Leuconostocaceae* = phylum Firmicutes. (Wild = control animals, 1-month algae = animals fed algae diet for 1 month, 3-month algae = animals fed algae diet for 3 months, 1-month fish feed = animals fed fish feed diet for 1 month, 3-month fish feed = animals fed fish feed diet for 3 months).

Discussion:

The understanding of the complex but interesting relationship between the diet, the GIT bacterial community, and the host enzyme activity is in a preliminary phase. While the number of studies for this interaction in humans has increased in the past decade, especially with the initiation of the Human Microbiome Project, the number of similar studies on aquatic vertebrate models is lacking. Among the few studies examining aquatic vertebrates, most have focused on the implications of this relationship on omnivorous fish. Hence, with this novel study on the herbivorous fish central stoneroller, we characterized the native GIT microbiome and measured the enzyme activity in this fish for the first time. Then we expanded our approach by altering the diet and exploring the response of the GIT bacterial community and the digestive/metabolic enzymes in this fish to further understand their role in maintaining the host's homeostasis.

5.1. GIT bacterial community reorganization in response to an altered diet:

The presence of the bacterial phyla Firmicutes, Bacteroidetes, and Proteobacteria (Figure 1) was expected as they have previously been identified as the most abundant members in the GIT-bacterial community in aquatic vertebrates (Welch and Huse 2011; Givens 2012; Bolnick et al. 2014; Clements et al. 2014; Ghanbari et al. 2015). Furthermore, Bacteroidetes accounted for a predominate proportion in the wild samples as predicted (Figure 1), as they are correlated with dietary cellulose content (Ley et al. 2006; Turnbaugh et al. 2006; Ni et al. 2014; Miyake et al. 2015).

A trend of GIT bacterial abundance change was observed within one month of the change in diet as predicted (Figure 1). It has previously been demonstrated that diet is among the exogenous factors that shape the GIT bacterial communities within their hosts (Muegge et al.

2010; Wu et al. 2015), shaping that is attributed to the changes in bacterial species abundance, rather than the changes in the bacterial species identity (Figure 1; Liu et al. 2016). The increase in the proportions of the GIT bacterial phylum Firmicutes with the continuation of the commercial fish feed diet for 3-month was consistent with the previously established association between Firmicutes abundance and the lipid content of the commercial diet (Turnbaugh et al. 2006; Parfrey et al. 2011; Angelakis et al. 2012; Carmody and Turnbaugh 2012; Givens 2012; Semova et al. 2012). In contrast, the abundance of the GIT bacterial phylum Proteobacteria with the algae diet (both 1- and 3-month) was not expected (Figure 1), as per Bacteroidetes correlations with dietary cellulose content (Ley et al. 2006; Turnbaugh et al. 2006; Ni et al. 2014; Miyake et al. 2015). As multiple parameters, including the diet's nutritional content (fat/protein/saccharide) and the habitat of the fish (source water vs. lab water) changed concurrently, it is difficult to pinpoint a single cause for this anomalous results in our current study. However, recovery in the Bacteroidetes abundance proportions by the end of the 3-month algae diet treatment (Figure 1) may support the predicted association and demonstrate an acclimation to the diet and lab conditions.

The decrease in the number of unique species OTUs from the wild treatment compared to the other dietary treatments was also anticipated (Figure 2.A), as host's feeding on a generalized diet typically decreased GIT bacterial OTU richness compared to that of host's feeding on heterogeneous diets (Bolnick et al. 2014). This prediction was validated successfully in this study, mainly because an overall decrease in the count of bacterial OTUs (Figure 2.A), rare bacterial species (Figure 2.B), phylogenetic distance between the bacterial species (Figure 2.C), and richness and evenness distribution between the bacterial species (Figure 2.D) was observed between the wild vs the other dietary treatments. Interestingly, although the altered diet impacted

the species count (Figure 2.A), rarer species (Figure 2.B), and phylogenetic distance between the species (Figure 2.C) in the 1-month fish feed versus the 3-month algae diet treatment; the distribution of the species was not impacted (Figure 2.D). This may due to the presence of a few, highly abundant bacterial species in these treatments regardless of an alteration in the diet.

The clustering of the diet treatments within the weighted beta-diversity analysis (Figure 3.A) also demonstrated the implications of the altered diet in reshaping the abundance of the GIT bacterial communities. The 1- and 3-month algae diet treatments clustered together (Figure 3.A), possibly due the abundance of similar bacterial species, while the 3-month fish feed diet treatment clustered separately possibly due to a different abundance of the GIT bacterial species compared to the other treatments (Figure 3.A). This clustering of the diet treatments apart from the wild treatment (Figure 3.A) is further suggestive of the implication of an altered diet in reshaping the abundance of the host's GIT bacterial communities. On the other hand, although the unweighted beta-diversity analysis exhibited a continued distinction between the treatments (Figure 3.B), especially between the wild and the diet treatment animals, the decreased support for the association (green line = < 50% support, Figure 3.B) indicated that the association between the diet and GIT bacterial community was driven more by the bacterial abundance than by the bacterial identity (Semova et al. 2012; Ringo et al. 2016).

The multivariate analysis in this study suggested similarity in the GIT bacterial composition between the treatments (Figure 4.A), however, the separation of the treatments in the redundancy quartiles based upon the abundance of the resident GIT bacterial species suggests that certain abundant bacterial species were specifically associated with the dietary treatments (Figure 4.B). Furthermore, the presence/absence data for the bacterial species with low weight and abundance was unique for each treatment due to the presence of a few, highly abundant

bacterial species (Figure 4.B). This separation of the treatments in the redundancy quartiles did not change even when the bacterial species with low weight and abundance were removed with a Hellinger transformation (Figure 4.B). This analysis once again suggests that the change in abundance, and not the identity of the bacterial species was strongly associated with the diet treatments. As initially proposed, the abundance of the bacterial genera *Bacilli* and *Leuconostocaceae* of phylum Firmicutes was associated discretely with the 3-month commercial fish feed diet treatment (Figure 4.B), and consistent with previously published literature (Givens 2012, pinfish, killifish; Semova et al. 2012, zebrafish; Carmody and Turnbaugh 2012, zebrafish). This finding further supported our initial hypothesis that a high fat diet transitions the abundance of the GIT bacterial community from the phylum Bacteroidetes to that of phylum Firmicutes in the central stoneroller GIT.

5.2. Integrated reaction of the GIT bacterial community reorganization and the digestive enzymes in response to an altered diet:

The vital process of digestion requires the combined efforts of the endogenous and exogenous components that comprise the host's overall GIT unit (Furne et al. 2005). The efficacy of this coordination to combat external challenges, such as an altered diet, is a crucial factor determining the robustness of the host's digestive and metabolic processes (Furne et al. 2005; Muegge et al. 2010; Carmody and Turnbaugh 2012). In our current study, there was a gradual decrease in the GIT cellulase enzyme activity with a reduction in the dietary plant material followed by a gradual decrease in the abundance of the Bacteriodetes (Figure 1, Figure 5, Figure 18). This is supported by previous studies, which conversely demonstrated an increase in exogenous cellulase activity through the activation of bacterial cellulosome system genes and this phenomenon was associated with an increase in the abundance of GIT Bacteroidetes

(Luczkovich and Stellwag 1993, pinfish; Saha and Ray 1998, Rohu). In terms of protein digestion, trypsin activity in the GIT of the wild-caught stonerollers was unexpectedly high (compared to the 3-month fish feed diet treatment samples) and there was a slightly elevated trypsin activity in both the algae diet treatment samples (compared to both the fish feed diet treatment samples; Figure 6). This may be indicative of maximal protein digestion and absorption efforts on the part of the host in response to a protein-deficient diet, regardless of the changes in the abundance of the GIT bacterial communities (Chan et al. 2004).

The association between lipid metabolism and the GIT bacterial community has previously been correlated with Firmicutes-mediated digestion of lipids into short-chained fatty acids (SCFA) (German 2009; Angelakis et al. 2012; Ghanbari et al. 2015; Koca et al. 2015; Miyake et al. 2015; Olsen et al. 2015), followed by the absorption of these SCFA through Firmicutes-mediated pathways (Carmody and Turnbaugh 2012; Semova et al. 2012; Boonmahome and Mongkolthanaruk 2013; Karsov et al. 2015). Furthermore, the increase in the abundance of the GIT bacterial phylum Firmicutes has been directly associated with an increase in lipase activity, especially in hosts feeding on a lipid rich commercial diet (Angelakis et al. 2012; humans, Semova et al. 2012; zebrafish, Dutta et al. 2015; *Catla catla*, Pujante et al. 2017; mullet). Perhaps the increase in the GIT lipase activity followed by an increase in the abundance of Firmicutes in the commercial fish feed diet treatment (1- and 3-month) in our study (Figure 1, Figure 7, Figure 18) is further suggestive of the synergistic response of the GIT bacterial community towards an altered diet (lipid aspect of the diet) in the central stoneroller GIT.

5.3. The synergistic response of an altered GIT bacterial community and the GIT/full body metabolic enzymatic physiology in response to an altered diet:

The basolateral membrane of the intestinal epithelial cells generates an electrochemical gradient that drives transcellular transport of water, ions, and nutrients (Hakim et al. 2006) and is created by NKA enzyme activity (Skulachev 1978; Dibrova et al. 2015). The impact of the GIT bacterial community on the NKA activity of the enterocyte is currently unclear, but previous studies have indicated the presence of a connection. For example, phylum Firmicutes has been implicated in the increased incorporation of unsaturated fatty acids into the phospholipid membrane of the enterocytes in the *D. rerio* GIT (Carmody and Turnbaug 2012; Semova et al. 2012). This could potentially impact NKA enzyme activity as the incorporation of the unsaturated fatty acids into the phospholipid membrane has been previously associated with an increase in the water permeability of the enterocytes, resulting in an increase in NKA activity in order to maintain the electrochemical gradient (Sundell and Sundh 2012). Interestingly, in our current study, a significant increase in GIT NKA activity was correlated with an increase in the abundance of phylum Firmicutes in the 3-month fish feed diet treatment (Figure 1, Figure 8, Figure 18). When examining the 3-month algae diet treatment, the increased GIT NKA activity may be the response of the host in response to an altered diet, regardless of the changes in the abundance of the GIT bacterial communities (Figure 1, Figure 8, Figure 18).

Diet and/or GIT bacterial communities are also thought to also influence the activity of enzymes involved in glycolysis through the manipulation of the host's metabolic processes (Kihara and Sakata 2001; Muegge et al. 2010; Ni et al. 2014; Konishi et al. 2015; Bucking et al. unpublished). For example, Muegge et al. (2010) observed that Firmicutes in the GIT of mammalian herbivores downregulated genes encoding for enzymes associated with oxaloacetate

production from phosphoenolpyruvate in response to a carbohydrate rich diet, further supporting the idea of the integral contribution of diet and/or GIT bacterial communities to the glycolysis pathway. An increase in the GIT PK activity followed by an increase in the abundance of Firmicutes in the 3-month fish feed diet treatment in our study suggests an increased breakdown of exogenous glucose obtained from a carbohydrate diet, modulated by the Firmicutes (Figure 1, Figure 12, Figure 18). In contrast, an increase in the GIT LDH activity followed by a decreased abundance of Firmicutes in the 3-month algae diet treatment in our study suggested that the host used its endogenous glucose reserves to compensate for dietary carbohydrate deficiency (Figure 1, Figure 10, Figure 18). Furthermore, the increase in LDH activity in the white muscle (Figure 11.A), and the increase in PK activity in the gill (Figure 13.C) indicated the possibility of differential use of exogenous or endogenous polysaccharides among tissues. Overall, the changes in the glycolysis enzyme activities in response to certain dietary treatments seem logical in that their catalytic capacity may be limited primarily by the availability of exogenous dietary polysaccharides, in addition to the abundance of certain GIT bacteria, which in turn may influence the activities of other downstream enzymes. Indeed, the significant decrease in the GIT CS activity with an increase in the abundance of Firmicutes in the 1- and 3-month commercial fish feed diet treatments (Figure 1, Figure 14, Figure 15, Figure 18**)** suggests the possibility of the presence of a Firmicutes-mediated pathway, allowing the direct flow of dietary carbon into the citric acid cycle as previously reported in mice (Cummins et al. 2014). The GIT activity of CS in the 3-month algae diet treatment (Figure 14) also decreased significantly regardless of the changes in the abundance of the GIT bacterial communities, however, this may be suggestive of the response of the host in response to the dietary impact.

A 6-fold increase in the GIT ammonia load following feeding and digestion (Bucking and Wood 2012; rainbow trout) is a frequent stressor faced by both the intestinal tract and the resident GIT-bacterial communities (Nazarchuk et al. 1981; Olsen et al. 2015). Interestingly, the production of exogenous GDH as a detoxification response to ammonia toxicity in the GIT, especially by Firmicutes has previously been proposed as a benefit of hosting a GIT bacterial community (Nazarchuk et al. 1981; Sanchez-Muros et al. 1998; Girinathan et al. 2016). Indeed, an increase in the GIT GDH activity concomitant with an increase in dietary protein (3-month fish feed diet treatment), and an increase in the abundance of Firmicutes in our study supported the bacteria-mediated ammonia detoxification theory that was previously reported by Nazarchuk et al. (1981) in termites (Figure 1, Figure 16, Figure 18).

In the current study, however, changes in NKA, CS, and GDH activity in the other tissues (white muscle, kidney, liver, and gill) did not exhibit any clear trends. This may indicate that diet manipulation and GIT bacterial community reorganization may not further dictate the activity levels of the enzymes beyond the GIT. Another possibility is that other internal and external factors such as habitat, genetic makeup, evolutionary traits, may compensate for changes brought on by the GIT. Therefore, future studies on the connection between an altered diet, GIT bacterial community organization, and internal and external factors in relation to the whole animal enzyme activities would further enhance our understanding of this intriguing physiological relationship. This remains an interesting avenue of investigation for future comparative studies.

Overall, the transition of the GIT bacterial community from the abundance of Bacteroidetes in the wild treatment, to the abundance of Firmicutes after 3-months of commercial fish feed diet treatment (Figure 1) supported our initial hypothesis that a high fat diet

would alter the composition of the GIT bacterial community in favor of an abundance of Firmicutes in the central stoneroller GIT. Additionally, the activity of the cellulase and lipase digestive enzymes were influenced by the altered diet, and correlated with the transition in the abundance of the GIT bacterial community from the phylum Bacteroidetes to that of phylum Firmicutes. In contrast, we deduced that the activity of trypsin in the GIT was influenced by the response of the host towards a protein-deficient diet, regardless of the changes in the abundance of the GIT bacterial communities. The activities of the metabolic enzymes in the GIT investigated in this study were influenced by the altered diet, changes in the abundance of the GIT bacterial community, and/or by the coordination of both factors supporting our hypothesis. However, in the white muscle, kidney, liver, and gill, evidence of the influence of diet, GIT bacterial community abundance composition changes, and/or both factors on the various metabolic enzyme activities was lacking. Therefore, our initial hypothesis that changes in the GIT converge to influence the full-body enzyme activities of the central stoneroller was not upheld as other exogenous and endogenous factors may or may not also dictate the enzymatic activities in these tissues.

Chapter 3: The response of the GIT bacterial community and the digestive/metabolic enzyme activity in response to an altered diet in the carnivorous fish **Etheostoma caeruleum**

Introduction:

6.1. GIT of carnivorous fish:

Carnivorous fish feed on a protein-rich diet, and are adapted to digesting protein through proteolytic enzymes (pepsin, trypsin etc.). The protein digestion pathways in carnivorous fish, which involves the breakdown of larger polypeptides into smaller peptides by peptidases in the GIT, has been previously demonstrated (e.g. Natalia et al. 2004; Asian arowana). Initially, the dietary protein polypeptides are partially hydrolyzed into smaller peptides in the stomach of the carnivorous fish by pepsin (secreted both by the GIT bacteria and host stomach gastric cells into the lumen; Moran and Saborido 1996; Solovyev et al. 2015). The digesta is then passed into the intestine where the smaller peptides are absorbed by the host and the remaining larger polypeptides are further digested by trypsin, again secreted by the GIT bacteria and the host's intestinal tissue into the lumen (Krogdahl et al. 1994; Al Hafedh 1999; Furne et al. 2005; Golchinfar et al. 2011). Meanwhile, lipid-digesting enzymes such as lipase are also secreted by the GIT bacteria and the host's intestinal tissue into the intestinal lumen which results in the digestion of dietary lipids (triglycerides broken down into monoglycerides; Golchinfar et al. 2011; Boonmahome and Mongkolthanaruk 2013). Finally, the hydrolyzed peptides and monoglycerides are absorbed by the host through the enterocyte. GIT bacteria aide in the digestion of proteins and lipids, and the colonization of the GIT of the carnivorous fish by bacterial communities is influenced by dietary factors. For example, the colonization of the GIT

of the *Gadus morhua* (Atlantic cod) and the *Oncorhynchus mykiss* (rainbow trout) by the bacterial species *Pseudomonas, Psychrobacter, Citrobacter, Carnobacterium,* and *Aeromonas* has previously been identified and associated with dietary protein concentrations (Spanggaard et al. 2000; Belanger et al. 2003; Lazado et al. 2012).

6.2. Effects of an altered diet on the GIT enzyme activity of a carnivorous fish:

Due to the uncertainty in the supply of fish meal and increasing competition from the horticulture sector, several efforts have been made to include plant based nutrients in commercial diets (De et al 2015). Furthermore, there is a trend in aquaculture towards an increase in the use of plant-based carbohydrates and proteins in the diets of farmed carnivorous fish as a cost-saving measure. However, in order to be a successful strategy, this requires an understanding of the response of the GIT of carnivorous fish towards this dietary challenge (Anderson et al. 1984; Buddington et al. 1987; Buddington et al. 1997; Geruden et al. 2007). Initial studies suggest that plant-based diets are poorly assimilated by carnivorous fish (Dhage 1968, Bairagi et al 2002) and the current supposition is that plant-based diets present a challenge for the capacity of the carnivorous fish GIT to modulate its enzyme activity (Chan et al. 2004). Indeed, the digestion of carbohydrates by carnivorous fish is less efficient when compared to their herbivorous and omnivorous counterparts as they lack the necessary enzymes to digest these nutrients (Kuperman and Kuzmina 1993; Buddington et al. 1997; Faccioli et al. 2014). However, the GIT of the carnivorous fish is thought to harbor a bacterial community that not only bolsters the digestion and absorption of proteins, but also carbohydrates and glucose from the ingested material that would otherwise be difficult if not impossible to digest (Givens 2012; Lazado et al. 2012; Ni et al. 2014). The question of whether the GIT bacterial community of a carnivorous fish responds to an altered diet remains poorly answered. Therefore, understanding the response of the GIT

bacterial community of the carnivorous fish *Etheostoma caeruleum*, to an altered diet serves as an initial step toward answering this question, and toward enhancing knowledge of the overall functioning of their GIT.

Proteobacteria is the abundant GIT bacterial phyla of many carnivorous fish, and as a protease-producing phylum, it is likely responsible for secreting proteolytic enzymes (trypsin and pepsin) in the GIT (Bairagi et al. 2002; Ghosh et al. 2002; Ray et al. 2012; Banarjee et al. 2016; Andrade et al. 2017). Furthermore, the detection of enzyme activity involved in carbohydrate digestion such as that of maltase, amylase, and glucokinase, provides a correlative basis for the exogenous release of these enzymes by the other residential GIT bacterial communities (Wilson 1994; Moran and Saborido 1996; Hidalgo et al. 1999; Bairagi et al. 2002; Ghosh et al. 2002; German et al. 2004; Geruden et al. 2007). However, we currently do not know the activity of these enzymes in the rainbow darter GIT, and other tissues. Furthermore, while the response of several enzymes (including pepsin, trypsin, amylase, glucokinase, lipase, GDH, PK, LDH, and CS) to plant based carbohydrate and protein diet in carnivorous fish species such as the rainbow trout have been studied (Kapoor et al 1976; Al Hafedh 1999; Krogdahl et al. 1999; Furne et al. 2005; Geurden et al. 2007; Golchinfar et al. 2011; Balasubramanian et al. 2016; Veron et al. 2016), it is not known if the rainbow darter will display the same responses. Hence, in this novel study, we characterized the native microbiome and measured the enzyme activity in several tissues (GIT, white muscle, liver, kidney, and gill) of the rainbow darter for the first time. We also explored for the first time how altering the diet by including more plant protein would affect both the bacterial communities and the enzyme activities in the rainbow darter.

6.3. Objectives and Hypothesis:

E.caeruleum is a benthic species, whose diet consists of larvae, fish eggs, and other smaller fish (Stewart 1988) which is high in protein (Stewart 1988). We explored the GIT of this species, both in terms of the microbiome but also the enzymatic activities. Furthermore, we wanted to understand how this species would react to an increase in plant based dietary protein and carbohydrate. We hypothesized that the composition of the GIT bacterial community in wild-caught rainbow darters would present an abundance of Proteobacteria. Furthermore, we predicted that Proteobacteria would continue as the most abundant phylum in the GIT bacterial community of the rainbow darter following exposure to a protein-rich (bloodworm) diet; and these treatments would show a high GIT trypsin activity. Finally, we predicted a transition from an abundance of the phylum Proteobacteria to an abundance of Firmicutes in response to the prolonged feeding of the commercial fish feed diet (lipid-rich), and this transition will influence the GIT enzyme activity in the rainbow darter in response to the above dietary changes; with a high GIT lipase activity. Additionally, we hypothesized that the enzyme activity in the reference tissues; that is, white muscle, liver, kidney, and gill, will be influenced by an array of factors, including the continued feeding of an altered diet, the transition of the GIT bacterial community from an abundance of Proteobacteria to an abundance of Firmicutes, accompanied by an overall change in the digestive and metabolic enzyme activities of the GIT.

Understanding the responses of the GIT enzymes such as cellulase, trypsin, lipase, NKA, LDH, PK, GDH, and CS, following exposure to an altered diet will provide a necessary link towards enhancing the knowledge of the overall contribution of the carnivorous fish GIT. Similarly, correlation with bacterial changes will allow us to discover whole animal responses to dietary shifts and will give us a better understanding of the whole animal homeostasis.

Materials and Methods:

7.1. Model fish species collection:

Rainbow darters, as summarized in Table 4 below, were collected using the same method and location as found above in section 3.1 of Chapter 2. 5 individuals (average mass= 2.5 ± 1 gram, average length= 3 ± 1.2 cm) were sacrificed aseptically on the same day as collection, using the method previously described above in section 3.4. The remaining fish were held under laboratory conditions and subjected to dietary treatments.

7.2. Diet treatments:

After 7-days of laboratory acclimation during which the animals were unfed, the fish were separated into two tanks, containing 20 and 15 individuals respectively. Individuals in the first tank were fed UV-sterilized bloodworms and individuals in the second tank were fed UV-sterilized commercial fish feed. The nutritional values of each diet are presented in Table 5. 7 individuals from each tank were then aseptically sampled 1-month and then 3-month following the first meal.

Sampling and analysis were performed identically to that presented in Chapter 2. The bacterial gDNA was extracted, quantified, pooled, sequenced, and the microbiome was then characterized with QIIME. Cellulase, lipase, and trypsin enzymes were measured in the GIT and NKA, LDH, PK, GDH, and CS enzymes were measured the GIT, white muscle, liver, kidney, and gill tissues. Finally, correlation between the diet treatments, microbiome abundance, and enzyme activity in the GIT was analyzed through a multivariate RDA analysis performed in R. All methods and analysis were previously described and replicated with this species as mentioned above in sections 3.4 to 3.16 of Chapter 2.

Table 4: Summary of *E.caeruleum* collected from the sampling site of Irvine Creek:

Date of collection	Number of individuals collected	Temperature of source water (C°)	Temperature of lab water (C°)
May 5th, 2016	40	14 ± 2	11 ± 2

Table 5: Summary of nutritional values in the *E.caeruleum* diet treatments:

Blood worm	Commercial fish feed
Protein = 55 % Fat = 3 % Fiber = 5 % Moisture = 10 %	Protein = 42 % Fat = 15 % Fiber = 2 % Moisture= 6.5 %

Results:

8.1. Transition of the GIT bacterial abundance and richness in response to an altered diet:

175,110 bacterial sequences were detected and a total of 513 unique OTUs were identified after the quality control procedure. Sequences and OTU's removed during the quality control procedure totaled only to be less than 1 % of the entire data set. The identified 513 OTUs belonged to the phyla Bacteroidetes, Proteobacteria, and Firmicutes. Initially, in the wild (control) samples, the GIT bacterial phyla Proteobacteria, Firmicutes, and Bacteroidetes made up the GIT bacterial community, with 61%, 36% and 3% of the total abundance respectively (Figure 19). The introduction of the bloodworm diet for 1-month resulted in an increase in Proteobacteria abundance to 85%, and a decrease in the abundance of Firmicutes to 4%, and Bacteroidetes to 0.7 % (Figure 19). Furthermore, continuation of the bloodworm diet for 3 months resulted in the dominance of the GIT bacterial community abundance by Proteobacteria (99% abundance), while Firmicutes and Bacteroidetes were reduced to trace levels, at 0.4 % and 0.1% abundance, respectively (Figure 19).

Proteobacteria abundance was maintained with the introduction of the commercial fish feed diet for 1-month at 55%, with an increase in Bacteroidetes abundance to 32% and a decrease in Firmicutes abundance to 1% (Figure 19). Continuation of the fish feed diet for 3 months resulted in a recovery in Firmicutes abundance to 10 % and Proteobacteria abundance to 72%, but Bacteroidetes abundance was reduced to 2% (Figure 19).

The rarefied observed species curves (Figure 20.A) showed the highest count of bacterial species (OTUs) in the wild treatment with 329 OTUs at 10,000 sequences, followed by the 1-month bloodworm diet treatment (251 OTUs), the 1-month fish feed diet treatment (235 OTUs), and the 3-month fish feed diet treatment (201 OTUs). The lowest GIT OTU richness was

observed in the 3-month bloodworm diet treatment, with 155 OTUs (Figure 20.A). Similarly, the phylogenetic distance between the bacterial species (PD whole tree) and the distribution evenness of the bacterial species (Shannon Index) was also the greatest in the wild treatment compared to the dietary treatments (Figure 20.C, 20.D). Interestingly, both the 1-month bloodworm and 1-month fish feed diet treatments had a greater number of rarer bacterial species (Chao1) compared to the other treatments (Figure 20.B).

Unweighted beta-analysis (similarity between the treatments based on species identity) showed that the 3-month fish feed treatment clustered separately from the other treatments (Figure 21.B). The 1-month bloodworm and 1-month fish feed diet treatments clustered together possibly due to similarity between the treatments based on species identity; however, this association was weakly supported (< 25 %; Figure 21.B). Furthermore, the separation of the wild and the 3-month bloodworm diet treatments from this clustering was also weakly supported (<50 %; Figure 21.B). The clusters of the treatment samples in the unweighted analysis was further supported by Bonferroni corrected p-values at $p \leq 0.01$ and a bootstrapped tree at a distance of 0.1 (Figure 21.B).

On the other hand, the weighted beta-analysis (similarity between the treatments based on species identity and abundance) showed that the wild and the 1-month fish feed diet treatments clustered together possibly due to the abundance of similar bacterial species (Figure 21.A). The 3-month fish feed and the 1-month bloodworm diet treatments clustered separately possibly due to a different composition of the bacterial species abundance compared to the other treatments (Figure 21.A). Overall, the 1-month bloodworm, 3-month bloodworm, and 3-month fish feed diet treatments demonstrated altered bacterial OTU communities compared to the wild samples

(Figure 21.A). The cluster of the treatment samples in the weighted analysis was supported by Bonferroni correction ($p \leq 0.01$) and a bootstrapped tree at a distance of 0.1 **(**Figure 21.A).

Identification of the bacteria species correlating with the diet treatments was achieved through the multivariate redundancy analysis (RDA). The axes that explained the most variations were RDA 1 (Eigenvalue=0.2008, proportion explained= 50.99%) and RDA 2 (Eigenvalue= 0.1329, proportion explained= 33.74%). These two axes combined explained 84.72% of the total variation seen in the bacterial species abundance in the treatments (adjusted R^2 =0.852). Furthermore, a comparison of the Hellinger-transformed data (Figure 22.B) to the non-transformed data (Figure 22.A) showed that filtering out the bacterial species with low weight and abundance did not alter the redundancy analysis results, therefore establishing that the treatments were distinguished by the abundance of a few highly abundant bacterial species, rather than the species richness (Figure 22).

Most bacterial species detected were initially scattered on the redudancy plot before the Hellinger transformation was performed (Figure 22.A). However, post-Hellinger transformation (Figure 22.B), only a few bacterial species were detected that correlated specifically with the treatments. Bacterial species of the genus *Ruminococcaceae* and genus *Ethanoligenens* (phylum Firmicutes) had a strong positive correlation (increased abundance) to the wild, 1- and 3-month fish feed diet treatments, but a negative correlation (decreased abundance) compared to the 3-month bloodworm diet treatment (Figure 22.B). Bacterial species of the genus *Burkholderia* and *Pseudomonas* (phylum Proteobacteria) demonstrated a strong positive correlation (increased abundance) with bloodworm diet for 1- or 3-month, respectively (Figure 22.B).

8.2. Enzyme activities

8.2.1. Cellulase enzyme activity in the GIT:

The highest cellulase activity was detected in the wild samples (0.637 ± 0.2 mg glucose min^{-1} mg^{-1} protein) but the dietary treatments (1-month bloodworm, 1-month fish feed, 3-month bloodworm, and 3-month fish feed) did not impact the enzyme activity **(**Figure 23**)**.

8.2.2. Trypsin enzyme activity in the GIT:

Trypsin enzyme activity was not impacted by the 1-month bloodworm, 1-month fish feed, and 3-month fish feed diet treatments. However, the enzyme activity increased significantly ($p < 0.05$, n= 5) in the 3-month bloodworm treatment compared to the wild (control) treatment (1842.72 ± 311.75 µmol min^{-1} g^{-1} protein; Figure 24).

8.2.3. Lipase enzyme activity in the GIT:

Lipase activity was not impacted by the 1-month bloodworm diet, however, the activity increased significantly ($p < 0.05$, n= 5) with the continuation of the bloodworm diet for 3-month to the highest level (61.214 ± 6.0293 nmol of glycerol min^{-1}). Contrastingly, the 1- and 3-month fish feed treatments significantly lowered the enzyme rates ($p < 0.05$, n= 5; Figure 25).

8.2.4.1. Na^+/K^+ ATPase enzyme activity in the GIT :

NKA activity in the GIT was not impacted by the dietary treatments averaging across all samples at 6.1 ± 3.02 µmol ADP mg^{-1} protein h^{-1} (1-month bloodworm, 1-month fish feed, 3-month bloodworm, and 3-month fish feed; Figure 26).

8.2.4.2. Na^+/K^+ ATPase enzyme activity in the white muscle, liver, kidney, and gill:

NKA activity in the white muscle (Figure 27.A), gill (Figure 27.C), and kidney (Figure 27.D) was not impacted by the diet treatments. Enzyme activity in the liver was significantly higher in the 1-month fish feed diet treatment ($p< 0.05$, n=6; 52 ±0.4 µmol ADP mg^{-1} protein h^{-1}) compared to the other treatments (Figure 27.B).

8.2.5.1. LDH enzyme activity in the GIT:

LDH activity in the GIT was not impacted by the 1-month or 3-month fish feed diet treatments. Activity however increased significantly ($p<0.05$, n= 6) with the introduction and continuation of the bloodworm diet for both 1-month (2367.57 ± 685.166 µmol min^{-1} g^{-1} protein) and 3-month (2536.27 ± 339.58 µmol min^{-1} g^{-1} protein) compared to the wild and the 3-month fish feed diet treatments (Figure 28).

8.2.5.2. LDH enzyme activity in the white muscle, liver, kidney, and gill:

LDH activity in the white muscle was not impacted by the dietary treatments (Figure 29.A). LDH activity in the liver decreased significantly ($p <0.05$, n=6) with the 3-month bloodworm diet treatment (390.41 ± 27.9355 µmol min^{-1} g^{-1} protein) compared to the wild and the 3-month fish feed diet treatments (Figure 29.B). Dietary treatments (1-month bloodworm, 1-month fish feed, and 3-month fish feed) caused a significant decrease in the gill enzyme rates compared to the wild and 3-month bloodworm diet treatments ($p <0.05$, n=6; Figure 29.C). Dietary treatments (1-month bloodworm and 3-month bloodworm) caused a significant decrease in the kidney enzyme rates compared to the wild and 3-month fish feed diet treatments ($p <0.05$, n=6; Figure 29.D).

8.2.6.1. PK enzyme activity in the GIT:

PK activity in the GIT was not impacted by the dietary treatments averaging at 332.55 ± 80 µmol min^{-1} g^{-1} protein across all treatments (1-month bloodworm, 1-month fish feed, 3-month bloodworm, and 3-month fish feed; Figure 30).

8.2.6.2. PK enzyme activity in the white muscle, liver, kidney, and gill:

The PK activity in the white muscle decreased significantly ($p<0.05$, n=6) between the wild and 1- and 3-month fish feed diet treatments (Figure 31.A). PK activity in the liver was not impacted by the diet treatments (Figure 31.B). Gill PK activity was significantly higher ($p<0.05$, n=6) in the 3-month bloodworm diet treatment (764.43 ± 154.32 µmol min^{-1} g^{-1} protein) compared to the other treatments (Figure 31.C). PK activity in the kidney was not impacted by the diet compared to controls, however, the 3-month fish feed diet treatment had a significantly higher rate ($p<0.05$, n=6) compared to the 1- and 3-month bloodworm diet treatments (Figure 31.D).

8.2.7.1. CS enzyme activity in the GIT:

CS activity in the GIT was not impacted by the dietary treatments. However, significantly higher activity ($p< 0.05$, n=6) was detected in the 1- and 3-month bloodworm diet treatments (115.05 ~ 122.93 µmol min^{-1} g^{-1} protein) compared to the 3-month fish feed diet treatment (15.13 ± 3.68 µmol min^{-1} g^{-1} protein; Figure 32).

8.2.7.2. CS enzyme activity in the white muscle, liver, kidney, and gill:

CS enzyme activity in the white muscle (Figure 33.A), liver (Figure 33.B), gill (Figure 33.C), and kidney (Figure 33.D) was not impacted by the diet treatments (Figure 33).

8.2.8.1. GDH enzyme activity in the GIT:

GDH activity in the GIT of the wild treatment was the lowest at 216.714 ± 89.32 µmol min^{-1} g^{-1} protein and was not impacted by the 1-month bloodworm, 3-month bloodworm, and 3-month fish feed diet treatments. Activity increased significantly ($p < 0.05$, n=6) with the 1-month fish feed diet treatment (2036.16 ± 15.69 µmol min^{-1} g^{-1} protein) compared to the wild treatment (Figure 34).

8.2.8.2. GDH enzyme activity in the white muscle, liver, kidney, and gill:

The GDH activity in the white muscle (Figure 35.A) and the gill (Figure 35.C) was not impacted by the dietary treatments. GDH rates in the liver decreased significantly with the introduction of the bloodworm diet for 1-month and continuation for 3-month in comparison to the wild and 1-month fish feed diet treatments (Figure 35.B). GDH activity in the kidney increased significantly ($p < 0.05$, n=6) in the 1- and 3-month fish feed diet treatments, as compared to the other treatments (Figure 35.D).

8.3. Multivariate association of the GIT bacterial abundance and the GIT enzyme activity in response to an altered diet:

Several prominent correlations between the distinct diet treatments, the GIT bacterial species abundance, and the GIT enzyme activity were observed when a multivariate analysis was performed. Firstly, GDH activity was correlated with the abundance of the bacterial species *Ethanoligenens* (phylum Firmicutes) in the wild and 1-month fish feed diet treatments (Figure 36). LDH activity was correlated with the abundance of bacteria species *Ruminococcacea* (phylum Firmicutes) in the 3-month fish feed diet treatment (Figure 36). CS and PK activities were correlated with the abundance of the bacterial species *Burkholderia* (phylum

Proteobacteria) in the 1-month bloodworm diet treatment (Figure 36). Finally, NKA, lipase, and trypsin activities were correlated with the abundance of the bacteria species *Pseudomonas* (phylum Proteobacteria) in the 3-month bloodworm diet treatment (Figure 36).

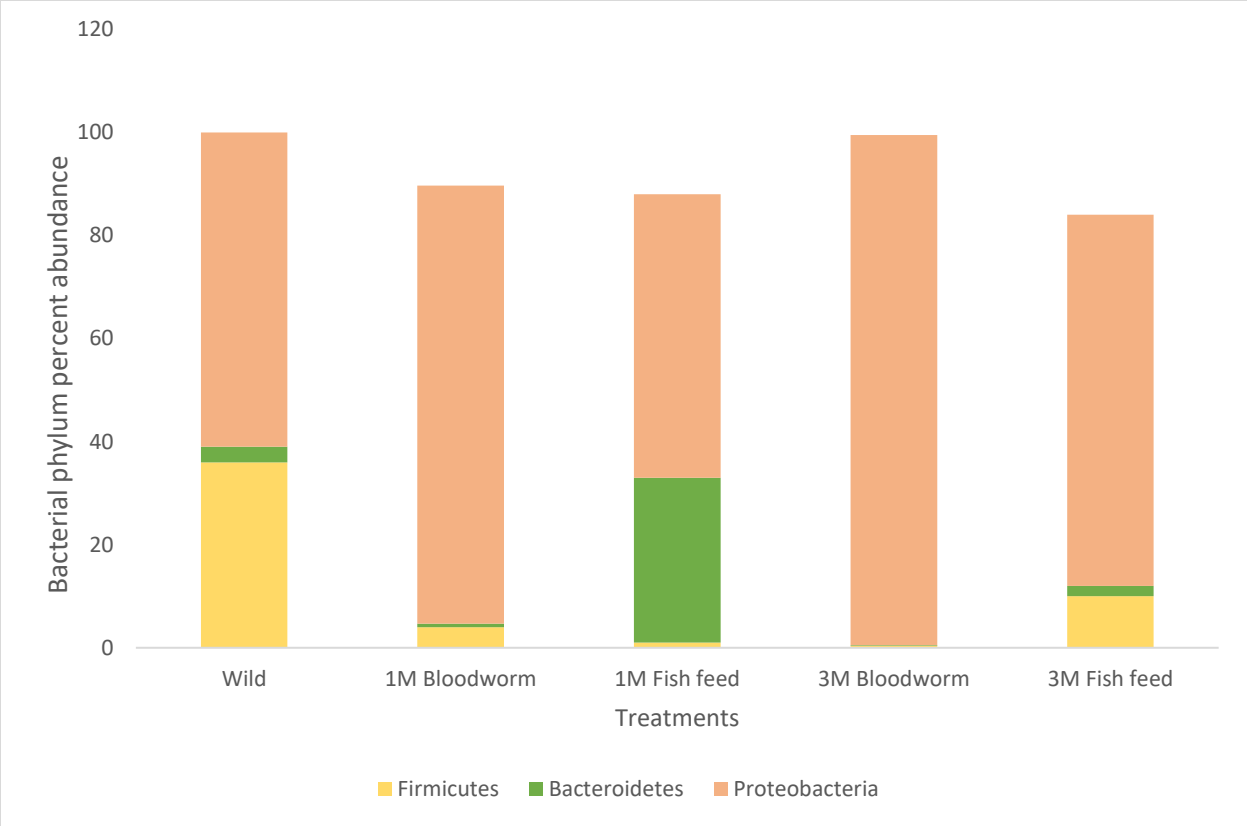

Figure 19: Percent GIT-bacterial community phylum abundance distribution in unique diet treatments over 3 months. GIT bacterial phyla Bacteroidetes (green), Firmicutes (yellow) and Proteobacteria (maroon) dominated the GIT bacterial abundance, however, one phylum dominated in expense of the other two based on the properties of the diet composition.

(Wild= control animals, 1M bloodworm= animals fed bloodworm diet for 1 month, 3M bloodworm= animals fed bloodworm diet for 3 months, 1M fish feed= animals fed fish feed diet for 1 month, and 3M fish feed= animals fed fish feed diet for 3 months).

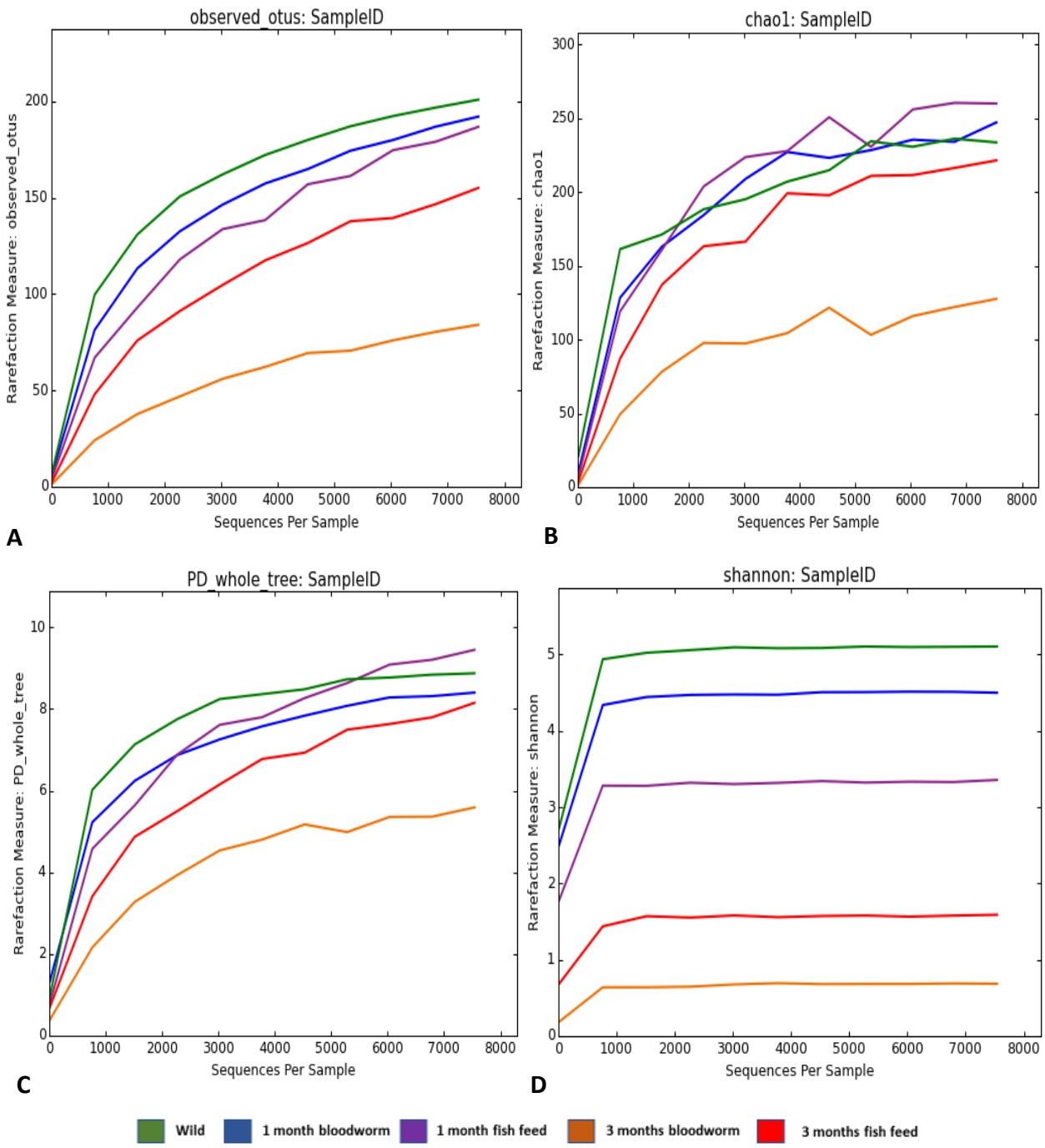

Figure 20: The observed alpha rarefaction plots (**A.** Observed Species, **B.** Chao1, **C.** PD whole tree, and **D.** Shannon Index) of the distinct treatments at an increment sequence depth of 1000 sequences per sample. Rarefaction was performed on 10,000 random sequences from each sample.

(Wild = control animals, 1-month bloodworm = animals fed bloodworm diet for 1 month, 3-month bloodworm = animals fed bloodworm diet for 3 months, 1-month fish feed = animals fed fish feed diet for 1 month, 3-month fish feed = animals fed fish feed diet for 3 months).

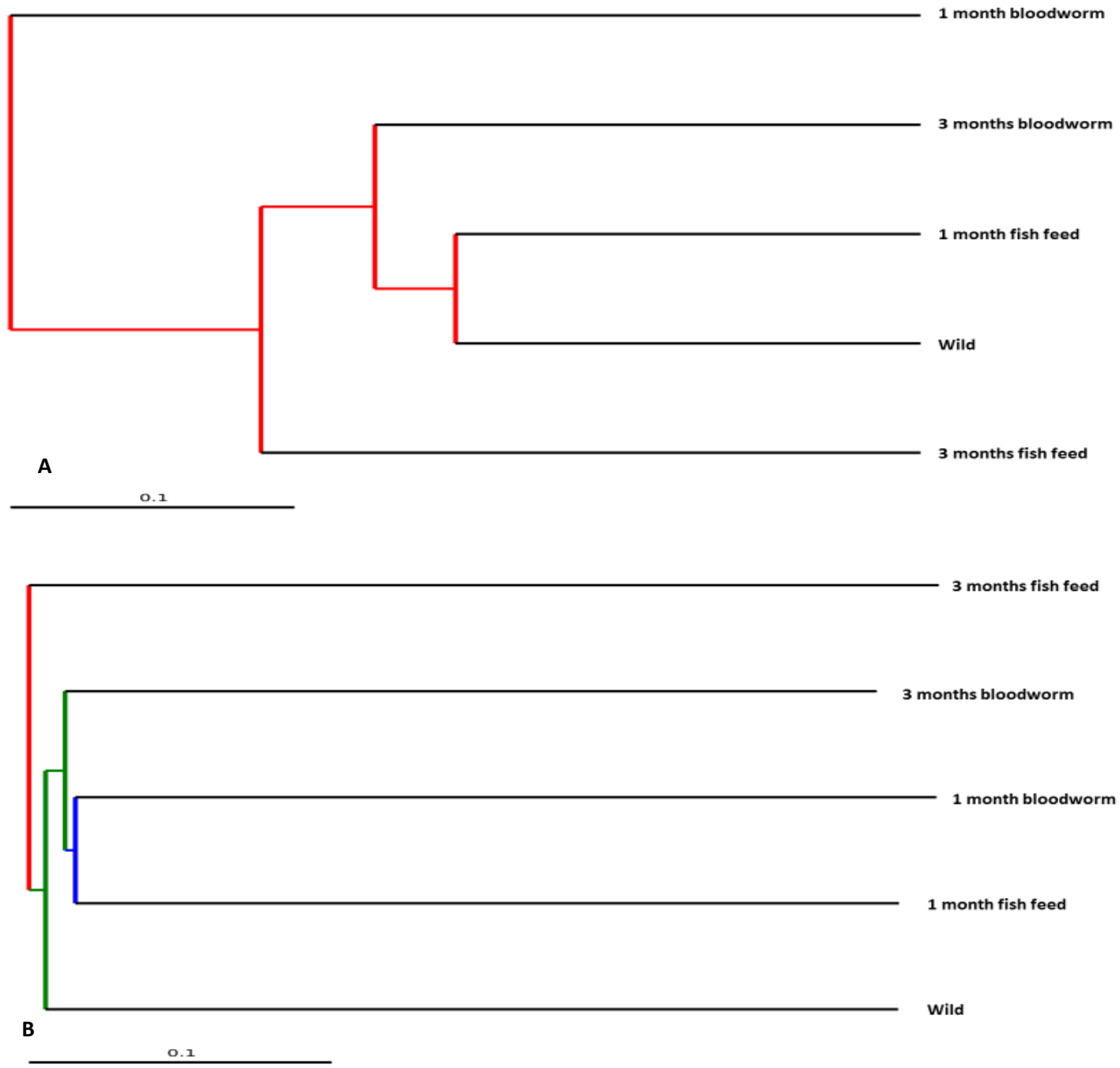

Figure 21: Jackknife weighted (**A**) and unweighted (**B**) bootstrap trees at a distance of 0.1. The red line resembles 75% or more support, the green line resembles 25-50 % support and the blue line represents less than 25 % support. Jackknife beta analysis was performed using 90 % of random sequences as a baseline adopted from the sample containing the least number of OTUs. (Wild = control animals, 1-month bloodworm = animals fed bloodworm diet for 1 month, 3-month bloodworm = animals fed bloodworm diet for 3 months, 1-month fish feed = animals fed fish feed diet for 1 month, 3-month fish feed = animals fed fish feed diet for 3 months).

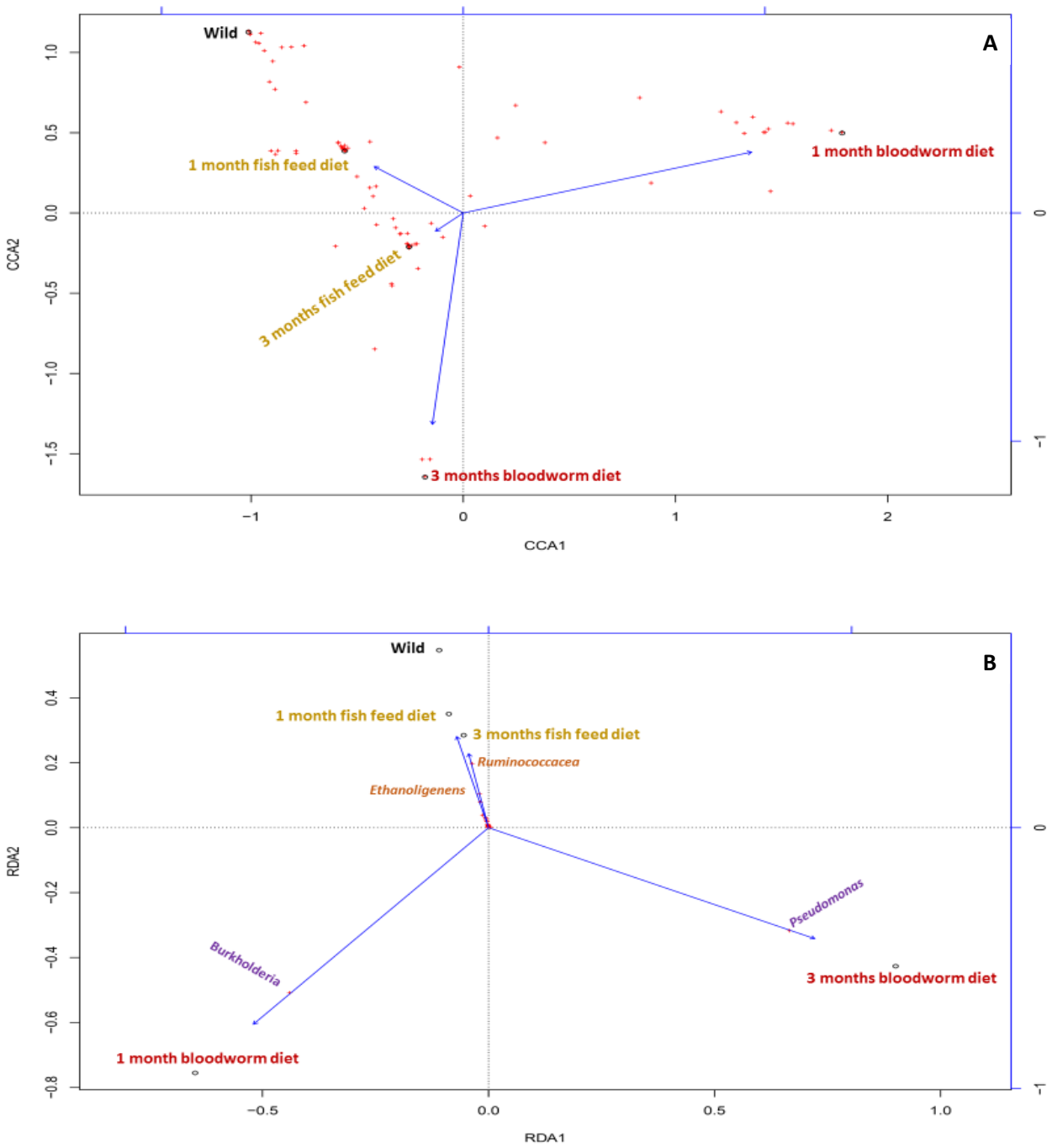

Figure 22: Multivariate RDA analysis showing the treatments seperated by the function of the abundance of the bacterial species present with **A** (RDA analysis without hellinger transformation) and **B** (RDA analysis with hellinger transformation). Bacterial species *Ethanoligenens* and *Ruminococcacea* = phylum Firmicutes and bacterial species *Burkholderia* and *Pseudomonas* = phylum Proteobacteria.
Wild = control animals, 1-month bloodworm = animals fed bloodworm diet for 1 month, 3-month bloodworm = animals fed bloodworm diet for 3 months, 1-month fish feed = animals fed fish feed diet for 1 month, 3-month fish feed = animals fed fish feed diet for 3 months.

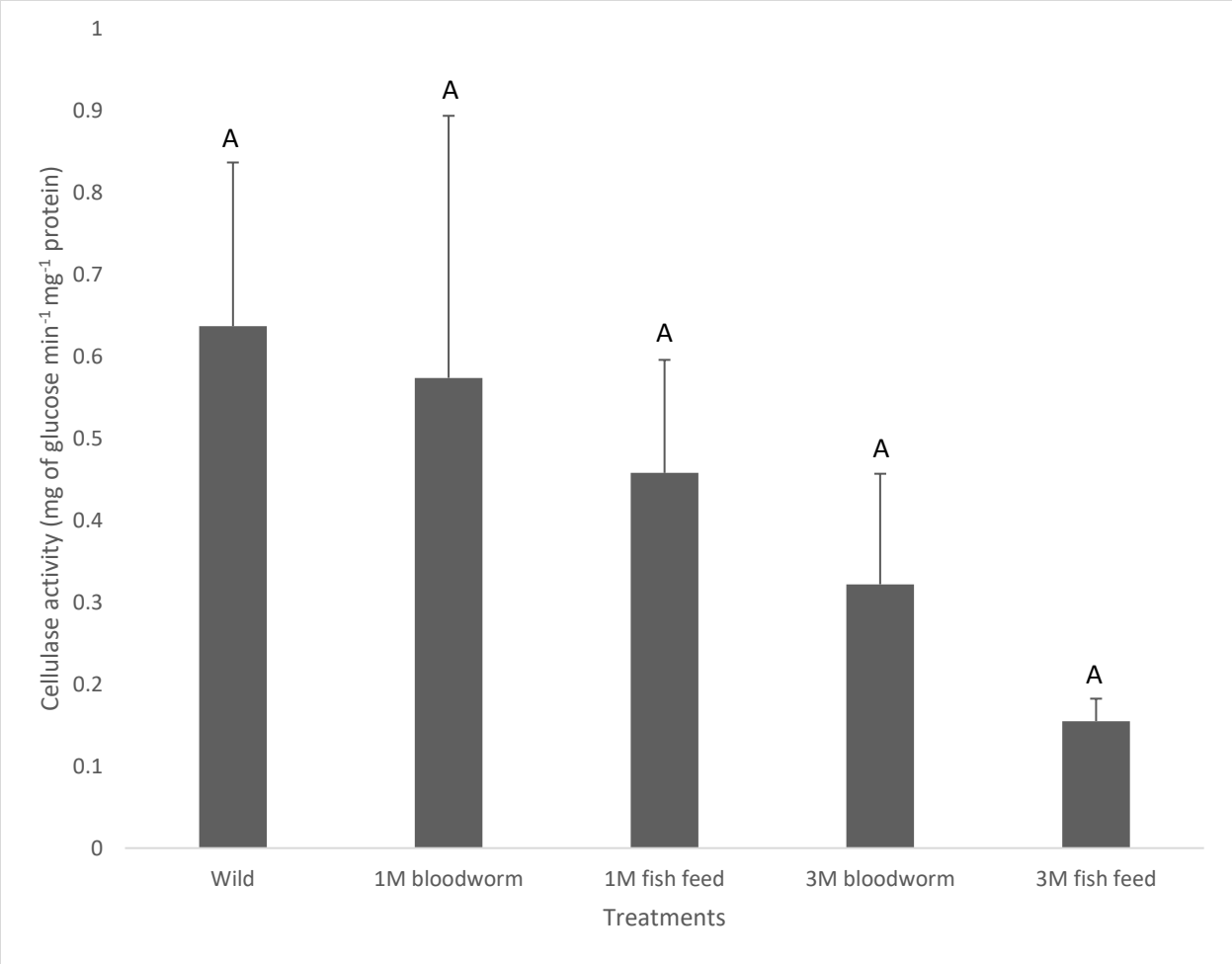

Figure 23: Cellulase enzyme activity (mg of glucose min^{-1} mg^{-1} protein) in the GIT with the introduction and the continuation of the distinct diet treatments. Bars that share the same letter are not significantly different (one-way ANOVA, $p < 0.05$, n= 5).
(Wild= control animals, 1M bloodworm= animals fed bloodworm diet for 1 month, 3M bloodworm= animals fed bloodworm diet for 3 months, 1M fish feed= animals fed fish feed diet for 1 month, and 3M fish feed= animals fed fish feed diet for 3 months).

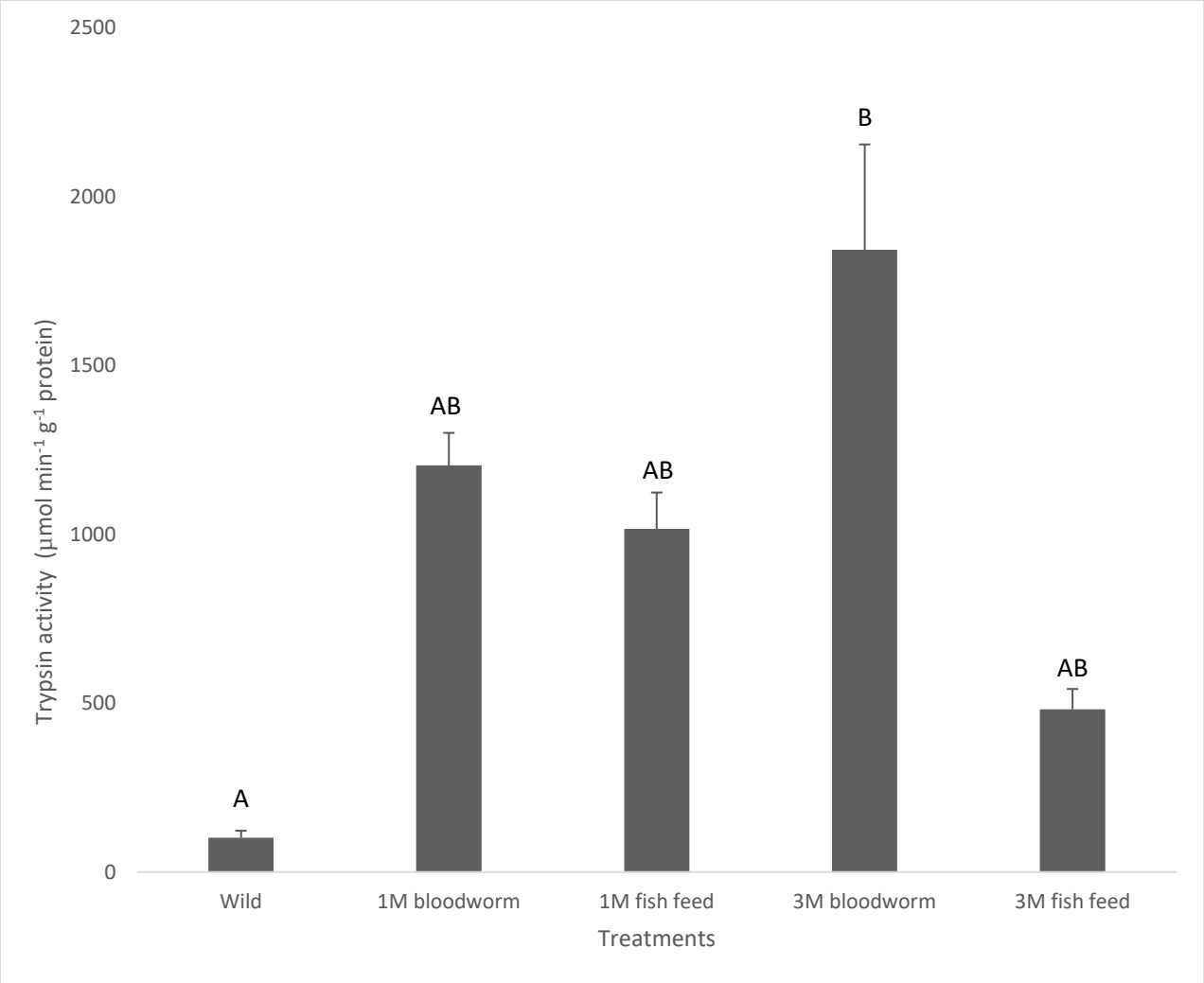

Figure 24: Trypsin enzyme activity (µmol min^{-1} g^{-1} protein) in the GIT with the introduction and the continuation of the distinct diet treatments. Bars that share the same letter are not significantly different (one-way ANOVA, $p < 0.05$, n= 5).
(Wild= control animals, 1M bloodworm= animals fed bloodworm diet for 1 month, 3M bloodworm= animals fed bloodworm diet for 3 months, 1M fish feed= animals fed fish feed diet for 1 month, and 3M fish feed= animals fed fish feed diet for 3 months).

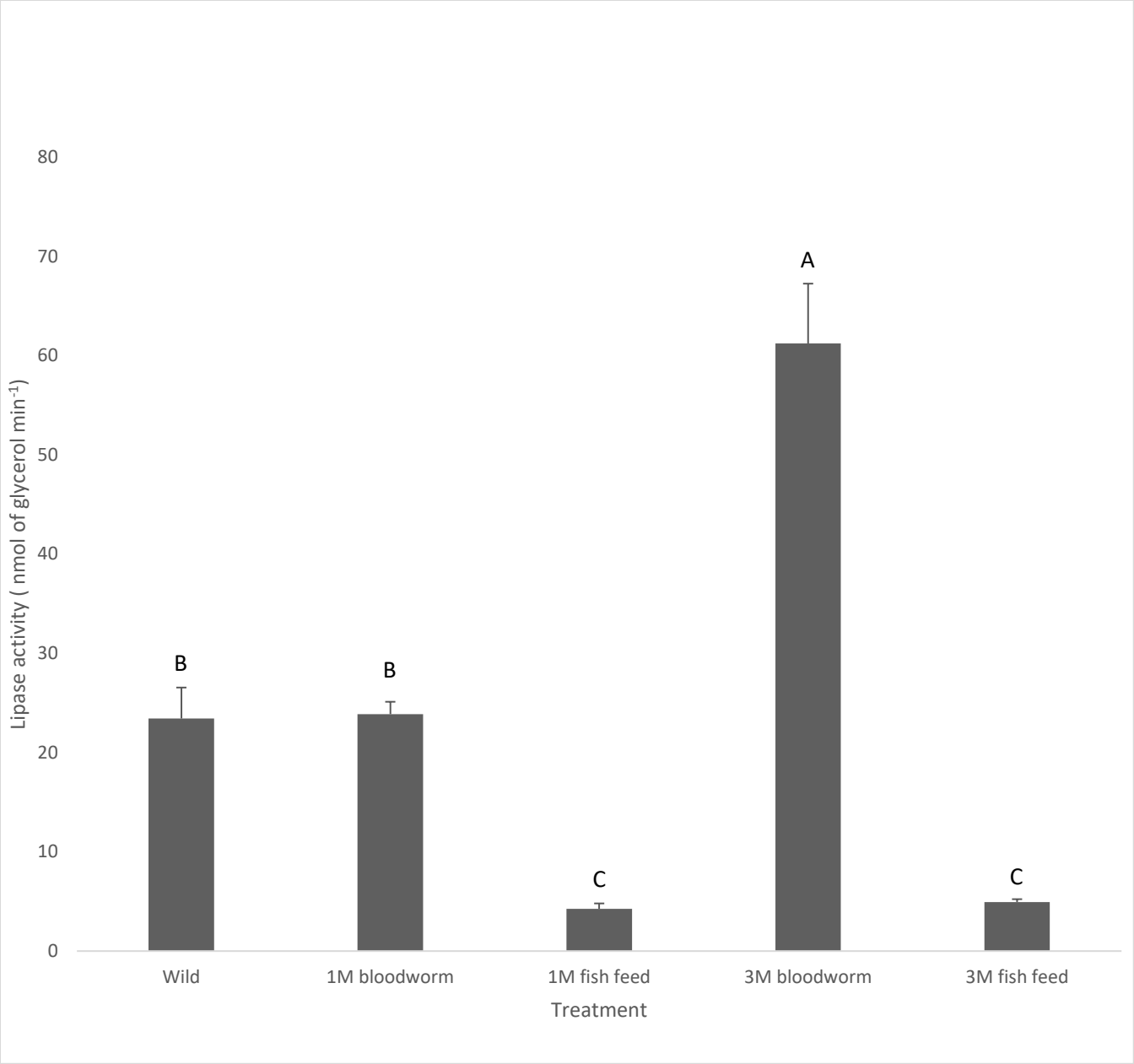

Figure 25: Lipase enzyme activity (nmol of glycerol min^{-1}) in the GIT with the introduction and the continuation of the distinct diet treatments. Bars that share the same letter are not significantly different (one-way ANOVA, $p < 0.05$, n= 5).
(Wild= control animals, 1M bloodworm= animals fed bloodworm diet for 1 month, 3M bloodworm= animals fed bloodworm diet for 3 months, 1M fish feed= animals fed fish feed diet for 1 month, and 3M fish feed= animals fed fish feed diet for 3 months).

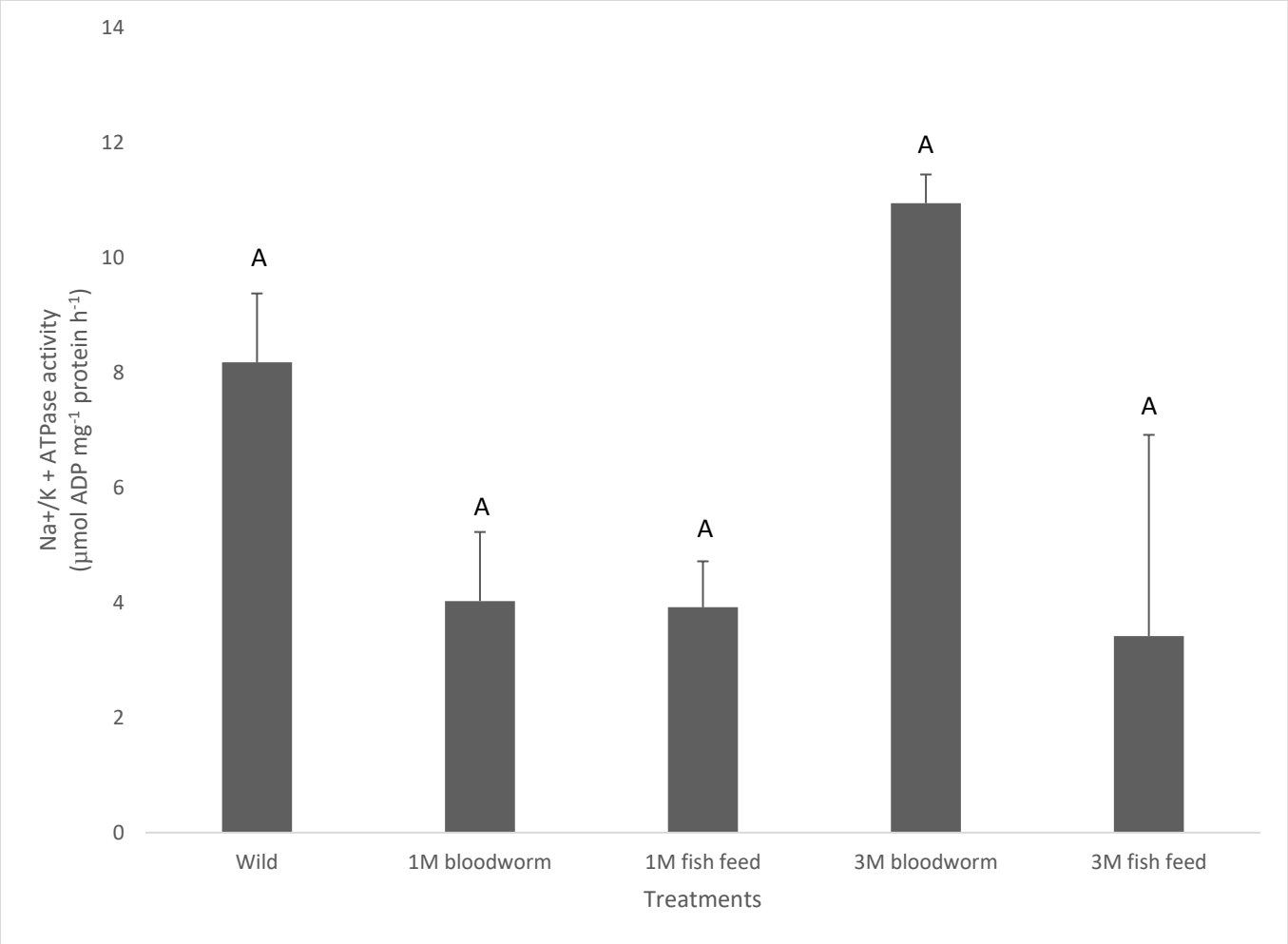

Figure 26: Na^+/K^+ ATPase enzyme activity (µmol ADP mg^{-1} protein h^{-1}) in the GIT with the introduction and continuation of distinct diet treatments. Bars that share the same letter are not significantly different (one-way ANOVA, $p < 0.05$, n= 6).
(Wild= control animals, 1M bloodworm= animals fed bloodworm diet for 1 month, 3M bloodworm= animals fed bloodworm diet for 3 months, 1M fish feed= animals fed fish feed diet for 1 month, and 3M fish feed= animals fed fish feed diet for 3 months).

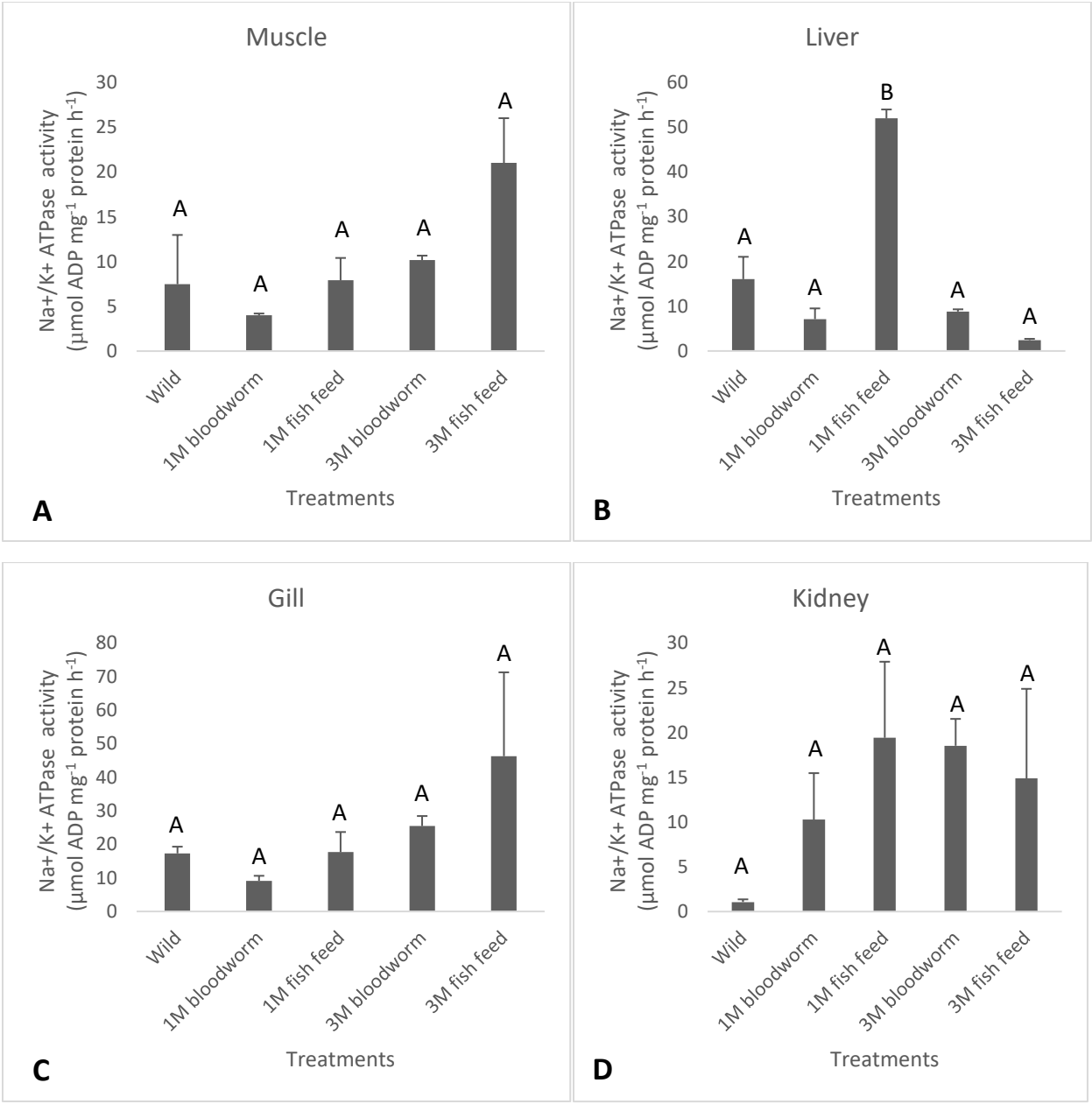

Figure 27: Na^+/K^+ ATPase enzyme activity (µmol ADP mg^{-1} protein h^{-1}) in the **A.** white muscle, **B.** liver, **C.** gill, and **D.** kidney with the introduction and continuation of the distinct diet treatments. Bars that share the same letter are not significantly different (one-way ANOVA, $p < 0.05$, n=6).
(Wild= control animals, 1M bloodworm= animals fed bloodworm diet for 1 month, 3M bloodworm= animals fed bloodworm diet for 3 months, 1M fish feed= animals fed fish feed diet for 1 month, and 3M fish feed= animals fed fish feed diet for 3 months).

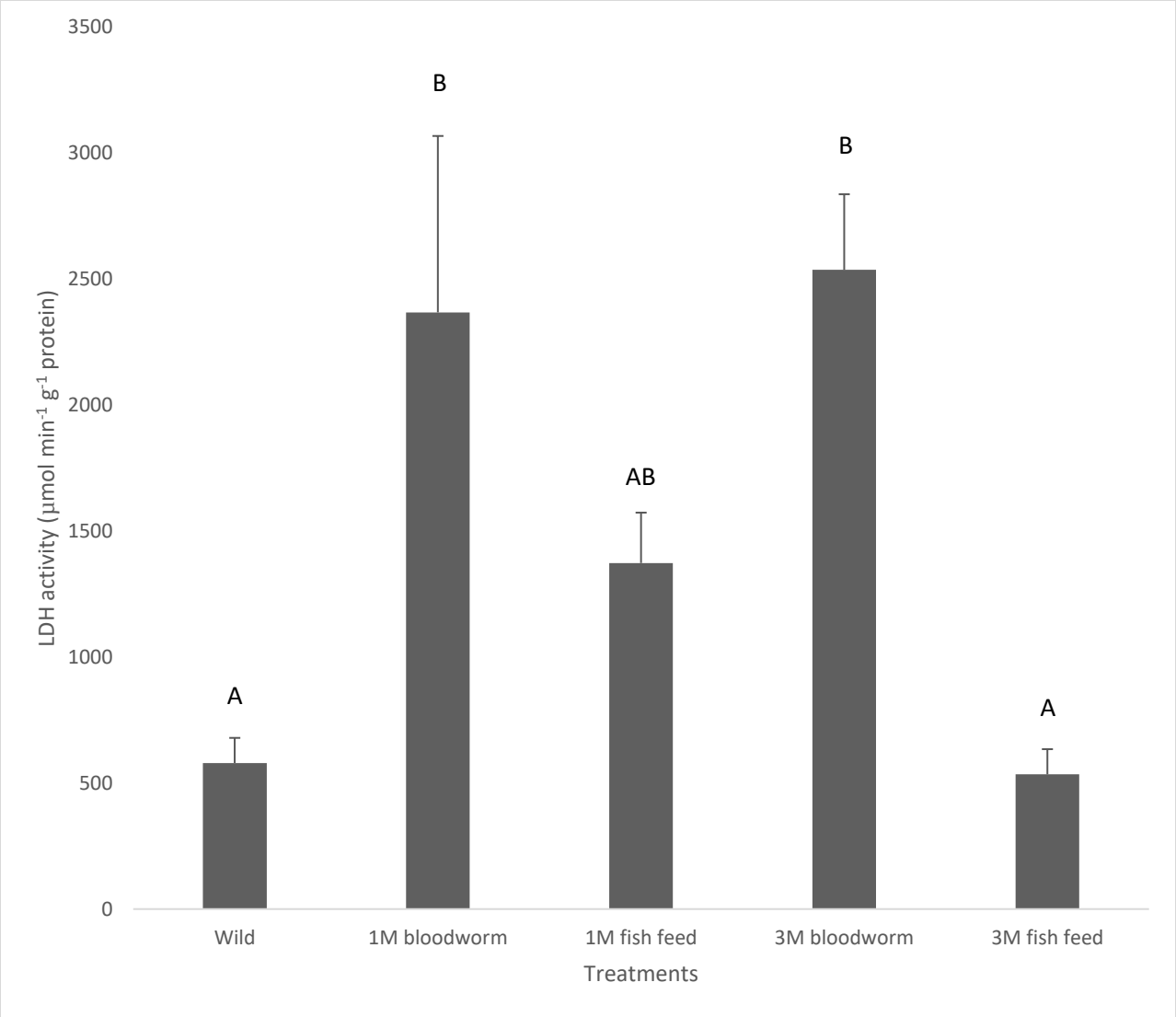

Figure 28: LDH enzyme activity (µmol min^{-1} g^{-1} protein) in the GIT with the introduction and continuation of the distinct diet treatments. Bars that share the same letter are not significantly different (one-way ANOVA, $p < 0.05$, n= 6).
(Wild= control animals, 1M bloodworm= animals fed bloodworm diet for 1 month, 3M bloodworm= animals fed bloodworm diet for 3 months, 1M fish feed= animals fed fish feed diet for 1 month, and 3M fish feed= animals fed fish feed diet for 3 months).

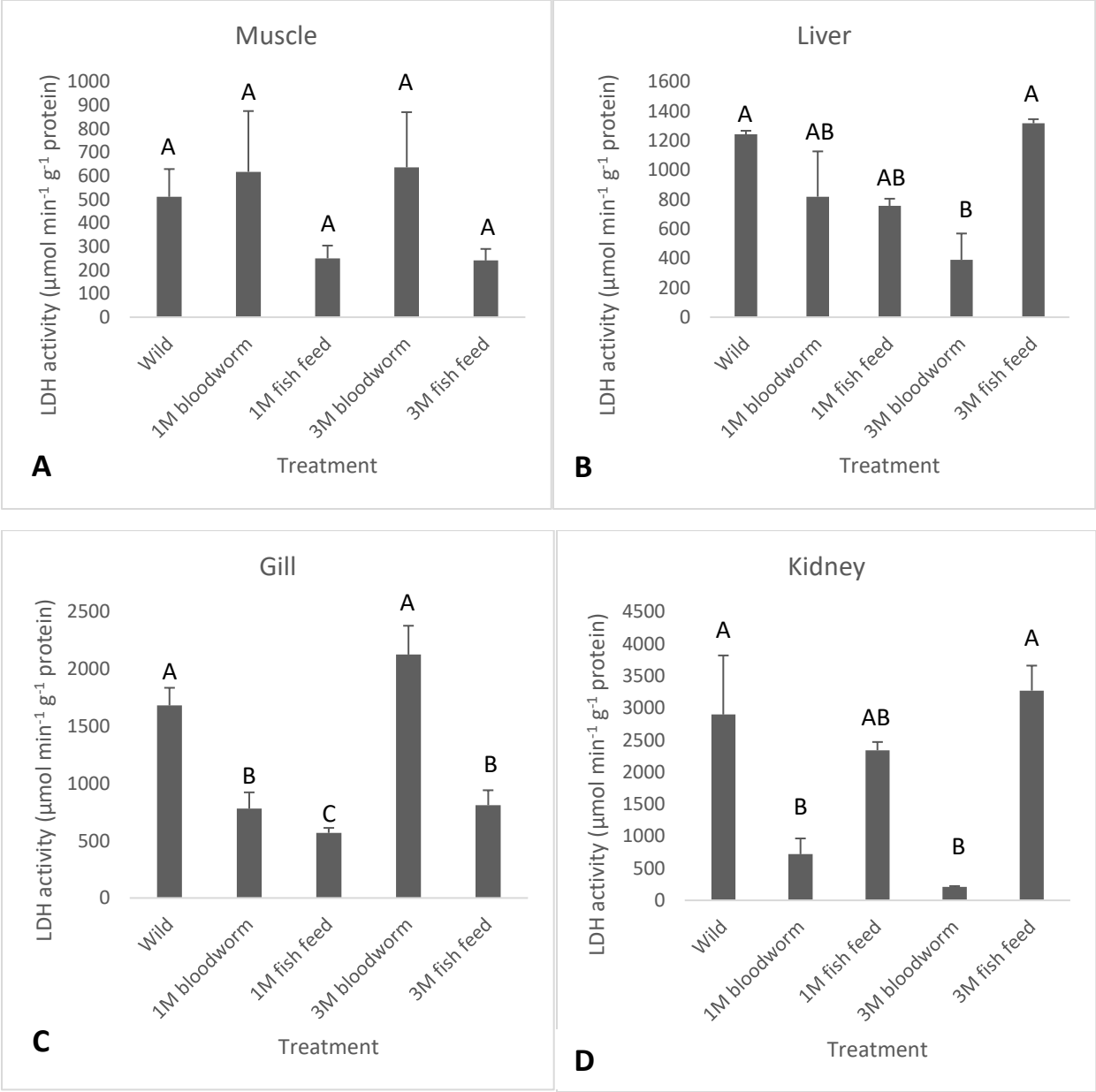

Figure 29: LDH enzyme activity (µmol min^{-1} g^{-1} protein) in the **A.** white muscle, **B.** liver, **C.** gill, and **D.** kidney with the introduction and continuation of distinct diet treatments. Bars that share the same letter are not significantly different (one-way ANOVA, $p < 0.05$, n= 6). (Wild= control animals, 1M bloodworm= animals fed bloodworm diet for 1 month, 3M bloodworm= animals fed bloodworm diet for 3 months, 1M fish feed= animals fed fish feed diet for 1 month, and 3M fish feed= animals fed fish feed diet for 3 months).

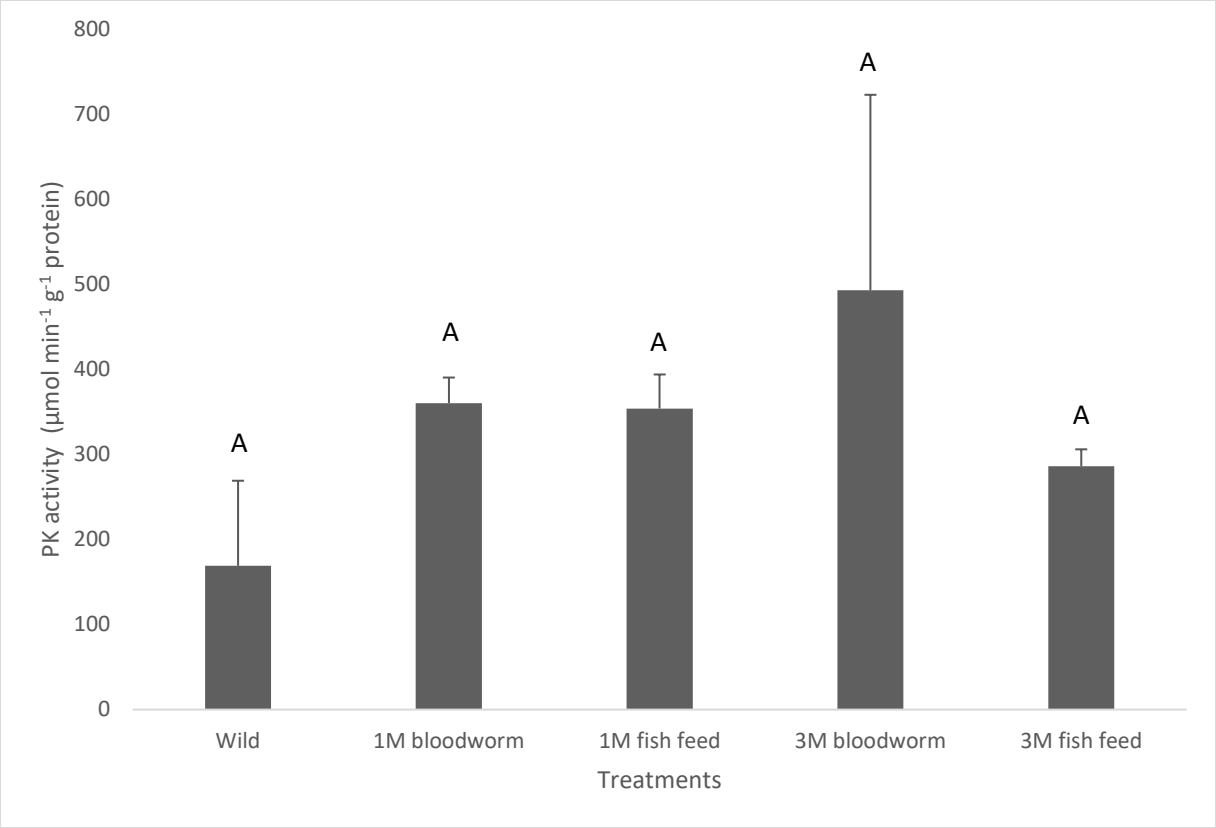

Figure 30: PK enzyme activity (µmol min^{-1} g^{-1} protein) in the GIT with the introduction and continuation of distinct diet treatments. Bars that share the same letter are not significantly different (one-way ANOVA, $p < 0.05$, n= 5).
(Wild= control animals, 1M bloodworm= animals fed bloodworm diet for 1 month, 3M bloodworm= animals fed bloodworm diet for 3 months, 1M fish feed= animals fed fish feed diet for 1 month, and 3M fish feed= animals fed fish feed diet for 3 months).

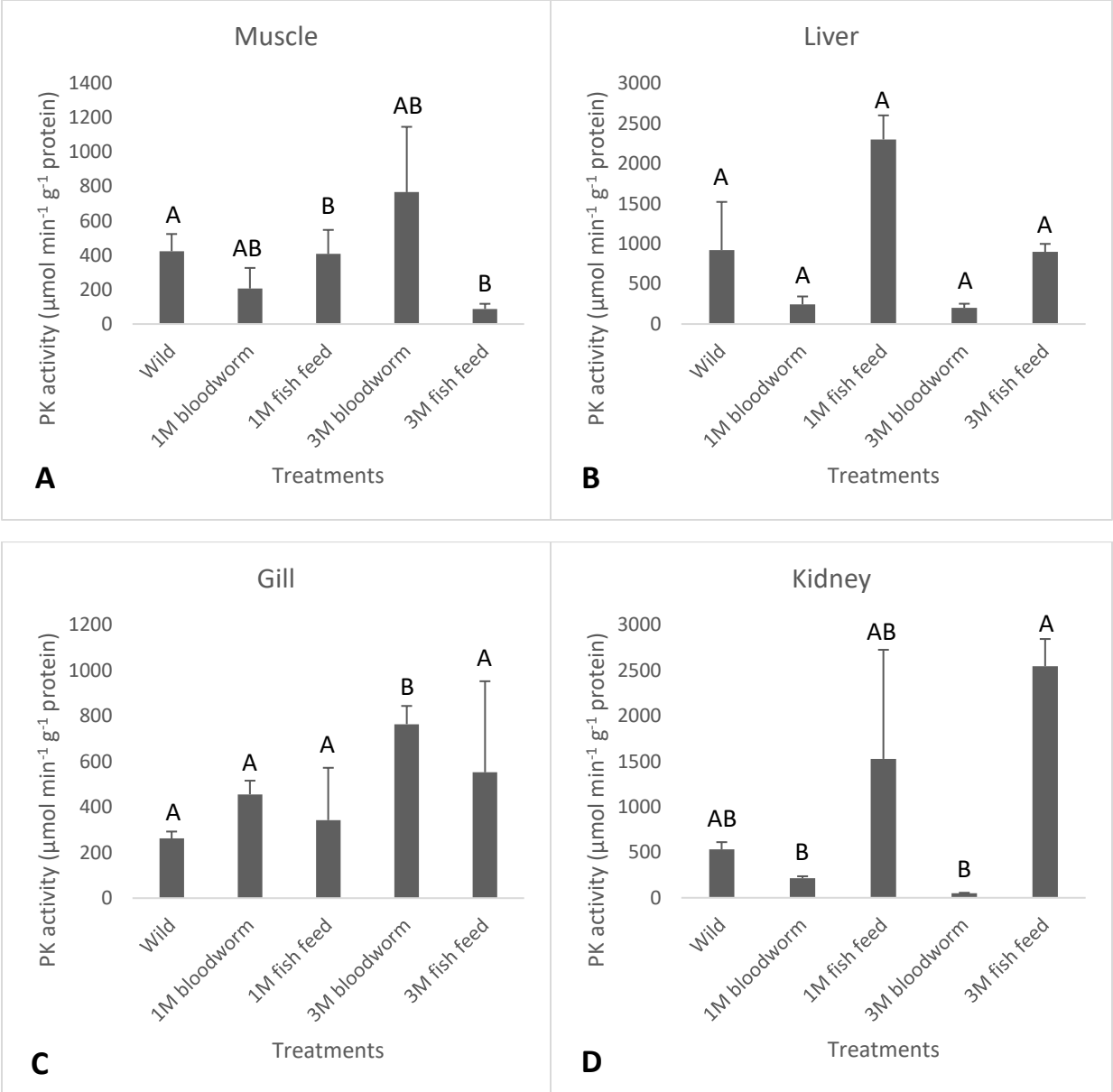

Figure 31: PK enzyme activity (µmol min^{-1} g^{-1} protein) in the **A.** white muscle, **B.** liver, **C.** gill, and **D.** kidney with the introduction and continuation of distinct diet treatments. Bars that share the same letter are not significantly different (one-way ANOVA, $p < 0.05$, n= 6).
(Wild= control animals, 1M bloodworm= animals fed bloodworm diet for 1 month, 3M bloodworm= animals fed bloodworm diet for 3 months, 1M fish feed= animals fed fish feed diet for 1 month, and 3M fish feed= animals fed fish feed diet for 3 months).

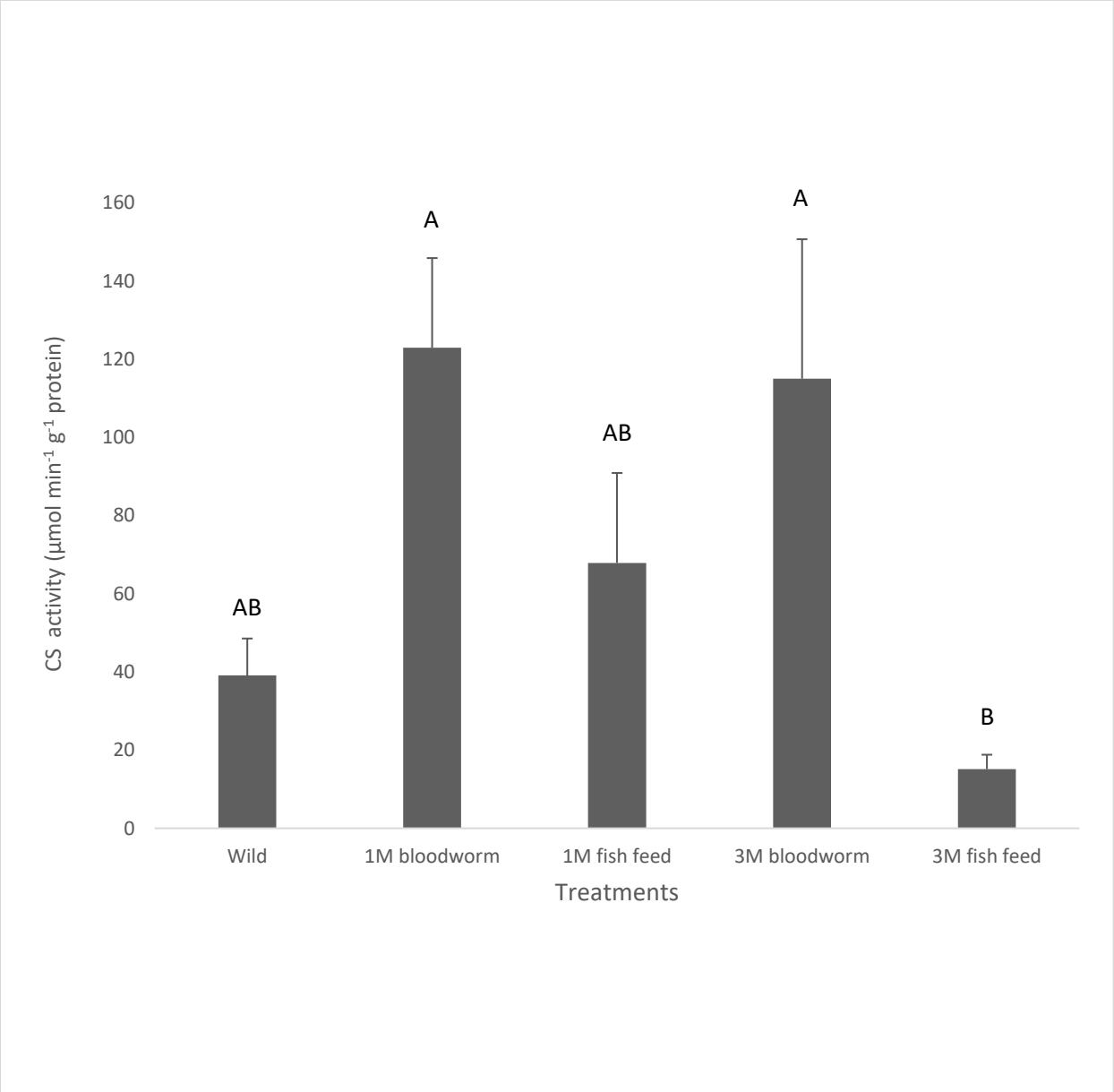

Figure 32: CS enzyme activity (µmol min^{-1} g^{-1} protein) in the GIT with the introduction and the continuation of the distinct diet treatments. Bars that share the same letter are not significantly different (one-way ANOVA, $p < 0.05$, n= 6).
(Wild= control animals, 1M bloodworm= animals fed bloodworm diet for 1 month, 3M bloodworm= animals fed bloodworm diet for 3 months, 1M fish feed= animals fed fish feed diet for 1 month, and 3M fish feed= animals fed fish feed diet for 3 months).

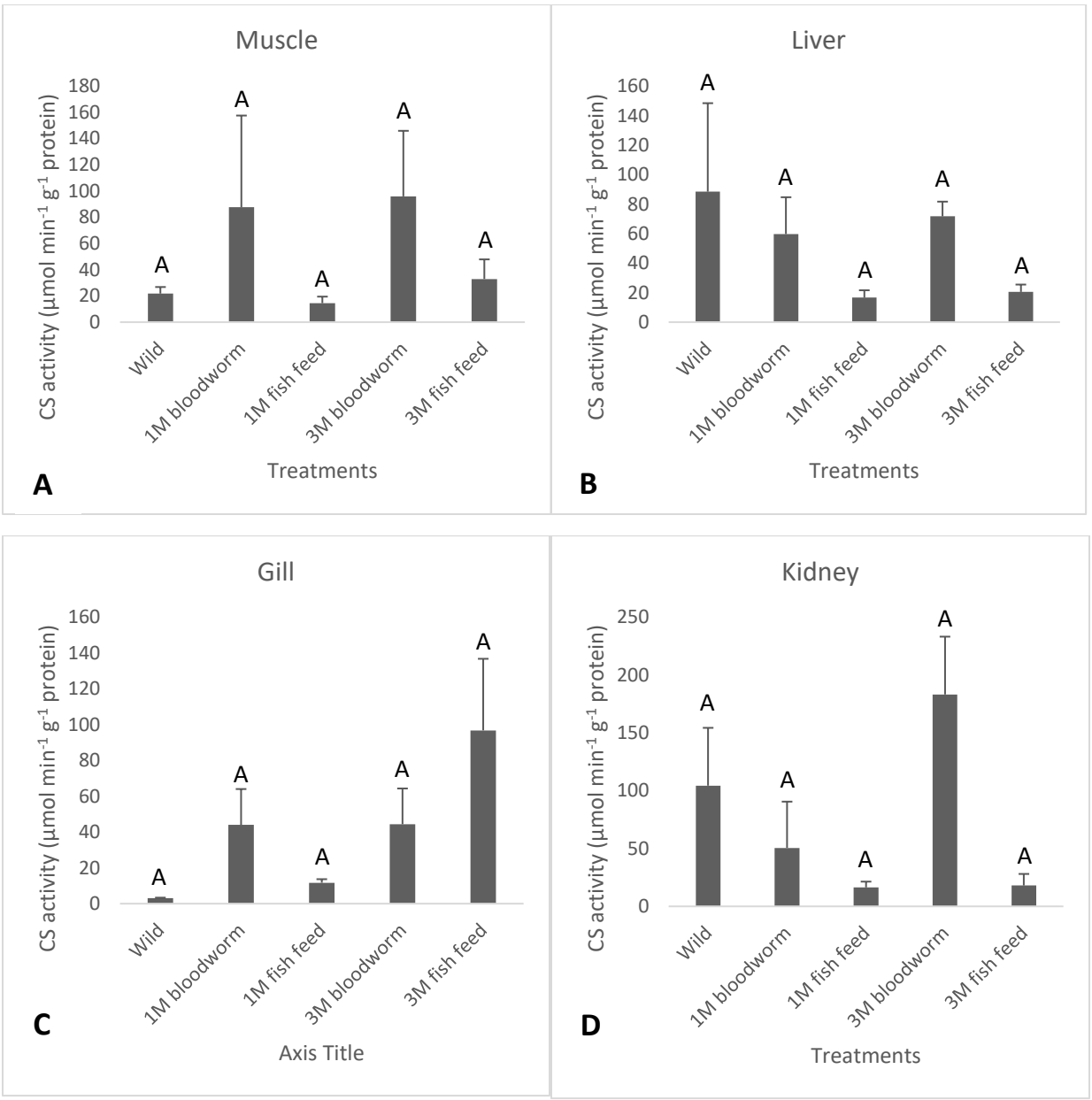

Figure 33: CS enzyme activity (µmol min^{-1} g^{-1} protein) in the **A.** white muscle, **B.** liver, **C.** gill, and **D.** kidney with the introduction and continuation of distinct diet treatments. Bars that share the same letter are not significantly different (one-way ANOVA, $p < 0.05$, n= 6).
(Wild= control animals, 1M bloodworm= animals fed bloodworm diet for 1 month, 3M bloodworm= animals fed bloodworm diet for 3 months, 1M fish feed= animals fed fish feed diet for 1 month, and 3M fish feed= animals fed fish feed diet for 3 months).

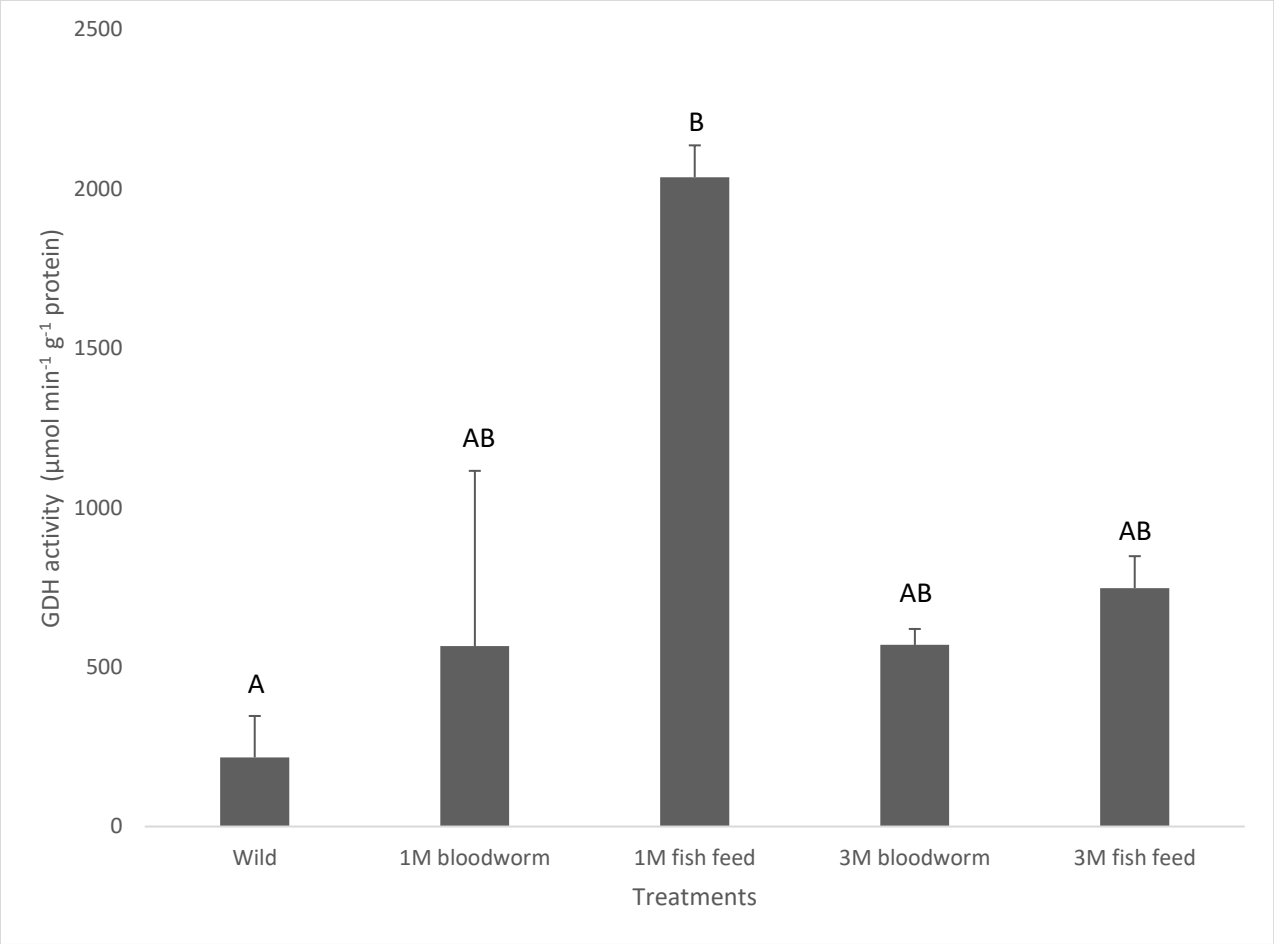

Figure 34: GDH enzyme activity (µmol min^{-1} g^{-1} protein) in the GIT with the introduction and continuation of distinct diet treatments. Bars that share the same letter are not significantly different (one-way ANOVA, $p < 0.05$, n= 6).
(Wild= control animals, 1M bloodworm= animals fed bloodworm diet for 1 month, 3M bloodworm= animals fed bloodworm diet for 3 months, 1M fish feed= animals fed fish feed diet for 1 month, and 3M fish feed= animals fed fish feed diet for 3 months).

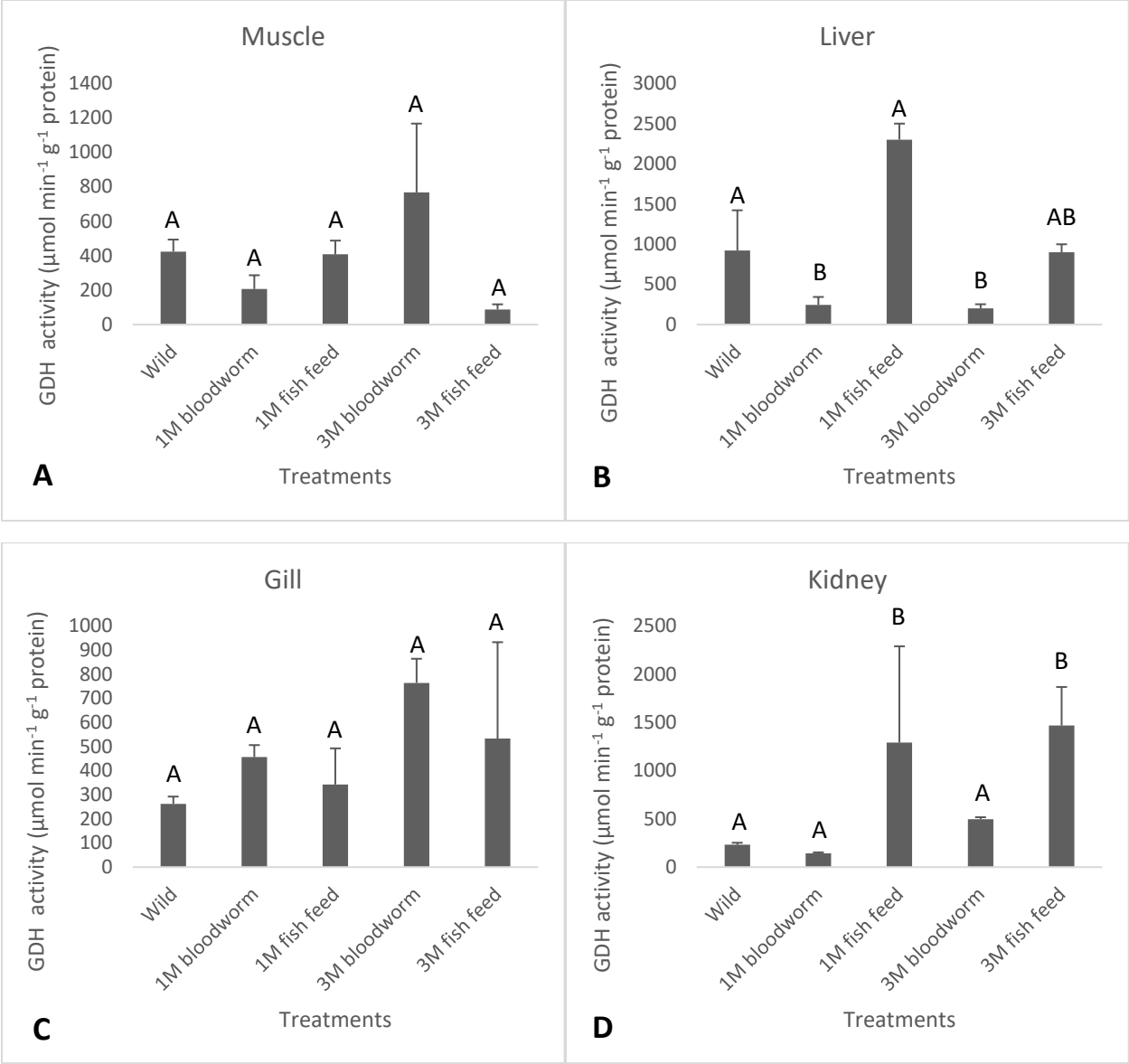

Figure 35: GDH enzyme activity (µmol min^{-1} g^{-1} protein) in the **A.** white muscle, **B.** liver, **C.** gill, and **D.** kidney with the introduction and continuation of distinct diet treatments. Bars that share the same letter are not significantly different (one-way ANOVA, $p < 0.05$, n= 6). (Wild= control animals, 1M bloodworm= animals fed bloodworm diet for 1 month, 3M bloodworm= animals fed bloodworm diet for 3 months, 1M fish feed= animals fed fish feed diet for 1 month, and 3M fish feed= animals fed fish feed diet for 3 months).

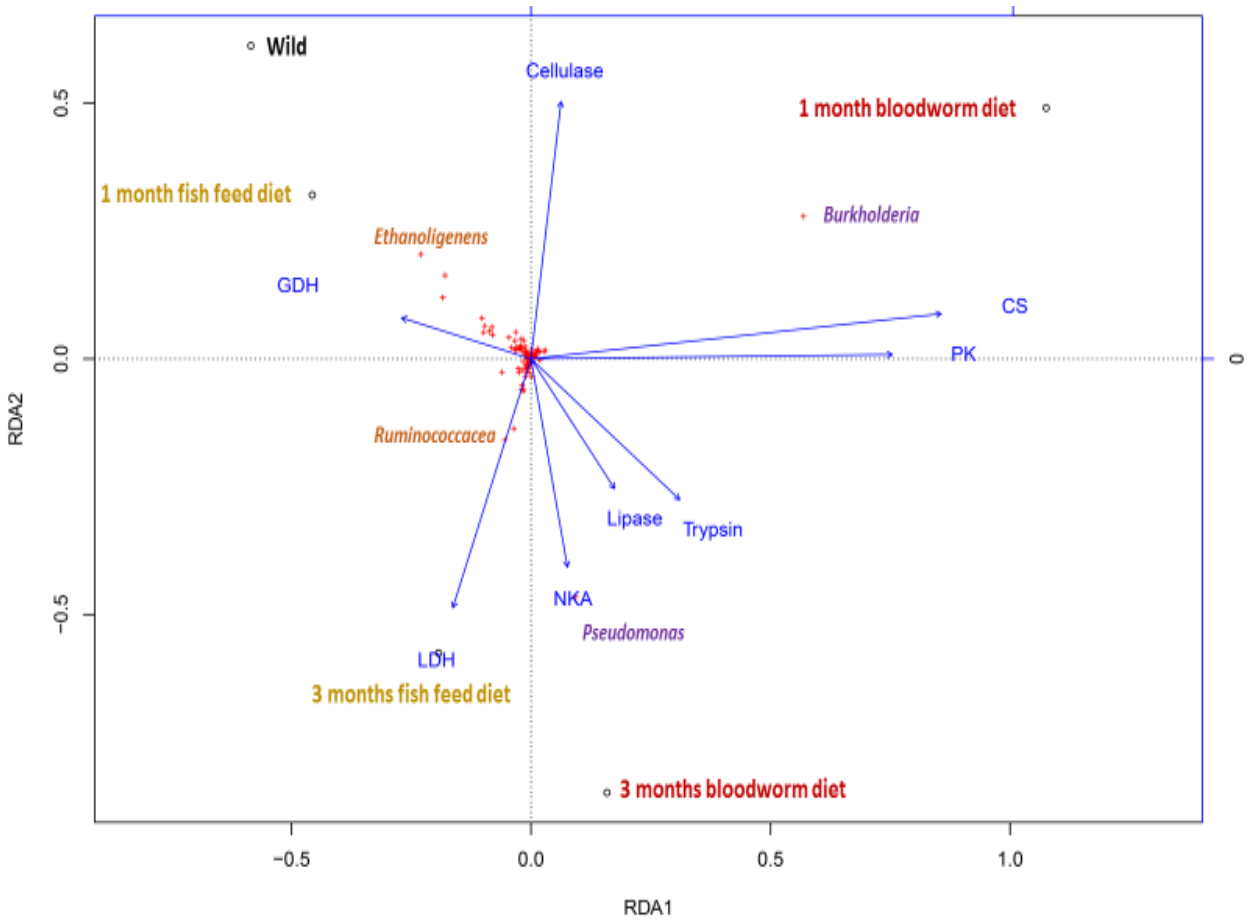

Figure 36: Multivariate RDA analysis showing the distinct separation and the correlation of the diet treatments with the abundance of the bacterial species and the enzyme activities. Enzymes are shown in blue. Diet treatments are shown in red and yellow. Bacterial species *Ethanoligenens* and *Ruminococcacea* = phylum Firmicutes and bacterial species *Burkholderia* and *Pseudomonas* = phylum Proteobacteria.
(Wild = control animals, 1-month bloodworm = animals fed bloodworm diet for 1 month, 3-month bloodworm = animals fed bloodworm diet for 3 months, 1-month fish feed = animals fed fish feed diet for 1 month, 3-month fish feed = animals fed fish feed diet for 3 months).

Discussion:

With this novel study on the carnivorous fish rainbow darter, we characterized the native GIT microbiome and measured enzyme activities in this fish for the first time. Then we expanded our approach by altering the diet and exploring the response of the GIT bacterial community and the digestive/metabolic enzymes in this fish to further understand their role in maintaining the host's homeostasis.

9.1. GIT bacterial community reorganization in response to an altered diet:

The GIT bacterial phyla Firmicutes, Bacteroidetes, and Proteobacteria have previously been identified as the more common members of the GIT bacterial communities in both terrestrial and aquatic vertebrates (Welch et al. 2011; Bolnick et al. 2014; Clements et al. 2014; Ghanbari et al. 2015), with an increase in one bacterial phylum at the expense of the other two, depending upon the diet, habitat, and/or genetics of the host (Ghanbari et al. 2015). In our current study, the dominant abundance of the Proteobacteria in the wild *E.caeruleum* as well as when fed with the bloodworm (high-protein) diet (Figure 19) is consistent with observations in the other carnivorous fish species (Bairagi et al. 2002; Ghosh et al. 2002, Ray et al. 2012; Banarjee et al. 2016; Andrade et al. 2017). This supports our initial hypothesis that Proteobacteria will be the most abundant phylum in the GIT bacterial community of the rainbow darter following exposure to a protein (bloodworm) diet (Figure 19).

The abundance proportions of Firmicutes in the rainbow darter GIT bacterial community recovered slightly with exposure to the fish feed diet for 3 months (Figure 19), which was expected, however, the continuous abundance of Proteobacteria was unexpected (Figure 19). Perhaps, the inability of this carnivorous fish GIT to effectively tolerate dietary changes, caused by host-specific selection of the GIT bacteria through the immune response of the gut-associated

lymphoid tissue (GALT; Estruch et al. 2015; Montalban-Arques et al. 2015; Martin et al. 2016) might be responsible for this maintenance of Proteobacteria abundance in the rainbow darter GIT even after a dietary alteration. This may be a species-specific effect as other carnivorous fish species have shown an abundance of Firmicutes in response to the exposure to a high fat diet (Desai et al. 2012, trout; Zarkasi et al. 2014, Atlantic salmon).

The overall declines in the number of bacterial species (Figure 20.A), phylogenetic distance between the bacterial species (Figure 20.C), and distribution of the bacterial species (Figure 20.D), in the dietary treatments compared to the wild treatment was expected as previous studies have shown that a host feeding on a generalized diet typically has a lesser GIT bacterial OTU richness compared to that of a host feeding on varying diets (Bolnick et al. 2014) (Figure 20). Although the 1-month bloodworm and 1-month fish feed diet treatments in this study showed a greater number of rarer bacterial species (Figure 20.B) compared to the wild treatment, their distribution and evenness of the bacterial species was lower compared to the wild treatment (Figure 20.C). This may suggest that these rarer species may have been introduced into the GIT of these particular treatment animals from their habitat; as habitat is also among the exogenous factors that shape the GIT bacterial communities within the hosts (Muegge et al. 2010; Wu et al. 2015).

The clustering of the specific diet treatments within the weighted beta-diversity (Figure 21.A) also suggested that the diet may be responsible for shaping the GIT bacterial communities. The wild and 1-month fish feed diet treatments clustered together (Figure 21.A), possibly due the abundance of similar bacterial species. The 1-month bloodworm and the 3-month fish feed diet treatments clustered separately possibly due to a different abundance of the GIT bacterial species compared to the other treatments (Figure 21.A). This clustering of the 1-month bloodworm and

3-month fish feed diet treatments apart from the wild treatment (Figure 21.A) is further suggestive of the implication of an altered diet in reshaping the abundance of the host's GIT bacterial communities. The unweighted beta-diversity analysis also highlighted the GIT bacterial community richness distinction between the dietary treatments, especially between the wild and diet-manipulated samples (Figure 21.B). However, the decreased support for the association (< 25 %) between the 1-month bloodworm and 1-month fish feed (Figure 21.B), and between the wild and the dietary treatments (< 50%; Figure 21.B) suggested that the association between the diet and GIT bacterial composition was driven more by the bacterial abundance than by the bacterial identity.

The separation of the treatments in the redundancy quartiles was unique for each treatment due to the presence of a few, highly abundant bacterial species (Figure 22.B). The separation of the treatments in the redundancy quartiles (Figure 22.A), even after subjecting the data to a Hellinger transformation did not change (Figure 22.B), further suggesting that the abundance, rather than the identity was strongly associated with the diet treatments (Figure 22.B). The correlation between the increased abundance of bacterial species *Burkholderia* and *Pseudomonas*, of Proteobacteria, with the bloodworm diet (Figure 22.B), as well as the correlation of the increased abundance of the bacterial species *Ethanoligenens* and *Ruminococcacea* of Firmicutes with the fish feed diet (Figure 22.B) is consistent with previous findings in other carnivorous fish species (Wilson 1994; Moran and Saborido 1996; Hidalgo et al. 1999; Bairagi et al. 2002; Ghosh et al. 2002; German et al. 2004; Geruden et al. 2007; Givens 2012; Lazado et al. 2012; Ray et al. 2012; Banarjee et al. 2016; Andrade et al. 2017).

9.2. Integrated reaction of the GIT bacterial community reorganization and the digestive enzymes in response to an altered diet:

As previously stated in chapter 1, the coordination of the overall GIT unit in order to manage an external challenge, such as an altered diet, may require a shift in digestive and metabolic processes as part of that adjustment (Furne et al. 2005; Muegge et al. 2010; Carmody et al. 2012). Unlike herbivorous or omnivorous fish, carnivorous fish harbor a GIT bacterial community that is abundant in the phylum Proteobacteria in order to enhance the digestion and absorption of proteins through the exogenous release of the protease enzymes such as pepsin and trypsin (Bairagi et al. 2002; Ghosh et al. 2002; Ray et al. 2012; Banarjee et al. 2016; Andrade et al. 2017). Indeed, the association between the GIT trypsin activity and an increase in the abundance of Proteobacteria with the 3-month bloodworm diet treatment in our study further supports this previous finding (Figure 19, Figure 24, Figure 36**)**. Furthermore, the small rate of cellulase activities detected in the GIT of the rainbow darter may also suggest the contribution of the lesser-abundant Bacteroidetes phylum in the digestion of trace amounts of plant content which may be present in the diet of the rainbow darter (Figure 19, Figure 23, Figure 36; Luczkovich and Stellwag 1993; Flint et al. 2008).

As previously mentioned in chapter 2, the association between lipid metabolism and the GIT bacterial community has previously correlated with Firmicutes-mediated digestion (exogenous lipase secretion into the lumen) and absorption (short-chained fatty acids) pathways (German 2009; Carmody and Turnbaugh 2012; Semova et al. 2012; Angelakis et al. 2012; Ghanbari et al. 2015; Karsov et al. 2015; Koca et al. 2015; Miyake et al. 2015; Olsen et al. 2015). However, in our current study, the elevated GIT lipase activities in the 3-month bloodworm diet treatments followed by a continued abundance of the Proteobacteria raises the

possibility that this might be an effort on the part of the fish host to maximize efficiency of lipid digestion and absorption from a lipid-deficient diet (Figure 19, Figure 25, Figure 36). Similarly, a decreased lipase activity in the 1- and 3-month fish feed diet treatments may suggest that this may be caused by the continued abundance of Proteobacteria, that is preventing the proliferation of Firmicutes in the GIT, and therefore causing a decreased secretion of exogenous lipase into the lumen, regardless of the changes in the dietary lipid content (Figure 19, Figure 25).

9.3. The synergistic response of an altered GIT bacterial community and the GIT/full body metabolic enzymatic physiology in response to an altered diet:

The electrochemical gradient in the basolateral epithelial cells is maintained by the NKA enzyme, and the impact of the GIT bacterial community on the enterocyte NKA activity is currently unclear (Skulachev 1978; Hakim et al. 2009; Dibrova et al. 2015). As pointed out in chapter 2, past studies have postulated a connection between the incorporation of unsaturated fatty acids into the phospholipid membrane mediated by the GIT bacterial phylum Firmicutes and an increase in the NKA activity (Carmody et al. 2012; Semova et al. 2012; Sundell and Sundh 2012). Unfortunately, in our current study on the rainbow darter, no distinguished connection between the NKA activity, the abundance of Firmicutes, and the diet was observed in the GIT nor the other tissues examined (Figure 26, Figure 27). This may be because of the continued abundance of Proteobacteria was preventing the proliferation of Firmicutes in the GIT. Therefore, this response of the rainbow darter GIT bacterial community and GIT NKA activity towards the dietary manipulation may be a species-specific response.

The influence of the diet and/or the bacterial community on the activity of enzymes involved in glycolysis, such as LDH and PK, has previously been correlated with the GIT

bacterial phylum Firmicutes (Kihara and Sakata 2001; Muegge et al. 2010, Ni et al. 2014; Konishi et al. 2015; Bucking et al. unpublished). In this study, the decrease in GIT LDH activity in the wild animals and 3-month fish feed diet treatment (Figure 28), correlating with an increase or recovery of Firmicutes abundance (Figure 19), may suggest a role for Firmicutes in increasing the breakdown of exogenous glucose obtained from the carbohydrate diet in the rainbow darter GIT (Figure 28, Figure 36). The increased GIT LDH activity in the 1- and 3-month bloodworm diet treatments (Figure 28) on the other hand may suggest that this might be an effort on the part of the fish host to utilize its endogenous glucose reserves in response to the dietary carbohydrate deficiency (Figure 28), regardless of the changes in the bacterial abundance.

The absence of a clear trend in the LDH activity in the reference tissues (Figure 29), an absence of significance in the GIT PK enzyme activity between the dietary treatments (Figure 30), and an absence of clear trends in the PK activity of the reference tissues (Figure 31) may suggest the rainbow darter GIT modulates the use of endogenous or exogenous glucose depending upon the diet. Additionally, an as-yet unknown ability of Proteobacteria to influence GIT LDH and PK production, either by producing those enzymes exogenously or by manipulating the host genes involved in the production of LDH and PK in response to dietary changes, may be present. The implications of these possibilities may further extend to downstream processes, like the Krebs cycle and CS activity. The decrease in the GIT CS activity with a slight recovery in the abundance of Firmicutes in the 3-month fish feed diet treatment (Figure 19, Figure 32) may suggest the possibility of the presence of a Firmicutes-mediated pathway, allowing the direct flow of dietary carbon into the citric acid cycle as previously mentioned in chapter 2. However, the unexpectedly elevated GIT CS activities in the bloodworm diet treatments (Figure 32) and an absence of significance in the CS activity in the

reference tissues (Figure 33) regardless of an alteration in the diet (Figure 32, Figure 33) suggests other factors must be considered. Hence, further study on this complex association is necessary in order fully understand the overall ramifications of the above-mentioned possibilities on the glycolysis and Krebs cycle pathways, and enzymes in this carnivorous fish.

As observed in the herbivorous fish central stoneroller (Chapter 2), the secretion of GDH into the lumen in order to decrease ammonia toxicity in the GIT is attributed to the GIT bacterial phyla Firmicutes (Nazarchuk et al. 1981; Sanchez-Muros et al. 1998; Girinathan et al. 2016). In our current study, a comparatively higher GIT GDH activity in the bloodworm diet treatments was anticipated, based on the possibility of an increased breakdown of the dietary amino acids into ammonia, particularly as an effort of the host to detoxify ammonia from its GIT (Figure 34). However, the observation of an increased GDH activity in the GIT with the 1-month fish feed diet, and an absence of a correlation with the GIT Firmicutes abundance, is suggestive of the effort of the host to alter its digestive physiology in response to the diet manipulation (Figure 34), regardless of the changes in bacterial abundance.

Finally, the seemingly random changes in NKA, CS, PK, LDH, and GDH activities in the reference tissues (Figure 35) may suggest the possibility that other factors not examined in this study such as habitat, genetic makeup, evolutionary traits, and other factors may further dictate the activity levels of these enzymes in the GIT and other tissues. Hence, future initiatives to understand this connection would further expand our knowledge about this physiological relationship.

Overall, our initial hypothesis that Proteobacteria will be the most abundant phylum in the GIT bacterial community of the wild rainbow darter and would correlate with dietary protein

content was supported. In contrast, our initial hypothesis that that the composition of the GIT bacterial community will transition from an abundance of the phylum Proteobacteria to an abundance of Firmicutes in response to the prolonged feeding of the commercial fish feed diet was not supported as Proteobacteria abundance was maintained in all phases of the distinct diet treatments. However, Firmicutes abundance did increase between 1- and 3-month feeding of a high fat commercial fish feed diet, partially supporting our hypothesis. Although the activity of trypsin, lipase, LDH, CS, and GDH in the GIT changed with the diet treatments, only the changes in the GIT trypsin, LDH, and CS provided enough evidence to suggest that the increase in phylum Firmicutes would influence the activity of enzymes in the rainbow darter GIT. This again only partially supported our initial hypothesis. Furthermore, evidence that an altered diet, bacterial community abundance, and/or both, can influence the various metabolic enzyme activities in the reference tissues also could not be established, due to the absence of a clear trend. Hence, our hypothesis that the enzyme activity in the reference tissues; that is, white muscle, liver, kidney, and gill, of the rainbow darter will be influenced by the diet and the GIT bacterial community composition was not upheld.

Chapter 4: Comparison/contrast between the response of the GIT of the **Campostoma anomalum** *and the* **Etheostoma caeruleum** *in response to an altered diet*

In this thesis, Illumina sequencing and enzyme analysis were used to characterize the influence of an altered diet on the composition of the GIT bacterial community and the enzyme activity in an herbivorous and a carnivorous fish host. The present study is novel, as it is the first time that the GIT bacteria was characterized, the first-time enzymes were measured, and the first-time association between the GIT bacterial abundance, enzyme activity, and an altered diet was examined in the native Ontario waterway fish *Campostoma anomalum* (central stoneroller) and *Etheostoma caeruleum* (rainbow darter). Throughout the course of this study, several similarities and differences were identified between these two fish species.

When contrasting the major phyla abundances between the wild-caught species, the startling differences in community structure highlight the importance of diet, as both animals inhabit the same ecological niche, and therefore environment cannot contribute to the observed dissimilarities (Figure 1 vs Figure 19). Additionally, the number of bacterial species (unique OTU's) was also higher in the central stoneroller compared to the rainbow darter before the dietary treatments (Figure 2.A vs Figure 20.A). In comparison, dietary treatments reduced the diversity of the GIT bacterial communities in both species, with a lower species (OTU) counts (Figure 2.A, Figure 20.A), phylogenetic diversity (Figure 2.C, Figure 20.C), and species evenness (Figure 2.D, Figure 20.D) distributions compared to the wild-caught animals (Figure 3, Figure 21). However, we found that the number of GIT bacterial sequences was higher in the central stoneroller (238,304 sequences) compared to the rainbow darter (175,110 sequences)

even after quality filtering, and the sheer number of OTUs was still higher in the central stoneroller (Figure 2.A vs Figure 20.A).

The transition of the bacterial community from the abundance of one phylum to another in response to the exogenous challenge of an altered diet was more distinct in the central stoneroller. For example, the transition of the bacterial community from an abundance of phylum Bacteroidetes to an abundance of phylum Firmicutes with the continuation of the commercial fish feed diet for 3 months was clearly observed in the central stoneroller (Figure 1, Figure 4). In contrast, the transition of the bacterial community composition from one phylum to another with the altered diet was obscure in the rainbow darter, as Proteobacteria was abundant in the GIT bacterial community of the rainbow darter through all phases of the diet treatments (Chapter 3, Figure 19). Based on these observations, it can be postulated that the GIT bacterial community of the herbivorous central stoneroller is more capable of effectively transitioning from the abundance of one phylum to another in response to an altered diet, as compared to the GIT bacterial community of the carnivorous rainbow darter.

As with the bacterial community composition, there were some notable similarities and differences in the activities of the digestive GIT enzymes in response to the dietary alteration in these two fish species. For example, the elevated GIT trypsin activity in the wild sample of the central stoneroller (Figure 6) and the elevated GIT lipase activity in the 3-month bloodworm treatment of the rainbow darter (Figure 25) may be representative of efforts of both the fish species to maximize the efficiency of the required nutrients from the nutrient-deficient diet. Moreover, when the overall digestive GIT enzyme activity between the two fish species were compared, we found that overall cellulase activity was higher in the central stoneroller (Figure 5) and that overall trypsin activity was higher in the rainbow darter (Figure 24). This may be further

indicative of the unique responses of the GIT of each fish species adapted to digest the host's primary diet, i.e. plant-cellulase diet in the central stoneroller and protein diet in the rainbow darter.

The activity of the electro gradient (NKA), glycolysis (LDH and PK), citric acid cycle (CS), and ammonia detoxification (GDH) enzymes also revealed patterns that were both distinct and similar between the two-fish species. For instance, we observed that the activity of the NKA enzyme in the GIT of the central stoneroller (Figure 8) was correlated with changes in bacterial abundance (phylum Firmicutes) and dietary alteration (lipid aspect of the diet) (Figure 18). This was not observed in the rainbow darter (Figure 26, Figure 36). On the other hand, both species demonstrated changes to the GIT LDH (Figure 10, Figure 28) and CS (Figure 14, Figure 32) enzyme activities in response to changes in the dietary carbohydrate content. A decrease in dietary carbohydrate resulted in an elevated GIT LDH activity (Figure 10, Figure 28), while an increase in dietary carbohydrate with an increased or recovery of Firmicutes abundance resulted in a decreased CS activity in the GIT of both fish species (Figure 14, Figure 18, Figure 32, Figure 36). In contrast, the response of the activities of the GIT PK (Figure 12) and GDH (Figure 16) to an altered diet was prominent in the central stoneroller only. For instance, the PK activity increased with increased dietary carbohydrate (Figure 12) and GDH activity increased with increased dietary protein (Figure 16), both followed by an increase in the abundance of Firmicutes (Figure 18) in the central stoneroller GIT. This is further suggestive of the ability of the GIT, GIT bacterial community, and GIT enzymes of the central stoneroller to efficiently modulate enzymatic activities in response to an altered diet, in comparison to the GIT, GIT bacterial community, and GIT enzymes of the rainbow darter.

Finally, there was a lack of any discernable trend in the enzymatic activities in the reference tissues of the white muscle, liver, kidney, and gill in both fish species (Figure 9, Figure 11, Figure 13, Figure 15, Figure 17, Figure 27, Figure 29, Figure 31, Figure 33, Figure 35), even after the introduction and continuation of the distinct diet treatments, and regardless of changes in the GIT bacterial abundance. This may be the result of other known factors, such as genetics or habitat, or of some other unknown factors that may or may not be influencing the enzymatic activities in these tissues. Therefore, more studies are required to fully understand the influence of these factors on the enzyme activities of these tissues in both of these fish species.

Perspective:

Fish make up almost fifty percent of all vertebrate life on Earth and many countries are dependent upon fisheries and aquaculture for both economical and dietary reasons. For example, half the GDP of island countries like the Marshall Islands, Kiribati, and Maldives is dependent upon fisheries and aquaculture (FAO 2016). Furthermore, the global consumption of fish products has increased steadily over the years, with reports suggesting a total of 146 million tons of fish products were consumed worldwide in 2016; up from the global consumption of 136 million tons in 2014 (FAO 2016). Finally, fish are important model organisms for research across many disciplines of biology, because of their diversity, phylogeny, genetic makeup, and ease of maintenance in the laboratory. Because of these economical, nutritional, and scientific roles, our effort to understand fish digestive physiology is an important preliminary step in the broader perspective of preserving global fish abundance, diversity, and production.

The interaction between the diet, host GIT bacterial community, and host enzymatic physiology is complex, concerning the implications of the diet on the GIT bacterial community,

the enzyme activities of the GIT, and/or the subsequent influence of the GIT bacterial community on the host physiology. The current novel study suggests that the GIT bacterial community, especially the GIT abundance of phyla Firmicutes and Bacteroidetes in the herbivorous fish central stoneroller, and the relative GIT abundance of phyla Proteobacteria and Firmicutes in the carnivorous rainbow darter are responsive to dietary changes. Furthermore, the change in activities of cellulase, lipase, NKA, PK, CS, and GDH in the GIT of the central stoneroller, and the activities of trypsin, LDH, and CS in the rainbow darter GIT may be reflective of the bacterial efforts to ensure homeostasis and beneficial for the host because of the bacteria taking care of the dietary response. Contrastingly, the activities of trypsin and NKA in the central stoneroller GIT, as well as cellulase, lipase, NKA, LDH, PK, CS, and GDH in the rainbow darter GIT were not responsive to the changes in the abundance of certain bacterial phyla, but rather responded to alteration in the diet. Therefore, an altered enterocyte activity for maximized endogenous enzyme production controlled by the host to maximize homeostasis may be responsible for the control of these enzymes in the GIT for these treatments. Overall, this study suggests that the optimal interaction of the GIT bacterial community and enzyme activity are important and beneficial for the homeostasis, health, and growth rate of both herbivorous and carnivorous fish, especially in response to the growing trends of using plant protein based diet in the commercial aquaculture industry sector.

Future directions:

In our current study, we established the implications of the diet on the GIT bacterial community, the GIT enzyme activity, and/or the subsequent influence of the GIT bacterial community on host physiology in the central stoneroller and the rainbow darter for the first time. However, we were unable to study the independent response of the endogenous or the exogenous

enzymes in response to the challenge of an altered diet. Although the response of the GIT to a challenge is coupled, i.e. endogenous plus exogenous response, it is essential to understand the individual contribution of the host and the bacterial community in order to fully understand this relationship. This understanding may be achieved in future studies by analyzing the response of the GIT enzymes mentioned in this thesis against an altered diet in the presence and absence of the GIT bacterial community by using antibiotics, or by rinsing the internal membrane of the GIT with antibacterial solutions to eliminate the bacterial community.

Our endeavor in this study to identify the influence of an altered GIT bacterial community and GIT enzyme activities in response to an altered diet on the reference tissues of the central stoneroller and the rainbow darter was unsuccessful. As previously mentioned, perhaps other internal and external factors such as genetics, habitat etc. may or may not be involved in controlling the enzyme activities in these tissues vs the challenge of an altered diet. Therefore, future studies that involves the interchanging of these factors and measuring the enzyme activities in the reference tissues by using an optimized approach to the methodologies used in this thesis may be able to identify and pinpoint the influence of these factors on the enzyme activities in the reference tissues.

Finally, in addition to members of domain Bacteria, the GIT is also inhabited by members of domains Archaea and the domain Eukaryota. Domain Archaea consists of ancient prokaryotes traditionally considered to thrive in harsh environmental conditions (Woese et al. 1978). However, with the transition from the traditional studies of plate culture analysis to methods enhanced by technological advancements such as the 16S rRNA sequencing, today it is known that Archaea are abundant in a wide range of terrestrial and aquatic systems (Maarel et al. 1998; Ohkuma et al. 2001), including in the GIT microbiome community (Maarel et al. 1998;

Maarel et al. 1999; Ohkuma et al. 2001; Zhang et al. 2009). Domain Eukaryota is a comparatively advanced domain which is also abundant in the GIT microbiome community. While the presence of bacteria in the GIT of vertebrates has been well-established, the presence of Archaea and Eukaryota in the GIT is a more recent discovery, therefore, our understanding of their function in the GIT is still in its infancy. Although it has been hypothesized that Archaea are responsible for methanogenesis and ammonia oxidation (Maarel et al. 1998; Maarel et al. 1999; Ohkuma et al. 2001; Zhang et al. 2009) and that Eukaryota are responsible for probiotic supplementation (Macfarland et al. 1993; Kelesidis et al. 2011; Parfrey et al. 2011; Dinleyici et al. 2012; Feizizadeh et al. 2014) within the host GIT, it is as yet unknown whether their actions are beneficial or harmful to the host in addition to whether the response of either of these domains in the face of external challenge, such as an altered diet, is beneficial or harmful for the host's digestive physiology, homeostasis, health, and growth. Therefore, with future studies that adapt similar techniques used in this thesis, a better understanding of the overall positive or negative contribution of these two domains in maintaining the physiological and homeostatic integrity of the host can be elucidated.

Appendix:

A. Command list with respective functioning description exerted to process the bacterial gDNA sequences in QIIME are listed below in chronological order:

1. Validate_mapping_file.py = This command was used to check the mapping file integrity. Initially, the barcode, linker-primer, and reverse-primer fields were checked for valid IUPAC DNA characters and barcode sequence characters being non-degenerate. Also, the mapping file was checked for the appearance of duplicate barcodes, no invalid characters in the headers, invalid alphanumeric characters, and same length of the barcodes used.
2. Split_libraries.py = This command was used to remove the bar-code and linker primer sequences from the total sequences and perform quality filter. Sequences from samples that are not found in the mapping file (no corresponding barcodes) and sequences without the correct primer sequences were excluded. This command also clustered sequences to the unique samples with the precise barcode for downstream processing. A new fasta file called seqs.fna was generated along with histograms.txt that contained the counts of sequences with a particular length and a split_library_log.txt file that contained a summary of information regarding the number of sequences that pass quality control and distribution across the different samples.
3. Pick_otus.py = This OTU (Operational Taxonomic Unit) picking step assigned similar sequences to OTUs based on a 97 % similarity threshold in reference with the green genes database using usearch (Edgar 2010) and closed-reference OTU picking protocol. Sequences in a sample that had 97 % or more similarity were clustered together as a single OTU or taxa. Chimeras were also removed from the sequences using uchime,

which is a part of the usearch algorithm (Edgar 2010; uchime as part of usearch v5.2.236). Multiple files were generated through this processing, however only seqs_otus.txt file was used.

4. Pick_rep_set.py = This command was used to pick a representative set of sequences where for each OTU; one sequence was generated to be used for a subsequent analysis. A fasta file containing one sequence per OTU was generated for effective representative downstream processing.
5. Align_seqs.py = This script was used to align the sequences in the representative fasta file based on the PyNAST alignment method (Caporaso et al. 2009). The NAST algorithm alignment provided a sequence (the "candidate" sequence) to the best-matching sequence in a pre-aligned database of sequences (the "template" sequence). Candidate sequences were not permitted to introduce new gap characters into the template database, so the algorithm introduced local alignments to preserve the existing template sequence.
6. Assign_taxonomy.py = This script was used to assign taxonomy to each OTU in the aligned representative sequence file. Taxonomy was assigned based on BLAST methodology. Taxonomy assignments were made by searching input sequences against a BLAST database of pre-assigned reference sequence set. Taxonomy was assigned from kingdom to the species level in a chronological order.
7. Filter_alignment.py = The OTUs were then filtered using this script for low quality reads (< quality score 25), short and long sequences (< 200 nt; >1000 nt), zero ambiguous base calls, zero primer sequence mismatch, and sequences with homopolymer runs exceeding 6nt. Also, OTU sequences with no BLAST hits for taxonomical assignments were filtered out in this step.

8. Make_phylogeny.py = This script was used to create a phylogenetic tree relating the OTUs using a multiple sequence alignment. Trees were constructed with a set of sequences that were representative of the OTUs in default using FastTree (Price et al. 2009). The resulting files from this script consisted of a Newick formatted tree (Computer readable raw phylogenetic tree) with a log file.
9. Make_otu_table.py = This script developed an OTU table dataset that predicted the number of times a unique OTU was found in each sample, generating a biom table file which was used for all subsequent downstream analysis.
10. Alpha_rarefaction.py: Alpha_rarefaction.py workflow script was used to generate alpha-rarified OTU tables, collate the alpha diversity results, and generate an observed species, Chao1, phylogenetic diversity, and Shannon Index plots. Alpha-rarefaction was done to compare the richness of GIT bacterial OTUs within each treatment samples (alpha diversity) with the sample containing the least number of sequences as a reference baseline.
11. Jackknifed_beta_diversity.py: This workflow script was used to compare the GIT bacterial composition of each individual treatment with other treatments (beta diversity). The analysis was done using 90 % of random sequences as a baseline adopted from the sample containing the least number of OTUs. A consensus tree was also generated by the script using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) distance matrices. Finally, make_bootstrapped_trees.py script was used to generate an unweighted-beta and weighted-beta bootstrapped trees with % support color identifier and beta_significance.py script was used to calculate the Bonferroni adjusted p-value between samples.

B. Command list with respective functioning description to generate the multivariate RDA plots in R are listed below in a chronological order:

1. Read.table: This function was initially used to read the user provided table as the primary input. Specifications to extrapolate the required columns or rows was provided to load the environmental dataset to identify the response and the predictor groups for downstream processing.
2. Library (vegan): This function was used to unpack and assign the vegan package for the diversity analysis, ordination methods, and analysis of dissimilarities.
3. Decostand (method= "Hellinger"): This function was used to perform a Hellinger transformation on the user provided table to remove the bacterial species with low counts and many zeros.
4. Rda (response, predictor): This function was used to perform the RDA multivariate analysis on the response variables (user provided table) by the user designated predictor variables.
5. Plot: This function was used to generate the graph obtained after performing the RDA analysis. Perimeters of display = "species" and display =" sites" was passed with this function to display the bacterial species, treatments, and enzymes in the generated graph.

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