NUTRITIONAL CONDITIONING DURING LARVAL DEVELOPMENT TO IMPROVE PRODUCTION AND FEED EFFICIENCY AND ESTABLISHMENT OF BENEFICIAL GUT FLORA IN TILAPIA CULTURE

Sustainable Feed Technology and Nutrient Input Systems/Experiment/16SFT02NC

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ABSTRACT

Feed constitutes 60-80% of total production costs of tilapia (*Oreochromis spp.*) with high quality protein being the most expensive component. Studies in mammals have demonstrated that early nutrient contributions can influence growth and other key physiological processes. This phenomenon, known as nutritional conditioning, was also shown in chickens and fish where limiting a nutrient in the early stages of development led to enhanced uptake and utilization later in life. We evaluated if this process could be applied to tilapia culture in the form of protein-restriction as this could reduce the cost of feed and thus production of tilapia for small-scale rural farmers in Bangladesh. It has yet to be determined in fish if protein restriction early in life might lead to improved growth later on. We first performed a tank trial to determine the effectiveness of protein-restriction in tilapia culture. Post-yolk sac fry were fed a 25% crude protein (CP) starter diet for 7, 14, or 21 days or a typical 48% CP diet for 21 days after which half the tanks from each treatment were fed a reduced 25% CP growout diet while the other half received a typical 38% CP growout diet. The results indicate that fish fed a reduced protein diet initially had significantly greater lengths and weights at the end of the 56-day study period relative to controls, however growth and survival decreased when fish remained on the 25% CP starter diet for longer than 14 days. Our RNA-seq analysis of tilapia intestinal tissue showed differential expression of genes involved in proteolysis and muscle function between groups receiving different levels of protein which may indicate compensatory mechanisms for maintaining function during protein restriction or enhancing muscle synthesis when dietary protein levels are increased. Additionally, tilapia fed an initial protein-restricted diet for 14 days had the greatest microbe diversity in their fecal matter. A more diverse microbiome is associated with enhanced health and feed efficiency and thus the use of nutritional conditioning in tilapia culture may be beneficial for more than simply increasing the growth of the fish. We also assessed whether this nutritional conditioning would also be effective for pond culture in Bangladesh. Here, post-yolk sac fry were fed a reduced 20% CP starter diet or a typical 40% starter diet for 15 days and then switched to a 20% CP or 40% CP growout diet through 90 days. Contrary to the tank study, we determined that fish fed a reduced protein diet initially had lower growth and production yields than those fed the 40% CP diet. It is likely that fish on the protein restricted diet had access to natural food sources within ponds and hence were not truly restricted enough to yield a nutritional programming response as was seen in tank trials. Early restriction of protein under more controlled conditions in hatcheries followed by growout in ponds should be tested. Interestingly, the fish that were switched from the 40% CP started diet to the 20% CP growout diet had higher growth and survival as well as the greatest net returns. Thus, while a nutritional conditioning response was not observed, it may still be possible to lower feed costs by reducing the amount of protein provided during the growout stage. These are the first studies to demonstrate that nutritional programming or protein restriction early in life can improve growth of fingerling tilapia under controlled conditions. Future work should evaluate whether improved feed efficiency and growth can be sustained throughout growout in fish subjected to early protein restriction.

INTRODUCTION

Global production of farmed Nile tilapia (*Oreochromis niloticus*) has increased exponentially since 1985, with over 2.4 million metric tons consumed in 2010 (FAO, 2013). In Bangladesh, Nile tilapia comprises a significant source of per capita caloric and protein intake, with production increasing 30-fold from 1999-2007 (Hussain, 2009). Total production is second only to carps (Apu, 2014). Feed ingredients typically include fishmeal, other animal meal or byproducts, and plant material (soybean products) as primary sources of protein for fish growth. High quality protein is critical for animal growth and health and requirements vary depending on age and size of the fish. Commercial feeds for pond growout contain ~30% protein while juvenile tilapia may require up to 40% protein for proper growth (El-Sayed, 2006). Work in mammalian models demonstrates that nutrient contributions early in pre- or postnatal development influence growth and immune function later in life (Lucas, 1998). This process, known as conditioning (also programming or imprinting), when established at critical periods in the animal's development, lead to life-long changes in the function of key elements of an organism's physiology. By altering early nutritional components, nutritional conditioning can result in more efficient uptake and utilization of nutrients from the diet thus increasing growth and health parameters in the organism later in life. The process of nutritional conditioning is also observed in poultry. Nutritional conditioning of energy and minerals can influence uptake and utilization in chickens (Ashwell and Angel, 2010; Ferket, 2013). Broiler chickens had increased retention of phosphorous from their diets following feeding of a phosphorous-deficient diet for the first 90 hours post-hatch (Ashwell and Angel, 2010).

A better understanding of how finfish acquire and utilize nutrient inputs is requisite for future improvements in aquaculture production efficiency as feed constitutes anywhere from 50% to 80% of total variable production costs. Nothing is known about the effectiveness of applying nutritional conditioning to tilapia culture, this despite strong evidence that the phenomenon is likely to occur across all vertebrate taxa. A few studies have looked at energy uptake and utilization following conditioning in rainbow trout (*Oncorhynchus mykiss*) and European sea bass (*Dicentrarchus labrax*), both carnivorous fishes. High dietary glucose diets fed to rainbow trout juveniles for a short period showed there was long-term modifications to carbohydrate metabolism (Geurden et al., 2007) while European sea bass juveniles fed a HUFA-deficient diet initially were able to metabolize lipids more efficiently than those fed a high HUFA diet (Vagner et al., 2007). Here we evaluated if nutritional conditioning can be applied to tilapia and assessed whether reductions in the amount of protein in the diet of post-larval tilapia would subsequently lead to improved feed or protein efficiency during later growout of fishes.

Currently, the underlying mechanisms explaining how larval nutritional conditioning strategies can potentially achieve equivalent production yields with less protein in the feed is poorly understood. Some evidence suggests that during periods of fasting, nutrient uptake efficiency in the intestine is intrinsically enhanced, leading to a more-efficient uptake of nutrients at the next feeding period (Ali et al., 2003; Picha et al., 2006). Thus, decreasing the amount of select nutrients early in the life of the fish may increase the uptake and utilization of those nutrients during the growout phase of fish culture. Using a transcriptomic approach, we evaluated the suite of genes in the intestine that are differentially expressed in fish fed a protein deficient diet early in larval development versus those receiving a diet containing traditional levels of protein to further our understanding of how protein assimilation and enhanced growth may be achieved for greater optimization of feeding protocols in the future.

Nutritional conditioning may also affect the microbial colonization of the gut. The establishment of beneficial microflora can affect nutrient availability and gut health (Marques et al., 2010). The emerging field of metagenomics has substantial implications for sustainable aquaculture, as diet, feeding strategy, and other environmental factors strongly influence the diversity and constitutive abundance of intestinal microbiota in both humans and fish (Al-Harbi and Uddin, 2004, 2005; De Filippo et al., 2010; Heikkinen et al., 2006). In cultured finfish, new research has shown that probiotic maintenance of beneficial gut flora

can promote growth, enhance nutrient availability, and increase overall stock health (Nayak, 2010; Welker and Lim, 2011). In our previous AquaFish Innovation Lab studies, we found that tilapia fed on alternate days in fertilized ponds produced similar growth and survival, but improved feed efficiency by 100%, compared with fish fed daily. Fish also had a higher diversity of microbes in their intestines that may benefit nutrient processing and uptake. Here we built on this work to determine whether tilapia intestinal microbial composition and diversity varies with nutritional conditioning and identify key microbes that may be associated with increased protein uptake and utilization. The identification of such microbes may benefit current research into the application of probiotic supplements in fish culture for further enhancement of feed efficiency.

This investigation targeted a method to improve production efficiency of tilapia, namely through reducing the amount and cost of feed needed to produce a kg of fish. Since >50% of the costs associated with feed is protein, practical approaches that improve its utilization has tremendous application to global tilapia production (El-Sayed, 2006). Previously, our research showed that Nile tilapia and milkfish (seacages and ponds; *Chanos chanos*) can be grown to market size in monoculture with significant cost savings through implementation of alternate-day feeding versus daily feeding (50% feed reduction; Bolivar et al., 2006; Borski et al., 2011; De Jesus-Ayson and Borski, 2012). Our work over the past year also indicates similar responses with tilapia grown in ponds in Bangladesh. Here we determined the proper length of time for nutritional conditioning of post-yolk sac Nile tilapia fry that can lead to enhanced feed efficiency with minimal impact on growth and survival of the fish in a laboratory setting and subsequently investigated whether this strategy could be applied to pond culture in Bangladesh where it would have the potential to provide substantial costs savings for tilapia farmers and also mitigate negative environmental impacts associated with excessive nutrient loading.

OBJECTIVES

1. Evaluate the effectiveness of nutritional conditioning on tilapia growth.
2. Identify key factors (gene networks) associated with improved growth in response to larval nutritional conditioning in tilapia.
3. Characterize changes in gut microbial communities in response to nutritional conditioning and identify those that may be associated with improved nutrient absorption and growth in fish.

MATERIALS AND METHODS

Study 1 - Evaluate the effectiveness of nutritional conditioning on tilapia growth

The initial laboratory investigations were performed at North Carolina State University. Restricted and normal protein diets were formulated by Integral Fish Foods Inc. (Albany, Indiana) using the nutritional profiles outlined by Mjoun et al. (2010) as a guideline (Table 1) and feed was produced by the Bozeman Fish Technology Centre (Bozeman, Montana). Post-yolk sac Nile tilapia fry were obtained from the Louisiana Speciality Aquafarm (Harvey, LA) and stocked in 36 experimental tanks (2.8 L) at the Grinnell's Fish Laboratory (NCSU). The study consisted of 8 treatment groups in which fry were fed a normal (48% CP) or restricted (25% CP) protein starter diet for 7, 14, or 21 days before being switched to a normal (38% CP) or restricted (25% CP) protein growout diet for the remainder of the 56-day study period (Figure 1). Each

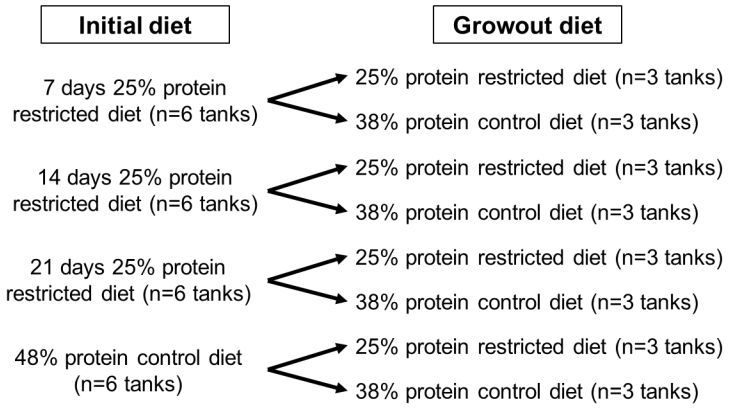

Figure 1. Experimental design for tank study.

treatment was replicated in 3 separate tanks. Fish were fed 3 times a day to satiation for the first 28 days after which this was reduced to twice a day to satiation and tanks were cleaned regularly to remove algae and feces that the tilapia could consume. Growth parameters (length and weight) were measured throughout the study period (sub-sampling) and survival rates for each treatment were calculated at the end of the experiment. Intestinal samples for RNA-Seq analysis were taken from a subset of fish at the end of 56 days and placed in RNA Later (Invitrogen). In the microbiome analyses tilapia fry up to 15 mm TL were too small to dissect out individual intestinal tissues for sampling. Instead, whole fry were rinsed in 70% ethanol and sterile deionized water to wash off external microbes that may be found on the skin of the fish. In animals > 15 mm, fecal material was collected from the posterior intestine (colon) and placed in a vial containing buffer from a Quick-DNA Fecal/Soil Microbe Kit (Zymo Research) and bullet homogenized with a portable homogenizer. Samples collected from fish were pooled together according to treatment group (samples from 2 fish per tank pooled, 2 pooled samples per tank, 3 tanks per treatment; N = 6 per treatment). The pooled sample design was used to offset potential variability of microbiota within individuals, instead focusing on common patterns, which may be more reflective of changes with treatment group among the population as a whole. Samples were collected at 7, 14, 21, 28, 35, and 56 days after start of the study.

Table 1. Nutrient composition of diets used in the tank study. ME = metabolizable energy.

	25% Starter	48% Starter	25% Growout	38% Growout
Protein	25.00%	48.00%	25.00%	38.00%
Fat	12.13%	8.58%	9.59%	8.00%
Carbohydrates	36.72%	15.88%	36.59%	23.80%
Fibre	3.64%	2.04%	4.78%	3.96%
Ash	4.47%	6.38%	5.17%	6.15%
Lysine	1.96%	3.80%	1.45%	2.19%
Methionine	0.45%	1.08%	0.44%	0.66%
Tryptophan	0.30%	0.50%	0.31%	0.48%
ME (Catfish)	2584 kcal/kg	1986 kcal/kg	2353 kcal/kg	2350 kcal/kg
Fishmeal	10.08%	24.67%	9.85%	8.47%
Poultry products	0%	19.74%	0%	14.05%
Wheat grain	25.19%	14.80%	14.77%	15.06%
Soybean seeds	1.68%	9.87%	9.85%	25.10%
Wheat flour	33.59%	9.87%	49.24%	23.42%

While the use of tanks for aquaculture is common practice in the US, developing countries such as Bangladesh primarily culture their fish in ponds. Thus, we evaluated whether restricted protein diets could be applied to pond cultures to help reduce feed costs for poor rural farmers. These studies were performed at the Fisheries Field Laboratory, Bangladesh Agricultural University. Normal and restricted protein feeds were produced by Krishibid Feed Ltd. (Mymensingh, Bangladesh) (Tables 2, 3). A total of 7500 Nile tilapia post-yolk sac fry were obtained from Reliance Aqua Farm (Mymensingh, Bangladesh) and stocked in 12 hapas at the field laboratory. Fry in 6 hapas received a 20% CP diet and the other 6

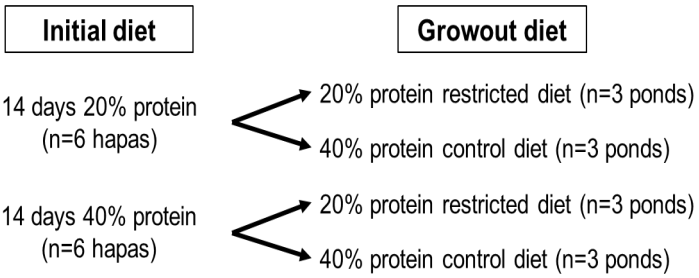

Figure 2. Experimental design for pond study.

received a 40% CP diet for 14 days at a rate of 15% BW per day, after which the fish from each hapa were transferred into one of 12 rectangular ponds, averaging 130 m^2 and 1.5 m deep. Ponds had previously been limed with $CaCO_3$ (1 kg/dec) and were fertilized with urea (28 kg N/ha) and triple super phosphate (7 kg P/ha) 7 days prior to fish stocking. Once in the ponds, tilapia were fed either a 20% or 40% CP growout diet as illustrated in Figure 2. Feed was provided at a rate of 15% BW initially before being reduced to 8% BW. Additionally, all ponds were fertilized weekly with urea and TSP. Water temperature, transparency, pH, dissolved oxygen, nitrates, nitrites, phosphates, total dissolved solids, alkalinity, conductivity, and chlorophyll-a were assessed weekly. Phytoplankton and zooplankton abundance were also measured. Data were analysed by one-way ANOVA followed by a Tukey's multiple comparison test.

Table 2. Composition of the starter feeds for the pond study.

	20% Crude Protein	40% Crude Protein
Fish meal	5.00%	25.00%
Meat and bone meal	9.00%	21.00%
Mustard oil cake	15.00%	15.00%
Rice bran	50.00%	18.25%
Chemical binder	5.00%	5.00%
Soybean meal	0.00%	15.00%
Maize	25.25%	0.00%
Salt	0.50%	0.50%
Vitamin premix	0.25%	0.25%

Table 3. Composition of the growout feeds for the pond study.

	20% Crude Protein	40% Crude Protein
Wheat flour	15.00%	19.00%
Corn products	22.80%	3.00%
De-oiled rice bran	30.00%	0.00%
Soybean meal	0.00%	30.85%
Full fat soybean	3.55%	10.00%
Rape seed meal	20.00%	20.00%
Meat and bone meal	0.00%	10.00%
Poultry oil	2.26%	1.70%
Methionine	0.11%	0.40%
Lysine sulphate	0.46%	0.79%
Fish vitamin	0.25%	0.25%
Salt	0.36%	0.25%

Study 2 – Identify key factors (gene networks) associated with enhanced nutrient utilization in response to larval nutritional conditioning in tilapia

Total RNA from tilapia anterior intestinal samples from Study 1 were extracted using TRI Reagent (Molecular Research Centre, Inc.) and sent to the NCSU Genomic Sciences Laboratory for library preparation and RNA-Seq analysis using the Illumina HiSeq platform. For each treatment, a bar-coded amplicon library (n=2-4) was constructed and the pooled sample was run on a single Illumina lane (125 bp, single end reads). The NCSU Bioinformatics Consulting and Service Core (BCSC) was then used for the High Performance Computing environment with the CLC Workbench license to identify differentially expressed genes (DEG) between each treatment group and the control (48% protein to 38% protein). The residuals of each highly significant DEG were put into a Modulated Modularity Clustering program (Stone and Ayroles, 2009) to group the highly significant differentially expressed genes while Gene Ontology (GO) term enrichment of each module determined cluster function using DAVID Bioinformatics suite (Huang et al., 2009) and Zebrafish Information Network (ZFIN) GO annotation.

Study 3 – Identification of key microbial factors promoting increased nutrient absorption by enterocytes in the tilapia gastrointestinal tract

Ribosomal RNA extraction

Extraction of ribosomal RNA from whole tilapia (9 mm to 15 mm TL) or tilapia posterior intestinal fecal samples (greater than 15 mm TL) was performed using a Quick-DNA Soil/Fecal DNA Microbial Kit (Zymo Research, Corp., Irvine, CA) following the included protocol. Up to 0.25 g of fecal sample was placed into a ZR BashingBead Lysis Tube with 750 µL Lysis/Stabilization Solution. The tube was secured in an Xpedition Sample Processor and processed for 30 s and stored at room temperature until extraction. The concentration and quality were determined by Nanodrop (Thermo Fisher Scientific, Inc., Waltham, MA). The extracted rRNA was stored at -20°C for sequencing library preparations.

Prokaryotic 16S rRNA sequencing library preparation

Prokaryotic 16S rRNA gene amplicons were prepared following the 16S Metagenomic Sequencing Library Preparation protocol for the Illumina MiSeq system with some modifications. Primers were designed to amplify the V3 to V5 regions of 16S rRNA (Muyzer et al. 1993; Sim et al. 2012) with overhang adapter sequences compatible with the Illumina index and sequencing adapters and allowed for double indexing to increase the accuracy of the multiplexed reads. Amplicon PCR was used to amplify the region of interest from the gDNA extracted from the tilapia fecal material samples. The PCR was performed: 1 cycle of 95°C for 3 min; 25 cycles each of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; 1 cycle of 72°C for 5 min; and hold at 4°C. Clean-up of the PCR amplicon products to remove free primers and primer dimers was performed using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA). Fresh 80% ethanol was prepared prior to clean-up. Following amplicon clean-up, index PCR was performed to attach indexes to the amplicon PCR products. Dual-index primers were designed so that samples could be multiplexed in one MiSeq lane. Index PCR was performed as follows: 1 cycle of 95°C for 3 min; 8 cycles each of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; 1 cycle of 72°C for 5 min; and hold at 4°C. Clean-up of the PCR index products was performed as above. All indexed amplicon concentrations were normalized and amplicons pooled into a single tube. The pooled library was checked for quality and quantified using an Agilent ScreenTape on the Agilent 2200 Tapestation (Agilent, Santa Clara, CA). The library was diluted and combined with a PhiX Control library (v3) (Illumina, San Diego, CA) at 10%. The library was sequenced on an Illumina v3 300PE MiSeq run, using standard sequencing protocols. Base calls were generated on-instrument during the sequencing run using the MiSeq Real Time Analysis (RTA 1.18.54) software and fastq generation; demultiplexing, adapter trimming, and quality filtering were performed by the MiSeq Reporter Software (2.4 and 2.5.1). The library was run on two lanes to increase the number of reads for each sample.

Sequence and statistical analyses

The resulting demultiplexed reads were processed using the QIIME 2 microbiome analysis package (Caporaso et al. 2010). Briefly, the paired end reads were joined together and Scikit-learn (Pedregosa et al. 2011) was used to taxonomically classify the resulting paired reads against the SILVA release 132 reference database (Pruesse et al 2007, Quast et al 2013, Yilmaz et al 2014) for 16S (prokaryote) analysis filtered at 99% identity. Reads not matching a reference sequence were removed from analysis. OTUs were assigned based on a database hit of 99% or greater sequence identity and taxonomy was assigned against the database. Core diversity analysis was used to perform α-diversity and rarefaction and β-diversity and rarefaction functions. Weighted and unweighted Unifrac distances (Lozupone and Knight 2005) were used to compute the between sample diversity which was visualized using principal coordinates analysis (PCoA) plots with Emperor (Vazquez-Baeza et al 2013).

RESULTS AND DISCUSSION

Study 1 - Evaluate the effectiveness of nutritional conditioning on tilapia feed efficiency and production

Results from the tank study suggest that restricting protein early in development (post-yolk sac absorption) enhances growth later in life (Figures 3, 4). Tilapia fry that were fed a diet containing 25% CP for 14 days and then transferred to a 38% CP diet had significantly higher lengths and weights relative to fry fed the control (48% CP) diets initially. Further, the growth of fry fed the 25% CP starter diet for 14 days that then remained on the 25% CP growout diet was not significantly different from either the controls or those transferred to the 38% CP diet indicating that it may be possible to restrict protein for up to 56 days provided they are switched to a growout diet. Survival rates were greatest (72%) in fry that received the 25% CP starter diet for only 7 days, however survival rates of fry that received the protein-restricted diet for 14 days (50%) were not significantly different from the control 48% protein diet (63% survival). Fry that were fed the 25% CP diet for 21 days had the lowest survival rate (28%) and thus it is recommended that tilapia be given a protein restricted starter diet for no longer than 14 days (Figure 5).

After obtaining positive results from the tank trials, we investigated whether this nutritional conditioning could also be applied to pond culture. Water quality was deemed suitable and within the range for fish culture throughout the study period and all parameters were relatively similar between treatments (Table 4). Contrary to what was observed in the tank study, tilapia fed a 40% CP diet initially (T3 and T4) were significantly larger after 93 days relative to those fed a 20% CP diet (Table 5). As tilapia are an omnivorous species, it is likely that natural food sources within the ponds negated any potential effects of the nutritional conditioning. Interestingly, the fish that were fed 40% CP initially and then switched to 20% CP (T3) had the highest survival, specific growth rates, and net production relative to all other groups (Table 5), leading to a significantly lower feed conversion ratio and higher net return (Figure 6). Thus, while nutritional conditioning per se may require nutrient restriction in tank systems to eliminate alternate food sources, it may still be possible to lessen feed costs for farmers in Bangladesh and other developing countries by reducing the amount of protein in growout diets. Further, despite growth and production being lowest in T1 (20% CP starter; 20% CP growout), this treatment had the second highest net profit and a similar benefit to cost ratio as T3 due to the lower cost of feed (Table 6). This suggests that providing low protein feeds throughout the culture period may be an acceptable option for farmers who have less money to invest and who can still market smaller fish. Introducing weekly fertilization as performed in this study could provide additional food sources and more diverse proteins to help compensate for the reduced levels in the formulated feed and may lead to enhanced growth.

Study 2 – Identify key factors (gene networks) associated with enhanced nutrient utilization in response to larval nutritional conditioning in tilapia

Nutritional conditioning is known to affect growth and immune function as well as enhance the uptake and utilization of specific nutrients in various vertebrates but little is known about the effects in fish and whether it can be applied to tilapia culture to enhance growth and feed efficiency. This study used a transcriptomic approach to identify changes in gene expression in the intestines of Nile tilapia fed a restricted 25% CP diet or a normal 48% CP diet for the first 14 days post-yolk sac absorption before being subjected to a 25% CP or 38% CP growout diet for 42 days. An RNA-seq analysis showed that tilapia fed 25% CP followed by 38% CP had 54 differentially expressed genes (DEG), those fed 25% CP followed by 25% CP had 1389 DEG, and those fed 48% CP followed by 25% CP had 105 DEG compared to the control diet (48% CP followed by 38% CP).

Gene Ontology (GO) analysis showed that in the 48% CP to 25% CP group, functions such as proteolysis (GO: 0006508), muscle contraction (GO: 0006936) and filament sliding (GO: 0030049), and immune responses (GO: 0006955) were enriched relative to the control (Table 7). The increase in genes associated with proteolysis (*klk2*, *map1b, tmprss15)* could indicate a restructuring of the intestine to provide amino acids for the production of more essential proteins given the decrease in dietary protein. This has been shown in neonatal pigs during weaning where providing a low protein diet suppresses protein synthesis (Deng *et al.*, 2009). One possibility for this restructuring could be to increase production of genes involved in muscle contraction such as myosin (*des, mylpf, myh11, myl3*) and actin (*acta1*) to help

maintain normal intestinal function despite the lack of protein availability. In the 25% CP to 38% CP group, muscle contraction and filament sliding and immune response processes were also enriched compared to the control group (48% CP to 38% CP), in addition to cell signaling (GO: 0007267) and negative regulation of proteolysis (GO: 0045861)(Table 8). This likely indicates a compensatory mechanism wherein protein and muscle synthesis were enhanced due to the increase of dietary amino acid availability. Interestingly, a KEGG pathway analysis also showed upregulation of tight junctions (upregulated genes: *cldn4, mylpf, myh7*) in this group (25% CP to 38% CP). Low protein diets are known to decrease intestinal permeability to unwanted materials (Magnusson *et al.*, 1990) suggesting that this pathway may have been upregulated after the switch to the higher protein diet to prevent an increase in intestinal permeability. There were 1389 DEGs in fish subjected to an initial protein restricted diet and a low protein growout diet (25% CP to 25% CP group) relative to the control, 48% CP to 38% CP fish. Principal component analysis revealed the low protein group (25% CP to 25% CP) formed a distinct cluster separate from samples of the other three groups indicating vast differences in the intestinal transcriptomes of these fish, suggestive of broad physiological effects associated with a state of limited dietary protein (data not shown).

Study 3 – Identification of key microbial factors promoting increased nutrient absorption by enterocytes in the tilapia gastrointestinal tract

This investigation assessed how the gut microbial flora is altered by nutritional programming strategies and whether these changes could benefit the growth and health of tilapia. The establishment of beneficial gut flora to increase nutrient absorption is an emerging research focus in human biology and aquaculture science (Welker and Lim, 2011), and may serve to augment existing practices of sustainable feeding and reduction in environmental footprint. A total of 1,153,339 reads were obtained following sequencing of the V3 to V5 regions of 16S rRNA (prokaryote) after quality filtering of the reads.

A total of 23 prokaryotic phyla, 44 classes, 112 orders, and 202 families were associated with the tilapia used in this study (100% Bacteria, no Archeae). The dominant phyla identified overall from these samples belonged to the Proteobacteria (70.0%), Fusobacteria (12.1%), reads that could not be classified beyond the Kingdom Bacteria (11.1%), Actinobacteria (3.1%), and Bacteroidetes (2.1%) (Figure 7). The proportions of the identified microbes varied between treatments and between collection time points. Bacteria from the class Fusobacteriaceae were most abundant in the fecal material of Nile tilapia used in our previous study of alternate feeding strategies in tilapia culture. *Cetobacterium somerae* had the highest number of reads or abundance of Fusobacteria found in this study. This species has been isolated from the intestinal tract of cultured freshwater fish (Tsuchiya et al. 2008) and thus may play an important role in the health of these fishes.

OTUs that made up the greatest proportion of the Proteobacteria belong to the class Gammaproteobacteria. These include bacteria in the genus *Aeromonas* (10.1% overall), genus *Rheinheimera* (4.6% overall), genus *Shewanella* (1.3% overall), family Burkholderiaceae (4.7% overall), genus *Cellvibrio* (1.4% overall), genus *Pseudomonas* (1.7% overall), and genus *Enterovibrio* (14.0% overall). Microbes found in the genus *Aeromonas* include many that are opportunistic pathogens of fishes. One OTU observed belongs to the species *A. hydrophila*, a major pathogen in freshwater fishes causing ulcers, “fin and tail rot”, and hemorrhagic septicemia (Subashkumar et al. 2007) along with antibiotic resistant strains found in Mozambique tilapia (*Oreochromis mossambicus*; Son et al. 1997). This OTU was found in our baseline samples and in our samples taken at day 14 whether fed the 25% or 48% initial diet but was found at proportions of less than 1% in these treatments. It was not observed in any of the later treatments. Organisms of the genus *Rheinheimera* include halotolerant freshwater and marine oligotrophs, mostly aquatic in nature (Baek and Jeon 2015). *R. aquatica* is an antimicrobial producing microorganism found in freshwater culture ponds (Chen et al. 2010; Sun et al. 2016). Bacteria of the genus *Shewanella* are normal components of surface floral of fish and have been implicated in fish spoilage (Adams and Moss 2008). Bacteria in the family Burkholderiaceae can be found in a wide variety

of niches, from soil and water to close associations with plants and animals (Coenye 2014). They also have a wide variety of functions including nitrogen fixation but many are highly pathogenic (Coenye 2014). We were not able to identify members of this family to the species level in this study but none of the genera identified include those of common pathogens. Members of the genus *Cellvibrio* are mainly cellulolytic organisms that produce xylanases and other hydrolases that break down cellulose and other polysaccharides (Wu and He 2015, Xie et al 2017). Of the members identified to the species level in this study within the genus *Pseudomonas*, *P. peli* is a nitrifying bacterium that has been found associated with aquaculture ponds, tanks, and aquaria (Vanparys et al 2006). Based on this, it is not unfounded that this organism was identified in this study. The genus *Enterovibrio* also includes species that have been found associated with fishes. One of these (not identified in the current study) has been isolated from the gut of larval turbot (*Scophthalmus maximus*; Thompson et al. 2002). This species *E. norvegicus*, was found in the guts of healthy cultured fish in Norway and may improve larval turbot survival and growth.

Alpha diversity measures are indices of the diversity within a community. The Observed OTUs index is commonly used to estimate the operational taxonomic unit (OTU) richness within a community and is based on the amount of unique OTUs in each sample. Alpha diversity and rarefaction curves were determined for all treatments evaluated. The treatment initially fed on a restricted diet for 14 days followed by the restricted growout diet had the highest measure of OTU richness of all treatments measured (Figure 8). There was no difference in OTU richness between fish fed high or restricted protein starter diets for 14 days. However, fish that had been fed the 48% CP diet initially showed a large decrease in OTU richness after 7 days on the growout diet, regardless of the protein content, while those fed the restricted 25% CP diet retained high OTU richness on both the 25% and 38% CP growout diets (Figure 8). Beta diversity, as illustrated by Principle Coordinates Analyses (PCoA) plots, is a measure of the microbial diversity between treatments. Our analyses indicate no differences in sample microbial communities between treatments (Figure 9). Principal Coordinate 1 explained 15.60% of the variability between samples, while principal coordinate 2 explained 14.15% of the variability.

There were 45 novel bacterial OTUs (15.7%) found in the fish fed the 25% protein restricted diet for 14 days compared to the fish fed the 48% protein diet for 14 days, and 124 novel bacterial OTUs (43.2%) found in the fish fed the 48% protein initial diet for 14 days versus those fed the 25% protein restricted initial diet for 14 days. Together they shared 118 (41.1%) of their bacterial OTUs (Figure 10). Many of these novel organisms were only identified to the family or genus taxonomic level so a complete idea of the roles these microbes may play in the tilapia fry is unknown. At the time of sampling (14 days after the start of the study), the fish fed the 25% protein diet were 0.093±0.091 g and 14.04±4.32 mm TL while the fish fed the 48% protein diet were 0.079±0.031 g and 13.87±2.89 mm TL (Figures 3, 4). Two organisms of notable significance and concern preliminarily identified in the analysis of the fry fed the 25% protein diet but not the 48% protein diet and also in the baseline samples were *Mycobacterium sp*. and *Flavobacterium columnare*. Both of these species are highly pathogenic in fishes and tend to proliferate in infected culture systems. Mycobacteriosis is common particularly in intensive aquaculture systems. It causes chronic infections leading to weight loss, scale loss, ulcers, and occasionally deep hemorrhagic skin lesions and is known to lead to zoonotic infections (Francis-Floyd 2011, Gauthier and Rhodes 2009). *F. columnare* is the causative agent of columnaris disease in fish. It is a serious disease of freshwater fishes. It is a highly pathogenic organism with infected fish showing symptoms of skin and fin erosion and lesions and gill necrosis, often leading to mortalities (Declercq et al. 2013). In our studies neither of these organisms were identified in any of the later samples.

CONCLUSION

It is estimated that 60-80% of total production costs for culturing tilapia is attributable to feeds, with high quality protein being the most costly ingredient. This study is the first to demonstrate that providing a reduced protein diet for the first 14 days post-yolk sac absorption can lead to enhanced growth later in life when fingerling tilapia are cultured in tank systems. A transcriptome analysis of tilapia intestinal tissue

showed differential expression of genes involved in proteolysis and muscle function between groups receiving different levels of protein which may indicate compensatory mechanisms for maintaining function during protein restriction or enhancing muscle synthesis when dietary protein levels are increased. We also observed an increased species diversity of microbes in fish fed a protein-restricted diet which may enhance the overall health of the animals. Despite a robust nutritional conditioning response seen in tank trials, early protein restriction was ineffective in enhancing growth of fingerlings in pond culture, likely due to the abundance of natural protein sources within the ponds (eg. plankton, algae, etc.) and inadequate early protein restriction. Future studies should test whether early restriction of protein under more controlled conditions in hatcheries yields greater growth rates and yield of tilapia during growout in ponds. Nonetheless, our results suggest that it may still be possible to reduce feed costs for fish farmers in Bangladesh by reducing the protein content of the diet during the growout phase rather than immediately following yolk-sac absorption as the treatment group provided with a 40% CP diet followed by a 20% CP diet had the greatest growth, production, and net returns relative to all other treatments.

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TABLES AND FIGURES

Table 4. Water quality parameters (mean ± SD) for the pond trials in Study 1. Values with different letters are significantly different (One-way ANOVA; $P < 0.05$). TDS = total dissolved solids.

	Treatment 1	***Treatment 2***	***Treatment 3***	***Treatment 4***
Temperature (°C)	28.08±2.58	28.05±2.54	28.40±2.03	28.28±1.90
Transparency (cm)	11.58±0.96^{a}	14.58±1.67^{b}	12.83±0.58^{c}	13.53±0.56^{d}
TDS (mg/l)	58.10±7.60^{a}	61.65±6.50^{a}	67.94±21.57^{a}	82.26±20.43^{b}
Alkalinity (mg/l)	45.88±7.70	48.15±7.87	46.30±5.02	49.76±3.95
pH	8.46±1.31	8.25±1.23	8.57±1.22	8.57±1.32
Dissolved Oxygen (mg/l)	5.95±1.18^{a}	5.22±1.06^{b}	5.19±1.04^{b}	5.45±1.32^{ab}
Nitrate (mg/l)	0.02±0.01^{a}	0.02±0.01^{a}	0.03±0.01^{b}	0.02±0.01^{a}
Nitrite (mg/l)	0.033±0.01^{a}	0.05±0.02^{b}	0.03±0.02^{a}	0.03±0.02^{a}
Ammonia (mg/l)	0.55±0.33	0.49±0.29	0.42±0.33	0.49±0.18
Phosphate (mg/l)	2.05±0.55^{a}	1.52±0.29^{b}	1.60±0.36^{b}	1.58±0.36^{b}
Chlorophyll-a (µg/l)	210.8±20.3^{a}	171.8±13.8^{b}	191.6±13.6^{c}	181.4±12.8^{d}

Table 5. Growth and production yield (mean ± SD) of tilapia (*O. niloticus*) for the pond trials in Study 1. Values with different letters are significantly different (One-way ANOVA; $P < 0.05$). SGR = specific growth rate; FCR = feed conversion ratio.

	Treatment 1 20%:20% CP	***Treatment 2 20:40% CP***	***Treatment 3 40:20% CP***	***Treatment 4 40:40% CP***
Initial Weight (g)	0.24 ± 0.00	0.24 ± 0.00	0.24 ± 0.00	0.24 ± 0.00
Final Weight (g)	50.4 ± 4.62^{a}	61.6 ± 3.86^{b}	120.0 ± 5.14^{c}	81.5 ± 5.10^{d}
Weight Gain (g)	50.2 ± 4.62^{a}	61.4 ± 3.86^{b}	120.1 ± 5.14^{c}	81.3 ± 5.10^{d}
SGR (%/day)	1.50 ± 0.05^{a}	1.55 ± 0.08^{b}	1.67 ± 0.05^{c}	1.48 ± 0.05^{a}
FCR	0.58 ± 0.01^{a}	0.45 ± 0.01^{b}	0.38 ± 0.01^{c}	0.48 ± 0.01^{d}
Survival Rate (%)	90.5 ± 1.31^{a}	92.6 ± 1.53^{a}	97.7 ± 1.05^{b}	93.3 ± 1.47^{a}
Net Yield (kg/ha)	8424 ± 263^{a}	10095 ± 268^{b}	11075 ± 251^{c}	10525 ± 257^{d}
Total Yield (kg/ha)	8900 ± 263^{a}	10571 ± 269^{b}	11500 ± 251^{c}	10950 ± 257^{d}

Table 6. Economic analysis of tilapia (*O. niloticus*) raised in ponds (Study 1). Values with different letters are significantly different (One-way ANOVA; $P < 0.05$).

	Treatment 1	***Treatment 2***	***Treatment 3***	***Treatment 4***
Expenditure (BDT/pond)				
Fingerlings	30,875	30,875	30,875	30,875
Feed	348,888[a]	486,111[b]	486,111[b]	523,333[c]
Lime	7,547	7,547	7,547	7,547
Fertilizers	24,013	24,013	24,013	24,013
Pond Lease	28,816	28,816	28,816	28,816
Labour	10,000	10,000	10,000	10,000
Operational Cost*	33,760	44, 052	44, 052	46,844
Total Expenditure	483,899	631,414	631,414	671,428
Income				
Gross Return (BDT/pond)				
Net Return (BDT/pond)				
Gross Return (BDT/ha)	712,000[a]	845,686[b]	920,000[c]	876,000[d]
Net Return (BDT/ha)	228,101	214,272	288,586	204,572
BCR (Benefit Cost Ratio)	1.47	1.34	1.46	1.30

*Operational cost is considered as 7.5% of total cost (ADCP, 1983).

Table 7. Significantly enriched (p<0.10) gene ontology (GO) biological processes and Kegg pathways associated with human homologues of genes that were significantly differentially expressed (FDR p-value <0.05) in the anterior intestine of fish fed a 48% protein diet for 14 days and a 25% protein diet for 42 days relative to the control fish (48% protein for 14 days, then 38% protein for 42 days)

Enriched GO Processes and Kegg Pathways 48% to 25% Protein			
GO Number	**Biological Process**	**P-Value**	**Differentially Expressed Genes**
GO:0006936	muscle contraction	0.0044319	DES, ACTA1, MYH11, MYLPF
GO:0030049	muscle filament sliding	0.0061573	DES, ACTA1, MYL3
GO:0006955	immune response	0.0095619	CCL3, CHIA, MYLPF, IL2RG, MR1, HLA-F
GO:0006508	proteolysis	0.0188999	KLK2, MEP1B, CAPN2, CAPN3, TMPRSS15, CAPN1
GO:0030259	lipid glycosylation	0.0214804	B4GALNT2, B4GALNT1
GO:0022617	extracellular matrix disassembly	0.0231663	KLK2, CAPN2, CAPN1
GO:0002674	negative regulation of acute inflammatory response	0.0245119	APCS, INS
GO:0006486	protein glycosylation	0.0478591	ST3GAL1, B4GALNT2, B4GALNT1
GO:0001574	ganglioside biosynthetic process	0.0543244	ST3GAL1, B4GALNT1
GO:0045861	negative regulation of proteolysis	0.0630925	KNG1, INS
GO:0051092	positive regulation of NF-kappaB transcription factor activity	0.0638669	CARD14, INS, CAPN3
GO:0055010	ventricular cardiac muscle tissue morphogenesis	0.0746595	MYL3, PKP2
GO:0002474	antigen processing and presentation of peptide antigen via MHC class I	0.0889214	MR1, HLA-F
KEGG Number	**Pathway**	**P-Value**	**Differentially Expressed Genes**
hsa00604	Glycosphingolipid biosynthesis - ganglio series	0.061196272	ST3GAL1, B4GALNT1

Table 8. Significantly enriched (p<0.10) gene ontology (GO) biological processes and Kegg pathways associated with human homologues of genes that were significantly differentially expressed (FDR p-value <0.05) in the anterior intestine of fish fed a 25% protein diet for 14 days and a 38% protein diet for 42 days relative to the control fish (48% protein for 14 days, then 38% protein for 42 days)

Enriched GO Processes and Kegg Pathways 25% to 38% Protein			
GO Number	**Biological Process**	**P-Value**	**Differentially Expressed Genes**
GO:0030049	muscle filament sliding	0.0000004	TNNT3, DES, ACTA1, MYL3, MYH7
GO:0006936	muscle contraction	0.0006582	DES, ACTA1, MYLPF, MYH7
GO:0006942	regulation of striated muscle contraction	0.0143819	TNNT3, MYL3
GO:0006955	immune response	0.0293124	CHIA, MYLPF, MR1, HLA-F
GO:0002026	regulation of the force of heart contraction	0.0301281	MYL3, MYH7
GO:0048545	response to steroid hormone	0.0316893	ACTA1, SST
GO:0045861	negative regulation of proteolysis	0.0332481	INS, CSTB
GO:0003009	skeletal muscle contraction	0.0379101	TNNT3, MYH7
GO:0055010	ventricular cardiac muscle tissue morphogenesis	0.0394592	MYL3, MYH7
GO:0032148	activation of protein kinase B activity	0.0410060	INS, TXN
GO:0002474	antigen processing and presentation of peptide antigen via MHC class I	0.0471691	MR1, HLA-F
GO:0007267	cell-cell signaling	0.0624496	INS, TXN, SST
GO:0060048	cardiac muscle contraction	0.0699428	MYL3, MYH7
GO:0007519	skeletal muscle tissue development	0.0789048	MYL3, MYLPF
GO:0007586	digestion	0.0965798	CHIA, SST
KEGG Number	**Pathway**	**P-Value**	**Differentially Expressed Genes**
hsa04530	Tight junction	0.0263817	CLDN4, MYLPF, MYH7
hsa04940	Type I diabetes mellitus	0.0762614	INS, HLA-F

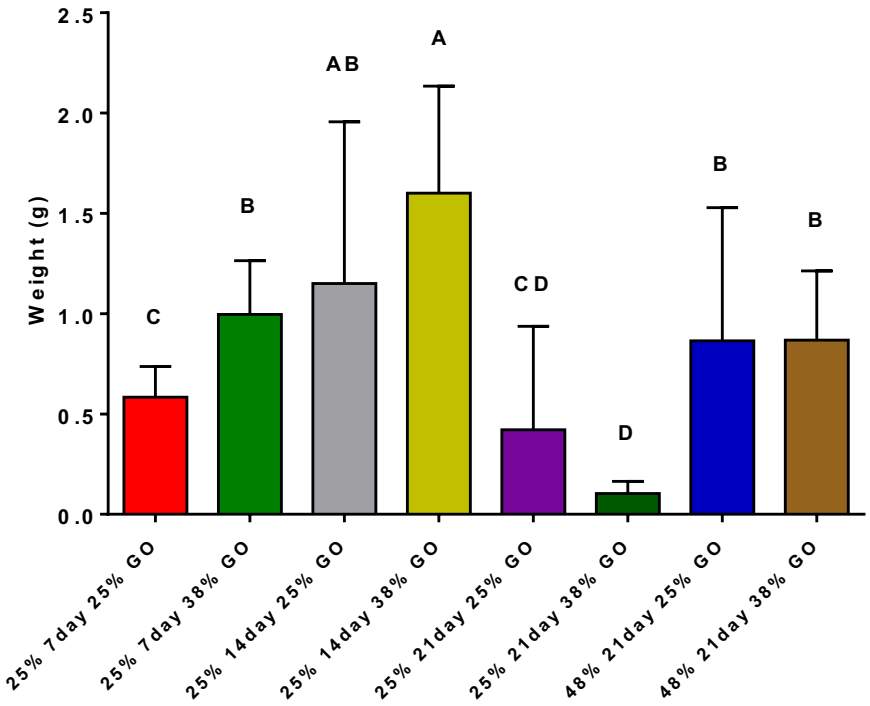

Figure 3. Final weights of Nile tilapia (*O. niloticus*) after 56 days culture in tank trials from Study 1. Data are means ± SD. Values with different letters are significantly different (One-way ANOVA; $P < 0.05$; GO, growout).

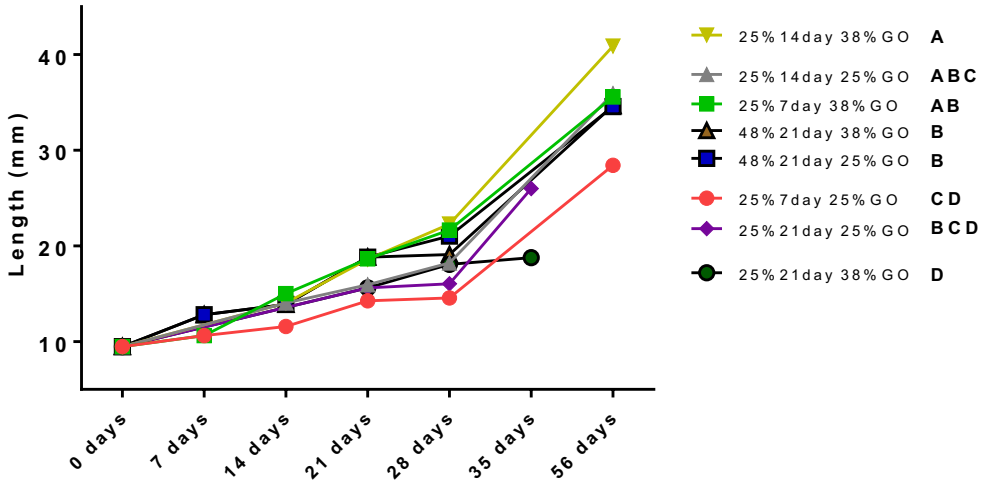

Figure 4. Mean lengths of Nile tilapia (*O. niloticus*) over the 56-day culture period for the tank trials in Study 1. Values with different letters are significantly different (Two-way ANOVA; $P < 0.05$). Error bars have been excluded for clarity.

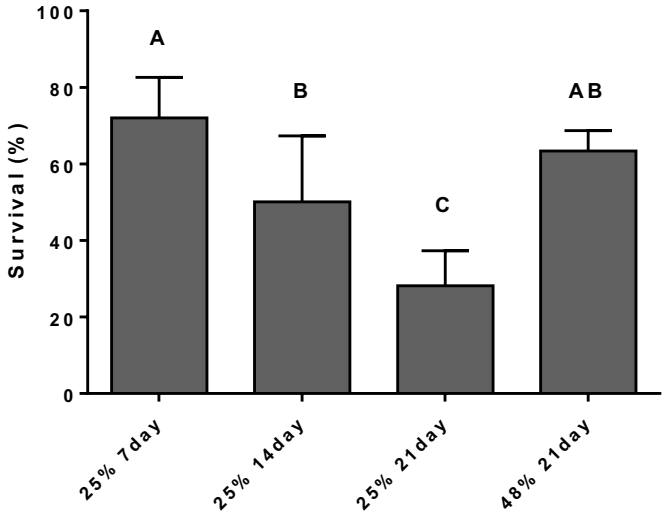

Figure 5. Survival rates (%) of Nile tilapia (*O. niloticus*) after 56 days in tank trials from Study 1. Data are means ± SD. Values with different letters are significantly different (One-way ANOVA; $P < 0.05$).

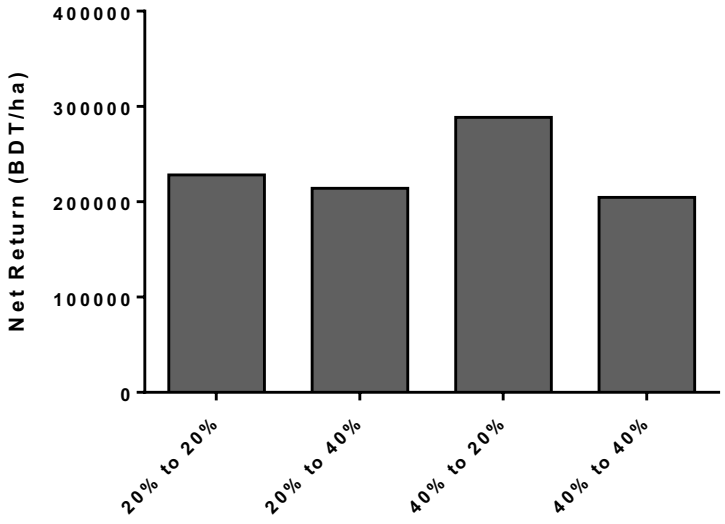

Figure 6. Net returns (BDT/ha) for Nile tilapia (*O. niloticus*) raised in ponds for Study 1.

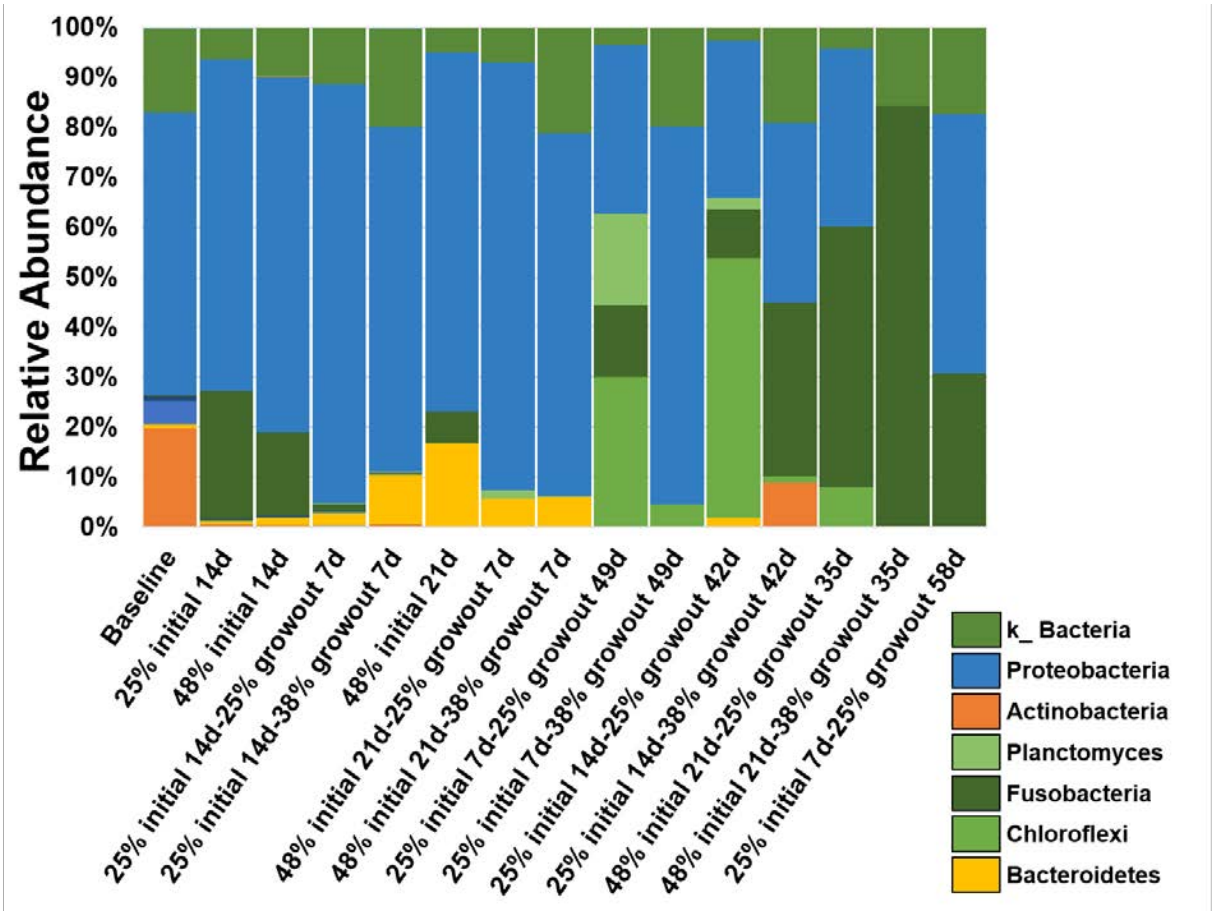

Figure 7. Relative abundance of phyla identified from Nile tilapia fry after feeding with diets containing restricted or normal protein levels. k_Bacteria = Kingdom Bacteria; these OTUs could not be identified beyond the Kingdom taxonomic level.

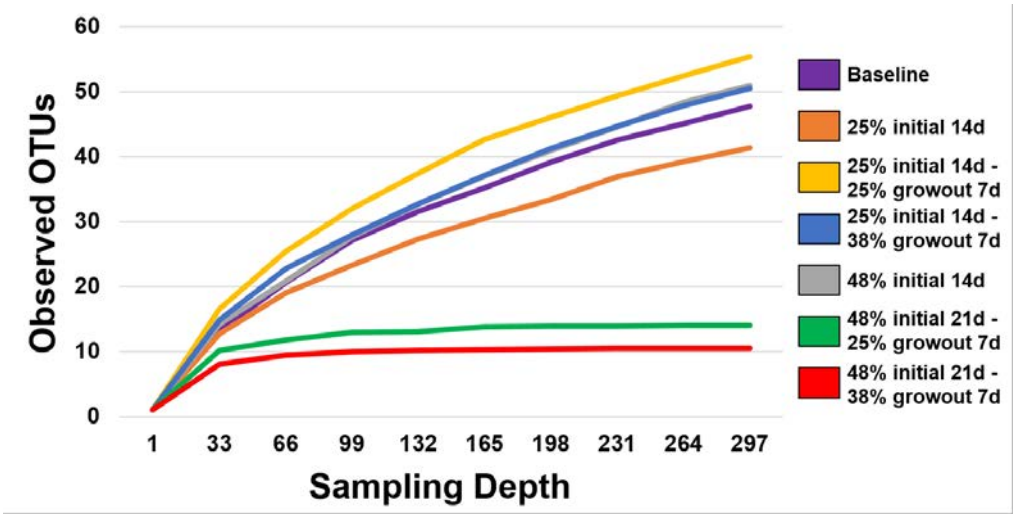

Figure 8. Alpha rarefaction plot to estimate species richness within bacterial communities found in Nile tilapia fry fed varying protein level diets.

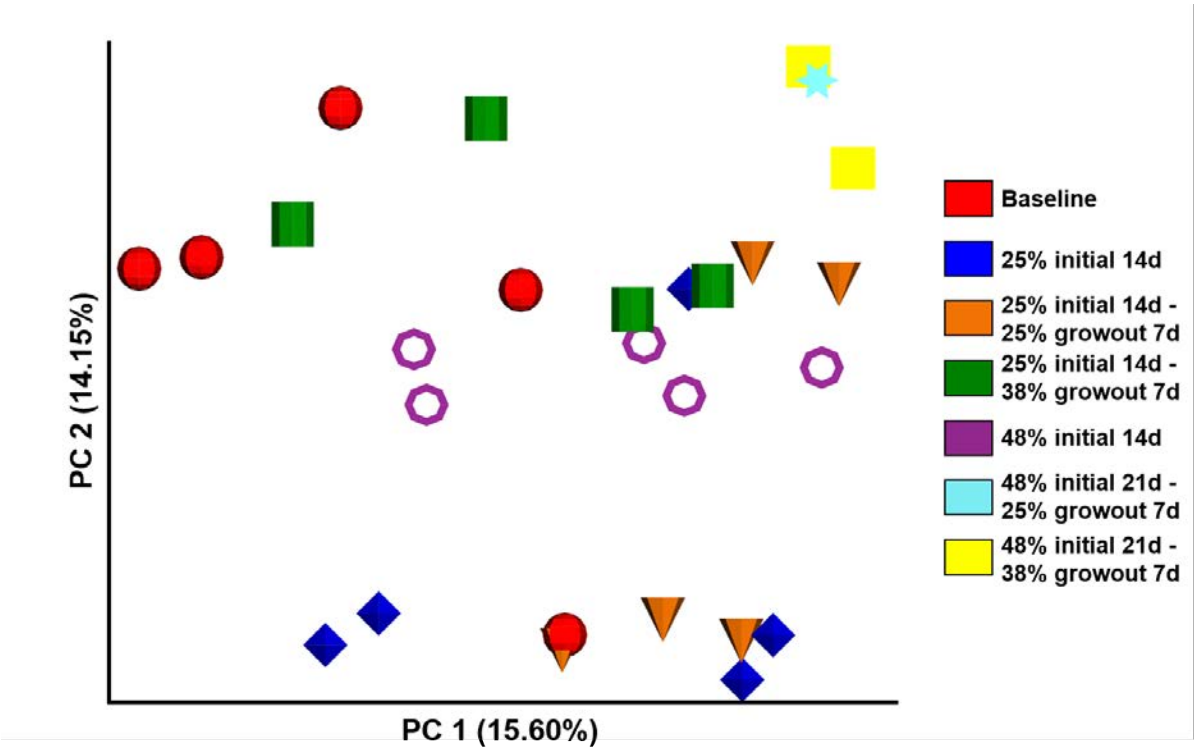

Figure 9. Beta diversity analysis of pair-wise differences in microbial communities in Nile tilapia fry fed varying protein level diets.

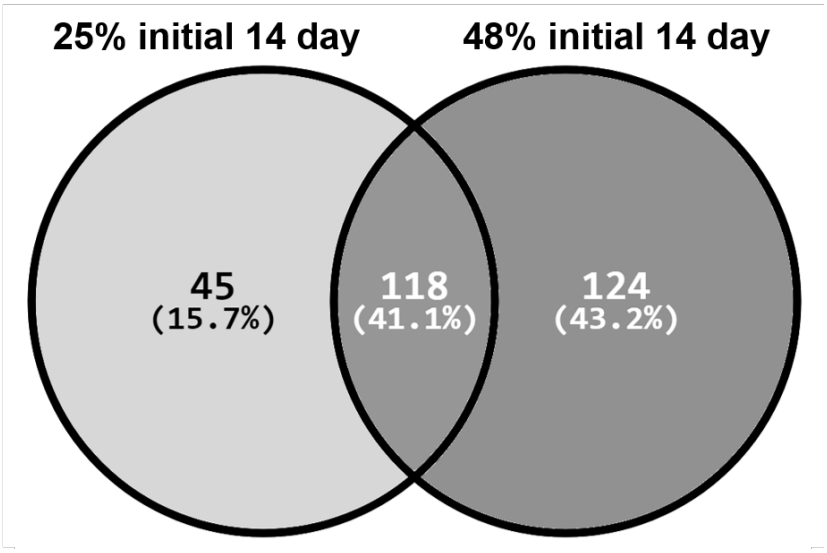

Figure 10. The numbers of novel OTUs identified when comparing Nile tilapia fry fed a 25% protein initial diet for 14 days and those fed a 48% protein initial diet for 14 days.