Pulsed Feeding Strategies to Improve Growth Performance, Gastrointestinal Nutrient Absorption Efficiency, and Establishment of Beneficial Gut Flora for Tilapia Pond Culture

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ABSTRACT

Feed constitutes 60%–80% of total production costs of tilapia (*Oreochromis* spp.). Reductions in quantity of feed used for fish growout and in the cost of formulated feeds are two approaches to containing feed costs. We evaluated pulsed-feeding strategies for improving production efficiency of Nile tilapia (*Oreochromis niloticus*). In a 12-week pond trial, fish were fed daily, every other day, every third day, or not at all. In all groups ponds were fertilized weekly to enhance natural foods. In the other group fish were fed daily without weekly pond fertilization. Fish fed daily with or without pond fertilization and fish fed every other day along with pond fertilization had greater weight gain, higher specific growth rates and survivability, and better net production than the other two treatments. There was no difference in feed conversion ratio in fish from ponds fed daily whether the ponds were fertilized, but feed efficiency was substantially improved in fish fed every other day. The benefit to cost ratio was highest for treatments fed in a pulsatile manner (i.e. fed every other day or every third day) with fish fed on alternate days providing the best net return among all groups. To determine if pulsed-feeding regimes affected nutrient uptake in the gut, gene expression of key solute-linked nutrient carriers was analyzed that included those for amino acids, fatty acids and simple carbohydrates (sugars). There were no significant differences in gene expression of these nutrient transporters in any treatment that incorporated both feeding and pond fertilization. Expression of the transporters was higher in those fish that were not fed and grown in fertilized ponds only and lower in fish that were fed daily without pond fertilization. Fish on alternate day feeding had more moderate expression levels of certain transporters which may allow for a more balanced and efficient nutrient uptake. Metagenomic analyses identified 145 different families of prokaryotic (all bacteria) and 132 genera of eukaryotic organisms in the fecal material of tilapia. The highest diversity of prokaryotes was found in fish fed either daily or every other day in fertilized ponds and the highest diversity of eukaryotes was found in fish fed every other day. Taken together, these studies along with those previously shown in the Philippines indicates feeding Nile tilapia on alternate days along with weekly pond fertilization has no deleterious effects on growth, survivability, or production versus daily feeding regimes and produces the greatest net return on investments. By feeding on alternate days, feed costs can be reduced by half without any decrease in production of farmed Nile tilapia. Our studies also suggest for the first time that combined feeding and fertilization produces the greatest biodiversity of microbiomes in the intestine that could contribute to enhanced feed efficiency and overall health of tilapia, particularly those subjected to more moderate feeding strategies.

INTRODUCTION

Global production of farmed Nile tilapia (*Oreochromis niloticus*) has increased exponentially since 1985, with more than 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 (Gupta et al. 1992, Hussain 2009). Currently, small-scale farmers often use extensive or improved extensive agricultural practices, a process where fertilizer is added (to stimulate pond primary production) but no feeds are used (Belton et al. 2011). As the addition of even modest amounts of feed (semi-intensive) can effectively quadruple production, the promotion of semi-intensive farming practices is a key target for increasing personal household income and fish consumption, and greater food security for impoverished farmers in Bangladesh (Belton et al. 2011, Dey et al. 2008). A significant hurdle for the implementation of semi-intensive farming is the cost of feed, comprising up to 50%–80% of total costs. Further, as local feed formulations often have low protein content, farmers compensate by overfeeding their fish, leading to poor water quality (Phillips 2013, USAID 2012). This study addressed these issues by demonstrating that equivalent production yields can be achieved with much less feed (50% reduction), through the implementation of pulsed feeding strategies, thereby reducing feed and labor costs and making the prospect of switching to semi-intensive culture more attractive to local farmers. Additionally, examined how alternate-day feeding strategies may enhance nutrient absorption by measuring nutrient transporter abundance and gut microbial diversity in response to different feeding regimes.

A better understanding of how finfish acquire and utilize nutrient inputs is requisite for future improvements in aquaculture production efficiency. Part of this investigation sought to further determine how intestinal nutrient absorption and gut microbial diversity change in response to the use of alternate feeding strategies, which have previously led to dramatic improvements in feed efficiency. Currently, the underlying mechanism explaining how alternate-day (pulsatile) feeding strategies can achieve equivalent production yields with less 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. Thus, fish being fed a daily regime have lower uptake efficiency and do not receive maximal dietary benefit. A similar phenomenon has been postulated, in part, to explain the compensatory growth (CG) response observed in some aquaculture species (Farmanfarmaian and Sun 1999, Picha et al. 2006). Additionally, reduced feeding may promote foraging on primary production within the ponds, leading to a more diverse diet (e.g., algae, insect larvae, plankton), enhancing nutrient recycling within the ponds, also may also directly influence intestinal absorption by promoting increases in nutrient transporters not utilized by fish with a constant and predictable diet (Heikkinen et al. 2006, Sigiura et al. 2009). Using the alternate-day feeding experiment, we will evaluate the mRNA expression of key nutrient transporters (involved in protein, lipid, and phosphate uptake) in response to pulsed-feeding strategies. This analysis will further our understanding of how greater nutrient uptake efficiency may be achieved for greater optimization of feeding protocols in the future, generating potential benefits not only for rural farmers in Bangladesh, but could also improve both US and global tilapia farming practices. Testing reduced feeding frequency (every third day) could provide an additional level of cost savings beyond alternate-day feeding, a protocol that could be adapted to studies in Bangladesh and elsewhere.

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 aquacultured finfish, new research has shown that probiotic maintenance of beneficial gut flora can promote growth, greater nutrient availability, and better stock health (Nayak 2010, Welker and Lim 2011). Early studies in channel

catfish (*Ictalurus punctatus*) and carp (*Cyprinidae*) identified several limiting nutrients (e.g., biotin, pantothenic acid, vitamin B12), which are produced by intestinal microbes, but may be limiting in lesser-quality feeds (Robinson and Luvell 1978, Kashiwada and Teshima 1966). In tilapia, the bacterium *Virgibacillus pantothenticus* stimulates intestinal production of alkaline protease, an enzyme involved in the digestion of dietary protein (Thillaimaharani et al. 2012). Naturally occurring lactic acids strains (such as *Leuconostoc mesenteroides*), appear to inhibit colonization of known fish pathogens (*Vibrio* and *Mycobacterium* sp.) through stimulation of the immune system (Zappata 2013). Interestingly, proper intestinal flora in tilapia may also positively impact human health as natural flora innoculates could theoretically out-compete non-natural pathogenic microbes. In Nile tilapia cultured in Saudi Arabia, fecal coliform bacteria (*E. coli*) comprised up to 10% of gut microbiota, which could be passed on to consumers through improper storage and handling practices (Al-Harbi and Uddin 2003, Mandal et al. 2009). We will test whether the tilapia intestinal microbiome differs in composition with alternate-day feeding, and identify key microbial factors associated with increased nutrient uptake and utilization. Identification of beneficial microbes that improve nutrient uptake will benefit current research into the application of probiotic supplements for the further enhancement of nutrient uptake in finfish cultivars. These investigations will lay the framework toward development of probiotic supplements that can be incorporated into diet formulations for improving growth and nutrient absorption. Ultimately, this work may have significant effects on tilapia culture, whose global production exceeds 2.4 million metric tons. The intensive tilapia culture practiced in the U.S. may also benefit substantially from this work.

OBJECTIVES

- Evaluate the effectiveness of pulsed feeding on tilapia production yields in fertilized ponds;
- Identify changes in key amino acids, sugars and fatty acid transporters in the intestine that may be linked to improved nutrient uptake efficiency;
- Characterize changes in gut microbial communities in response to pulse-feeding strategies; and
- Identify changes in key microbial communities that may be associated with increased nutrient availability or production efficiency in tilapia and that could potentially be used as probiotic dietary supplements for enhancing nutrient uptake in fish.

MATERIALS AND METHODS

Study 1 — The effects of alternate feeding strategies on tilapia growth performance. This study design was composed of 5 treatment groups as shown in Table 1.

This investigation follows previous studies from the Philippines (Bolivar et al. 2006, Borski et al. 2011) where a 50% reduction in feeding frequency resulted in equivalent production yields. This study was reproduced at the Fisheries Field Laboratory, Bangladesh Agricultural University, Mymensingh, Bangladesh with additional reductions in feeding (every third day) utilized. All-male sex reversed Nile tilapia (~3.5 g) were stocked at 5 fish/m^2 in 16 ponds (0.1 ha, 4 replicates per treatment), with weekly pond fertilization at a rate of 28 kg N and 7 kg P/ha/week for all treatment groups. Fish were fed with formulated feed (CP Bangladesh, 30% crude protein) initially at 10% and then down to 3% body weight/day based on a standard tilapia feed schedule.

Growth (length and weight) was monitored at 2-week intervals (sub-sampling) over a twelve-week growing period. Feed rates were adjusted accordingly based on this biweekly sampling. Samples of tilapia anterior intestinal tissue and fecal material from the colon were collected for further analysis at NCSU (see Studies 2 and 3). Water temperature (°C), transparency (cm), pH and dissolved oxygen (mg l–1) were measured weekly and total alkalinity (mg l–1), ammonia-

nitrogen (mg l–1), nitrate-nitrogen (mg l–1), nitrite-nitrogen (mg l–1), phosphate-phosphorus (mg l–1) and chlorophyll *a* (µg l–1) were measured biweekly. Production parameters (FCR, growth rate, yield) were determined at the end of study. A marginal cost-return analysis was also conducted. Significant differences between treatment groups were determined by two-way ANOVA analysis using JMP (SAS Institute, Cary, NC).

To collect tilapia gastrointestinal samples, tilapia from all 20 ponds were sampled using a seine net. Five fish were randomly collected from each pond, anesthetized, and decapitated. Samples for gene expression analyses was taken from a 1 cm section of the anterior intestine 5 cm posterior of the duodenal bulb (stomach sphincster where intestine meets the stomach) and placed in RNALater. Tilapia fecal material was collected from the posterior intestine (colon) and placed in a vial containing buffer and bullet homogenized with a portable homogenizer.

Study 2 — Assessment of tilapia nutrient uptake efficiency in response to alternate feed strategies. From data obtained from the publicly available tilapia genome assembly (http://cichlid.umd.edu), we identified 6 candidate transporters putatively involved in the digestive transport of amino acids, dietary sugars, and lipids across the intestinal epithelium: (1) facilitated glucose/fructose transporter (*slc2a5*), (2) facilitated glucose transporter (*slc2a6*), (3) long-chain fatty acid transporter (*slc27a4*), (4) Na^+-amino acid transporter 2 (*slc38a2*), (5) Na^+-amino acid transporter 4 (*slc38a4*), and (6) large neutral amino acid transporter subunit 3 (*slc43a1*). Samples (n=12; N=60) of tilapia intestine were collected at week 12 of the growth trial (see Study 1).

Tilapia gut transporter mRNA expression was quantified using real-time quantitative PCR (qPCR) performed as previously described with a few exceptions (Tipsmark et al. 2008). Briefly, total RNA was isolated from the anterior intestine using Tri Reagent (Invitrogen, Carlsbad, CA). One microgram of the total RNA was used to synthesize cDNA using a High Capacity cDNA Synthesis kit (Applied Biosystems, Carlsbad, CA) following treatment with Turbo DNA-free (Ambion, Foster City, CA). RNA was quantified and checked for quality at each step using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE) and agarose gel electrophoresis, respectively. Gene expression (mRNA) of the different transporter forms was measured in the tilapia cDNA using SYBR Green chemistry. Transporter gene-specific primers (Integrated DNA Technologies, Inc., Coralville, IA) were designed using the IDT PrimerQuest Tool (Integrated DNA Technologies, Inc.). Optimization for appropriate annealing temperature, primer concentrations, and cycling parameters was performed using pooled cDNA from the above reverse transcription reactions. One hundred ng of starting total RNA was used for qPCR analysis with Brilliant II QPCR Master Mix (Agilent Technologies Inc., Clara, CA) containing 1.5 µM gene-specific primers. No template controls and no reverse transcription controls were incorporated into the assay. Pooled cDNA was used to produce the cDNA for creating the standard curves. All qPCR assays were run in triplicate wells on a CFX384 real-time PCR system (BioRad Laboratories Inc., Hercules, CA). Cycling conditions were: 1 cycle, 50°C for 2 min; 1 cycle, 95°C for 10 min; 40 cycles, 95°C for 15 s, 60°C for 1 min. Melting curve analysis was performed to determine primer specificity. Data was normalized to the starting total RNA concentration. Gene copy number was predicted by comparing the mean cycle threshold (Ct) to the serially diluted cDNA standard curve ($R^2 = 0.98$). The gene expression data were then normalized to the expression of 18S ribosomal RNA, whose levels were found to be similar across treatment groups. Significant differences between treatment groups were determined by 2-way ANOVA analysis using JMP (SAS Institute, Cary, NC).

Study 3 — Characterize changes in gut microbial communities in response to pulse-feeding strategies for identifying microbes that may be associated with increased nutrient availability and production efficiency in tilapia. Samples of tilapia fecal material were collected from the Fisheries Field Laboratory, Bangladesh Agricultural University field study (see Study 1) and analyzed at North Carolina State University. Samples were collected from fish following 12 weeks of growout, with samples from 2 fish per pond pooled together from 2 sample replicates (total of 4 fish/pond). Samples were take from all 4 replicate ponds for all treatment group (n = 8 pooled samples per treatment; N = 40). A 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 (pond) as a whole.

Ribosomal RNA extraction. Extraction of ribosomal RNA from tilapia fecal samples was performed using an XpeditionTM Soil/Fecal DNA MiniPrep 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 Xpedition 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 and eukaryotic 18S rRNA sequencing library preparation. Prokaryotic 16S and eukaryotic 18S 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) and the V9 region of 18S rRNA (Amaral-Zettler et al. 2009, Chariton et al. 2010) 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. A Nile tilapia-specific blocking primer was introduced during the 18S amplification step to reduce the amplification of tilapia 18S rRNA and increase the amplification of lower abundance eukaryotic rRNA present in the 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, 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, 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 a High Sensitivity DNA chip on the Agilent 2100 Bioanalyzer (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 1.9.1 toolkit (Caporaso et al. 2010). Briefly, the paired end reads were joined together and uclust (Edgar 2010) was used to search against Greengenes 13_8 reference database (DeSantis et al 2006) for 16S (prokaryote) analysis and SILVA release 119 reference database (Pruesse et al 2007, Quast et al 2013, Yilmaz et al. 2014) for 18S (eukaryote) analysis both filtered at 97% identity. Reads not matching a reference sequence were removed from analysis. OTUs were assigned based on a database hit of 97% or greater sequence identity and taxonomy was assigned against the appropriate 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 — The effects of alternate feeding strategies on tilapia growth performance. Results suggest that the optimal benefit: cost ratio and net return for tilapia production occurs when fish are fed on alternate days in fertilized ponds. Studies also suggest that tilapia production is similar with fish fed daily in non-fertilized ponds to those fish fed daily in fertilized ponds, suggesting there is no added benefit of fertilizing ponds when providing a full daily ration of formulated feeds. In previous studies with lower stocking densities tilapia production was similar with half satiation and pond fertilization as feeding alone to full satiation (Diana et al. 1994).

Water quality parameters for each treatment are provided in Table 2. Ammonia and nitrites were highest in the feed-plus-fertilization group and lowest in the fertilization alone group likely a result of the relative amounts of nitrogen input provided from feeds and/or fertilizers in these relative to the other groups. Nonetheless, these and all other water parameters were well within the range for suitable growth of tilapia.

There was no significant difference in weight gained, specific growth rate, survival and production of Nile tilapia between groups fed daily with (Treatment 1) or without (Treatment 5) pond fertilization and those fed on alternate days with pond fertilization (Treatment 2, Figure 1). These treatments had the highest fish growth and net production followed by those fish fed every third day (Treatment 3, Figures 2 and 3). Fish in the Treatment 4 ponds showed the worst growth of all groups.

The fish produced with pond fertilization alone (Treatment 4) had the lowest survival relative to the other groups. The feed conversion ratio (FCR) was lowest for Treatment 3 (group fed every third day with pond fertilization), followed by Treatment 2 (Figure 2). The worst FCRs were found for Treatments 1 and 5, both of which had greater fish feed inputs, but had similar growth to Treatment 2.

Economic analyses from this study suggest feeding on alternate days along with weekly pond fertilization (Treatment 2) gives the best return on investment (Table 4). Feed is the most costly aspect of fish farming, representing the majority of the total production costs for tilapia. Here we show it constitutes almost 80% of total variable expenditures. This is noteworthy as both Treatments 1 and 5, which were fed daily, had the highest expenditures in our study while Treatment 4 with only pond fertilization had the lowest cost input. Net return values were highest for Treatment 2, followed by Treatment 3 (Figure 2). Overall, Treatment 2 had the highest benefit:cost ratio (BCR) but was not significantly different from Treatments 3 or 4; all three of these treatments had significantly higher BCRs than Treatments 1 and 5.

Although the BCR for Treatment 2 was not significantly different from that of Treatments 3 and 4, the fish showed much better growth and total production was highest. Based on these results we conclude that feeding tilapia on alternate days in fertilized ponds provides for the best return on investment and has little impact on growth of fish compared with fish fed daily. Feed efficiency is improved by 100% with no loss in production yield.

Small-scale tilapia farmers in Bangladesh can see marked reduction in production costs due to decreased feed costs by following the alternate-day feeding strategies. These findings support previous CRSP work done in the Philippines (Bolivar et al. 2010, Borski et al. 2011) and suggests that alternate day feeding is likely a cost-effective strategy that can be used for semi-intensive culture throughout the world.

Study 2 — Assessment of tilapia nutrient uptake efficiency in response to alternate feed strategies. Pulsatile feeding strategies can achieve equivalent production yields with less feed, however the mechanisms are poorly understood. This investigation assessed if differences in feed efficiency observed with feeding strategies might be assoicated with altered expression of nutrient transporters in the gastrointestinal tract of Nile tilapia. Absorption of nutrients from feed is facilitated through membrane bound transporters located within enterocytes of the gastrointestinal lumen (Broer 2008, Titus 1991). We evaluated how alternate-feeding strategies may change the gene expression of key solute-linked nutrient carriers, whose abundance may impact nutrient absorption efficiency (Broer 2008). The mRNA expression of six intestinal nutrient transporters was measured via real-time PCR in the intestine of fish from the growth trial. The nutrient transporters evaluated were:

- Facilitated glucose/fructose transporter (*slc2a5*);
- Facilitated glucose transporter (*slc2a6*);
- Long-chain fatty acid transporter (*slc27a4*);
- Na+-amino acid transporter 2 (*slc38a2*);
- Na+-amino acid transporter 4 (*slc38a4*); and
- Large neutral amino acid transporter subunit 3 (*slc43a1*).

The efficiency of all real-time PCR reactions were determined to be between 90%–110% and the standard curve correlations were greater than 0.97 for all assays performed. Our resulting gene expression values were normalized to 18S gene expression (no significant difference between treatments was observed in the 18S gene).

Results indicate no significant difference in gene expression of the six nutrient transporters analyzed between the treatments with both feeding and pond fertilization (Treatments 1, 2, and 3) regardless of the frequency of food applied (Figure 4). There was, however, a significant difference in gene expression between Treatments 4 and 5, fertilization only and daily feeding only, respectively, for the transporters *slc2a5*, *slc2a6*, *slc27a4*, and *slc38a2*. For these transporters, gene expression was significantly higher in fish from Treatment 4 than Treatment 5. This could signify that the fish in Treatment 4 that were not fed commercial diet are not getting the nutrients necessary to maintain growth and healthy nutrition from natural flora and fauna boosted by fertilization application alone, while the commercial diet provided the necessary nutrients for these purposes. Indeed, the upregulation of gene expression for nutrient transporters may be induced by a lack of certain nutrients present. This upregulation may have the effect of preparing the intestinal cells for rapid uptake of nutrients once they become present (Diamond and Karasov 1987). Nutrient transporters in tilapia that are grown in ponds that are fertilized, therefore, may be upregulated due to lack of nutrients that would otherwise be available with commercial diets. Interestingly, the nutrient transporters were most downregulated with fish that

were raised in unfertilized ponds on a pelleted diet, suggesting they had adequate nutrition. Fish grown in ponds that are both fed and fertilized had an intermediate expression of certain solute transporters suggesting they may be more efficient at nutrient uptake and utilization, particularly when commercial feeds are only periodically available.

Gene expression of *slc38a2* increases in response to an absence of amino acids. This increase leads to the translation of the gene to make the SNAT2 protein which has been shown to the recovery of cell volume following amino acid starvation or hypertonic stress (Franchi-Gazzola et al 2004, Franchi-Gazzola et al 2006, Gaccioli et al 2006, Nardi et al 2015). In addition, amino acid transporters may serve as sensors of nutrient supplies to regulate expression of the transporters and thus nutrient uptake by the cells (Taylor 2014). The results show that gene expression of this gene is upregulated in Treatment 4 with only pond fertilization and lower in fed treatments regardless of fertilization. This signifies that natural biota increased through fertilization of ponds may lack the full complement of amino acids necessary for the intestine to maintain cellular osmolality. In this case again the *slc38a2* would be upregulated to improve the efficiency of absorbing amino acids under limiting conditions.

The results show tilapia subsisting on endemic flora and fauna alone (fertilization only group) have increased gene expression of nutrient transporters. This may signify their intestine is conditioned for enhanced uptake in a nutrient limiting environment. By providing a diet of fish feed along with fertilization, which leads to an intermediate nutrient transporter gene expression, intestinal cells may be more efficient at nutrient uptake and utilization.

Study 3 — Characterize changes in gut microbial communities in response to pulse-feeding strategies for identifying microbes that may be associated with increased nutrient availability and production efficiency in tilapia. This investigation assessed how the gut microbial flora is altered by feeding/fertilization strategies that could potentially identify microbes beneficial to tilapia growth and health. 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 715,725 reads (330,883 reads for 16S, 384,842 reads for 18S) were obtained following sequencing of the V3 to V5 regions of 16S rRNA (prokaryote) and the V9 region of 18S rRNA (eukaryote) after quality filtering of the reads.

Prokaryotic assessment. A total of 20 prokaryotic phyla, 43 classes, 92 orders, and 145 families were associated with the fecal material of tilapia used in this study (100% bacteria, no archea). The dominant phyla identified from these samples belonged to Fusobacteria (80.4%), Firmicutes (13.8%), Cyanobacteria (2.4%), Bacteroidetes (1.3%), and Proteobacteria (1.3%) (Figure 5a). The proportions of the identified microbes varied between treatments with an increase in the proportion of Fusobacteria (62.6% to 84.6%) and a decreasing proportion of Firmicutes (32.1% to 11.5%) with decreasing rates of feed along with fertilization (treatments 1 to 3). The fish fed a commercial diet only with no fertilization (treatment 5) had the highest proportion of Fusobacteria (90.8%) and lowest proportion of Firmicutes (6.5%) of all treatments. An increase in the relative abundance of Firmicutes is indicative of obesity in mammals and positively correlated with caloric intake in bony fishes (Jumpertz et al. 2011, Ley et al. 2006, Turnbaugh et al. 2009). The fact that we also saw a higher proportion of Firmicute bacteria in tilapia from treatments fed daily in fertilized ponds indicates that there may be an abundance of high-calorie food resources available to these fish.

Bacteria from the classes Fusobacteriaceae and Clostridiaceae were most abundant in the fecal material of Nile tilapia used in our study. These groups include many pathogenic strains that that they may cause disease with immunosuppression or injury to the gut epithelium (Olsen 2014, Stackebrandt 2014). *Cetobacterium somerae* (a Fusobacteriaceae) had the highest number of reads or abundance in our study with no apparent trend in regulation by feeding or fertilization (Figure 5b). This species has been isolated from the intestinal tract of cultured freshwater fish (Tsuchiya et al. 2008).

Alpha diversity measures are indices of the diversity within a community. The Chao 1 index is commonly used to estimate the total number of species within a community and is based on the number of rare OTUs in that community (Chao 1984). Alpha diversity and rarefaction curves were determined for all treatments evaluated. Treatment 1 had the highest Chao 1 diversity index of all treatments. The bacterial diversity in the fecal material in tilapia guts decreased with increasing times between feeding when paired with pond fertilization (Figure 6a). Although there was no significant difference in species richness between treatments due to sample variation within treatments ($P = 0.2555$), the trend was for those treatments given a greater variety (feed and fertilization) to have higher diversity. The Chao 1 index was highest for treatments 1 and 2 (994.6 ± 162.1 SEM and 942.3 ± 98.9 SEM, respectively). Treatments 3 (668.0 ± 136.6 SEM), 4 (685.4 ± 176.2 SEM), and 5 (692.9 ± 62.4 SEM) had similar diversity indices.

Beta diversity, as illustrated by Principle Coordinates Analyses (PCoA) plots, is a measure of the microbial diversity between treatments. Our results indicate that there are some community differences between the treatments in our study (Figure 7). Treatments 1 and 5 had the most similar profiles while treatment 4 was the most dissimilar.

There were 41 and 40 bacterial OTUs identified in treatment 1 that were not identified in treatments 4 (Table 5) and 5 (Table 6), respectively. In addition, there were 37 and 53 OTUs identified that were unique to Treatment 2 versus Treatments 4 (Table 7) and 5 (Table 8). As described in Study 1, Treatment 2, feeding on alternate days with pond fertilization, had the best benefit to cost ratio of all treatments. It can be assumed that those OTUs identified in Treatment 2 that were not identified in Treatment 4 (pond fertilization only) were due to the feed given to Treatment 2 and those OTUs identified in Treatment 2 that were not identified in Treatment 5 (feed only) were due to fertilization. Of the differences between Treatment 2 and Treatments 4 and 5 the bacterial strains *Methlyobacterium* sp. and *Methylobacterium hispanicum* were present in Treatment 2 but not Treatments 4 and 5. *Methylobacterium* sp. are found in a wide variety of environments including freshwater sources and can be associated with terrestrial and aquatic plants (Austin et al.1978, Yoshimura 1982, Corpe and Rheem 1989, Trotsenko et al. 2001, Lidstrom and Chistoserdova 2002). Other bacteria identified include common gut colonizers in many vertebrates, many of which can become pathogenic if there is an increase of that bacterial strain due to the animal having a depressed immune system. The identified unique species and strains of bacteria found in Treatments 1 and 2 (food from formulated diet and pond microbiota boosted by fertilization) versus Treatments 4 and 5 (fertilization only and fed only groups), may be promising candidates for isolation that may be beneficial to increased growth and feed efficiency and reduced mortality in tilapia. We are currently conducting deeper analyses to determine the function of these bacteria.

Eukaryotic assessment. A total of eight major eukaryotic groups and 132 genera were associated with the fecal material of tilapia used in this study. Treatment 2 (fed alternate days with pond fertilization) had the greatest number of reads for all groups (58,450) followed by Treatments 4 (fertilization only, 49,112), 1 (feed every day with fertilization, 38,378), 5 (feed every day only, 31,758), and 3 (feed every third day with fertilization, 25,752). The dominant groups identified

from these samples belonged to the Opisthokonta (Metazoans and Fungi) (34.8%), Archaeplastida (green plants and red algae) (34.2%), and SAR supergroup (Stramenopiles, Alveolates, and Rhizaria; Dinoflagellates, Diatoms, Oomycetes, etc.) (30.7%) (Figure 8a). There were no significant differences in abundance of particular eukaryotic groups among the treatments.

Class Chlorophyceae (green algae), Class Mediophycae (diatoms), Phylum Rotifera, and group Magnoliophyta (flowering plants) made up the highest proportion of identified eukaryotic organisms (Figure 8b). Treatments 1 and 3 had the highest proportions of rotifers (31% and 35%, respectively). The largest proportion of Magnoliophyta were in Treatments 2 (19%) and 3 (20%) and Chlorophycae were in Treatments 1 (27%) and 4 (24%), while Treatment 5 had the highest proportion of Mediophycae (21%) of any other treatments. However, there were no significant differences in abundance of eukaryotes among the treatment groups.

The most abundant identified groups overall include the rotifers (50,705 counts), green algae (34,755 counts), and flowering plants (19,951 counts). We used blocking primers to eliminate amplification of tilapia 18S that might come from the intestine and that would reduce our capacity to detect low abundant reads of other eukaryotes of interest in this studies. Previous studies have shown that without the use of blocking primers in the amplification step of the 18S DNA library preparation from eukaryotic sources, the source DNA overwhelms the amplification of lower abundant DNA present in the samples making analysis of overall eukaryotes impossible (Levine and Salger, not published). Teleost 18S made up a low proportion of identified OTUs (11%), in our samples, indicating the success of the blocking primers. The teleost 18S that was detected aligned with Atlantic Salmon (*Salmo salar*). Whether this was derived from the fishmeal in the diet fed to tilapia is uncertain.

Alpha and beta diversity measures were also determined for the 18S amplicons. Similar to the prokaryotic analyses there was no significant difference in the Chao 1 index between treatments ($p = 0.1298$). Although this was true, treatment 2 had the highest diversity (677.5 ± 93.8 SEM), followed by treatment 1 (593.7 ± 112.1 SEM), treatment 5 (483.7 ± 96.1 SEM), and treatment 4 (444.1 ± 88.0 SEM). Treatment 3 had the lowest diversity measure at 363.2 ± 43.3 SEM (Figure 6b). There was significant overlap in the PCoA plots between treatments. Treatments 2 and 4 were most different from all treatments showing that the eukaryotic communities of these two treatments were different from the other treatments (Figure 9).

CONCLUSION

It is estimated that 60-80% of total variable costs for growing tilapia is attributable to formulated feeds. This study demonstrates that alternate-day feeding reduces the costs of feeds for growout of tilapia by 50%, increases feed efficiency by almost 140%, and has little impact on growth, survival or yield of fish farmed in Bangladesh. These findings can be readily adopted by small-scale farmers in Bangladesh for enhancing growout of tilapia. Insofar as our work supports that of previous Collaborative Research Support Program research in the Philippines, the study suggests that the alternate-day feeding strategy can be practically applied anywhere in the world for improving the livelihood of tilapia farmers.

Our work also suggests that:

- Tilapia grown in fertilized ponds without supplemental feeds may be nutritionally impaired as key nutrient transporters in the gut are enhanced in preparation for increased uptake of solutes should food become available, a process that is mitigated when animals are provided supplemental feeds alone; and

- The intermediate expression of gut nutrient transporters in alternate-day fed tilapia may reflect a condition for most efficient uptake of nutrients from the gastrointestinal tract.

Finally, our work shows that fish on the alternate-day feeding strategy have the greatest diversity of intestinal microbiota that may function in promoting growth and nutrient assimilation in these fishes. The presence of unique species and strains of bacteria in tilapia on supplemental feeds may serve as promising candidates for isolation and development of probiotics beneficial to increase growth, feed efficiency and health in tilapia.

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TABLES AND FIGURES

Table 1. Experimental design of this study

Treatment / Factors	*Treatment 1*	*Treatment 2*	*Treatment 3*	*Treatment 4*	*Treatment 5*
Stocking Density	5 fish/ m^2	5 fish/ m^2	5 fish/ m^2	5 fish/ m^2	5 fish/ m^2
Feeding Strategy	daily feeding	feeding every other day	feeding every 3rd day	no feeding	daily feeding
Pond Fertilization	4:1 (N:P), weekly	4:1 (N:P), weekly	4:1 (N:P), weekly	4:1 (N:P), weekly	no fertilization
Replicates (n)	4	4	4	4	4

Table 2. Water quality parameters (mean ± SD) for all treatments.Values with different letters are significantly different ($P < 0.05$).

	Treatment 1	*Treatment 2*	*Treatment 3*	*Treatment 4*	*Treatment 5*
Temperature (°C)	30.11±0.01	30.15±0.02	30.17±0.10	30.10±0.15	30.07±0.08
Transparency (cm)	16.31±2.13	14.49±0.88	20.51±7.78	17.92±6.76	15.18±1.28
TDS (mg/l)	119.34±11.13	113.97±8.43	95.42±7.39	106.23±8.56	103.90±12.06
Alkalinity (mg/l)	127.70±7.44^{a}	128.95±9.61^{a}	107.00±6.37^{b}	119.55±4.10^{ab}	116.55±14.72^{ab}
pH	7.72±0.06^{b}	7.84±0.04^{ab}	7.75±0.10^{b}	7.95±0.11^{a}	7.82±0.11^{ab}
Dissolved Oxygen (mg/l)	5.64±0.30^{b}	5.96±0.13^{ab}	6.10±0.17^{a}	6.15±0.27^{a}	5.60±0.28^{b}
Nitrate (mg/l)	0.34±0.07^{b}	0.31±0.01^{b}	0.33±0.07^{b}	0.25±0.05^{b}	0.17±0.04^{a}
Nitrite (mg/l)	0.13±0.03^{ab}	0.13±0.04^{ab}	0.14±0.05^{ab}	0.16±0.05^{a}	0.08±0.05^{b}
Ammonia (mg/l)	0.83±0.36^{a}	0.52±0.19^{ab}	0.48±0.30^{ab}	0.38±0.12^{b}	0.48±0.12^{ab}
Phosphate (mg/l)	1.12±0.18^{b}	1.14±0.11^{b}	1.11±0.23^{b}	1.51±0.06^{a}	0.82±0.19^{c}
Chlorophyll *a* (µg/l)	123.74±22.24	116.20±36.21	109.62±62.39	91.49±28.28	129.40±21.42

Table 3. Growth and production performances of tilapia (*O. niloticus*). Values are mean±SD. Values with different letters are significantly different ($P < 0.05$).

Parameters	*Treatment 1*	*Treatment 2*	*Treatment 3*	*Treatment 4*	*Treatment 5*
Initial Weight (g)	3.55 ± 0.90^{a}	3.55 ± 0.90^{a}	3.55 ± 0.90^{a}	3.55 ± 0.90^{a}	3.55 ± 0.90^{a}
Final Weight (g)	127.63 ± 2.75^{a}	120.17 ± 5.44^{a}	85.10 ± 11.13^{b}	43.15 ± 4.28^{c}	129.53 ± 8.59^{a}
Weight Gain (g)	124.08 ± 2.75^{a}	116.62 ± 5.44^{a}	81.55 ± 11.13^{b}	39.60 ± 4.28^{c}	125.98 ± 8.59^{a}
SGR (%/day)	3.14 ± 0.02^{a}	3.09 ± 0.04^{a}	2.78 ± 0.12^{b}	2.19 ± 0.09^{c}	3.15 ± 0.06^{a}
FCR	1.64 ± 0.10^{c}	0.93 ± 0.09^{b}	0.68 ± 0.15^{a}	--	1.61 ± 0.10^{c}
Survival Rate (%)	93.44 ± 6.26^{a}	91.66 ± 8.00^{a}	90.70 ± 9.74^{ab}	76.79 ± 2.68^{b}	97.71 ± 2.11^{a}
Production (kg/pond)	92.98 ± 10.29^{ab}	86.40 ± 9.33^{b}	58.93 ± 7.17^{c}	27.50 ± 4.57^{d}	102.63 ± 10.66^{a}
Net Production (kg/ha)	6091.42 ± 354.52^{a}	5658.26 ± 527.83^{a}	4086.39 ± 640.07^{b}	1796.04 ± 233.89^{c}	6392.81 ± 461.41^{a}
Total Production (kg/ha)	6282.09 ± 354.52^{a}	5837.84 ± 527.83^{a}	4179.08 ± 640.07^{b}	1950.35 ± 233.89^{c}	6578.53 ± 461.41^{a}

Table 4. Economic analysis of tilapia (*O. niloticus*) production among five treatments. Leasing cost for pond is not included. Values are mean±SD. Values with different letters are significantly different ($P < 0.05$).

	Treatment 1	*Treatment 2*	*Treatment 3*	*Treatment 4*	*Treatment 5*
Expenditure (Tk/pond)					
Fingerlings cost	925.00±50.00	925.00±50.00	881.25±177.22	881.25±177.22	975.00±50.00
Feed cost	8,783.57±555.26^{b}	4,613.61±166.49^{c}	2,303.69±588.95^{d}	--	9,567.44±631.31^{a}
Lime Cost	44.40±2.40	44.40±2.40	42.30±8.51	42.30±8.51	46.80±2.40
Fertilizers cost	354.76±19.18	378.41±20.45	360.51±72.50	360.51±72.50	--
Operational cost*	758.08±46.15^{a}	447.11±17.42^{b}	269.08±63.36^{c}	96.30±19.37^{d}	794.19±50.48^{a}
Total Expenditure	10,865.81±661.50^{a}	6,408.52±249.71^{b}	3,856.83±908.16^{c}	1,380.36±277.59^{d}	11,383.44±723.59^{a}
Income					
Gross return (Tk/pond)	11,157.00±1,234.60^{ab}	10,368.00±1,120.01^{b}	5,892.50±716.82^{c}	1,925.00±319.76^{d}	12,315.00±1,279.39^{a}
Net return (Tk/pond)	291.19±691.31^{c}	3,959.48±979.04^{a}	2,035.67±629.63^{b}	544.64±197.83^{c}	931.56±763.10^{c}
Gross return (Tk/ha)	752,270.83±42,542.75^{ab}	700,291.67±63,339.67^{b}	426,388.89±64,007.44^{c}	138,147.92±16,372.06^{d}	788,437.50±55,369.36^{a}
Net return (Tk/ha)	18,041.18±43,978.87^{c}	266,987.40±62763.26^{a}	154,640.31±77,869.53^{b}	40,249.82±16,372.06^{c}	58,739.54±47,129.35^{c}
BCR (Benefit Cost Ratio)	1.03±0.06^{b}	1.62±0.14^{a}	1.58±0.33^{a}	1.41±0.17^{a}	1.08±0.07^{b}

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

Table 5. Prokaryotes (16S rRNA OTUs) identified in Treatment 1 but not Treatment 4.

k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;Other;Other
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;g__;s__
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;g__Rathayibacter;s__caricis
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micromonosporaceae;g__Actinoplanes;s__
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Mycobacteriaceae;g__Mycobacterium;s__
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__[Amoebophilaceae];g__Candidatus Cardinium;s__
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__Flavobacterium;s__
k__Bacteria;p__Chloroflexi;c__SHA-26;o__;f__;g__;s__
k__Bacteria;p__Cyanobacteria;c__Chloroplast;o__;f__;g__;s__
k__Bacteria;p__Cyanobacteria;c__Chloroplast;o__Chlorophyta;f__Chlamydomonadaceae;g__Chlamydomonas;s__reinhardtii
k__Bacteria;p__Cyanobacteria;c__Chloroplast;o__Chlorophyta;f__Trebouxiophyceae;g__;s__
k__Bacteria;p__Cyanobacteria;c__Nostocophycideae;o__Nostocales;f__Nostocaceae;g__Cylindrospermopsis;s__
k__Bacteria;p__Cyanobacteria;c__Oscillatoriophycideae;Other;Other;Other;Other
k__Bacteria;p__Cyanobacteria;c__Oscillatoriophycideae;o__Chroococcales;Other;Other;Other
k__Bacteria;p__Cyanobacteria;c__Oscillatoriophycideae;o__Oscillatoriales;f__Phormidiaceae;g__Microcoleus;s__
k__Bacteria;p__Cyanobacteria;c__Synechococcophycideae;o__Pseudanabaenales;Other;Other;Other
k__Bacteria;p__Fibrobacteres;c__TG3;o__TG3-1;f__TSCOR003-O20;g__;s__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__;g__;s__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Lactococcus;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__02d06;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridium;s__intestinale
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptostreptococcaceae;g__Clostridium;s__ruminantium
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Mogibacteriaceae];g__;s__
k__Bacteria;p__Planctomycetes;c__Planctomycetia;o__Planctomycetales;f__Planctomycetaceae;g__Planctomyces;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;Other;Other
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Devosia;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Pedomicrobium;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__Methylobacterium;s__hispanicum

Table 5. Continued

k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylocystaceae;g__Pleomorphomonas;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Anaerospora;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhodospirillaceae;g__Azospirillum;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Aeromonadales;f__Aeromonadaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Plesiomonas;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Plesiomonas;s__shigelloides
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Legionellales;f__Legionellaceae;Other;Other
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Legionellales;f__Legionellaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Legionellales;f__Legionellaceae;g__Legionella;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Methylococcales;f__Methylococcaceae;g__Methylomonas;s__
k__Bacteria;p__TM7;c__TM7-3;o__EW055;f__;g__;s__

Table 6. Prokaryotes (16S rRNA OTUs) identified in Treatment 1 but not Treatment 5.

k__Bacteria;p__Actinobacteria;c__Acidimicrobiia;o__Acidimicrobiales;f__C111;g__;s__
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micromonosporaceae;g__Actinoplanes;s__
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Mycobacteriaceae;g__Mycobacterium;Other
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__[Amoebophilaceae];g__Candidatus Cardinium;s__
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__Flavobacterium;s__
k__Bacteria;p__Chlamydiae;c__Chlamydiia;o__Chlamydiales;f__;g__;s__
k__Bacteria;p__Chloroflexi;c__Anaerolineae;o__WCHB1-50;f__;g__;s__
k__Bacteria;p__Cyanobacteria;c__Chloroplast;o__Chlorophyta;f__Chlamydomonadaceae;g__Chlamydomonas;s__reinhardtii
k__Bacteria;p__Cyanobacteria;c__Nostocophycideae;o__Nostocales;f__Nostocaceae;g__;s__
k__Bacteria;p__Cyanobacteria;c__Nostocophycideae;o__Nostocales;f__Nostocaceae;g__Cylindrospermopsis;s__
k__Bacteria;p__Cyanobacteria;c__Oscillatoriophycideae;Other;Other;Other;Other
k__Bacteria;p__Cyanobacteria;c__Oscillatoriophycideae;o__Chroococcales;Other;Other;Other
k__Bacteria;p__Cyanobacteria;c__Oscillatoriophycideae;o__Chroococcales;f__Cyanobacteriaceae;g__;s__
k__Bacteria;p__Cyanobacteria;c__Oscillatoriophycideae;o__Chroococcales;f__Gomphosphaeriaceae;Other;Other
k__Bacteria;p__Cyanobacteria;c__Synechococcophycideae;o__Pseudanabaenales;Other;Other;Other
k__Bacteria;p__Cyanobacteria;c__Synechococcophycideae;o__Pseudanabaenales;f__Pseudanabaenaceae;g__Leptolyngbya;s__
k__Bacteria;p__Cyanobacteria;c__Synechococcophycideae;o__Synechococcales;f__Synechococcaceae;g__Paulinella;s__
k__Bacteria;p__Fibrobacteres;c__TG3;o__TG3-1;f__TSCOR003-O20;g__;s__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__;g__;s__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Bacillus;s__flexus
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__02d06;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridium;s__intestinale
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptostreptococcaceae;g__Clostridium;s__ruminantium
k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Fusobacteriaceae;g__u114;s__
k__Bacteria;p__Nitrospirae;c__Nitrospira;o__Nitrospirales;f__[Thermodesulfovibrionaceae];g__HB118;s__
k__Bacteria;p__Planctomycetes;c__Planctomycetia;o__Planctomycetales;f__Planctomycetaceae;g__Planctomyces;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Pedomicrobium;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__;s__

Table 6. Continued

k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__Methylobacterium;s__hispanicum
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylocystaceae;g__Pleomorphomonas;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Rhodobacter;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhodospirillaceae;g__Azospirillum;s__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;Other;Other;Other;Other
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__;f__;g__;s__
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Syntrophobacterales;f__Syntrophobacteraceae;g__Syntrophobacter;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Legionellales;f__Legionellaceae;Other;Other
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Methylococcales;f__Methylococcaceae;g__Methylomonas;s__

Table 7. Prokaryotes (16S rRNA OTUs) identified in Treatment 2 but not Treatment 4.

k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micromonosporaceae;g__Actinoplanes;s__
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Nocardioidaceae;g__;s__
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;Other;Other
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__Blvii28;s__
k__Bacteria;p__Cyanobacteria;c__;o__;f__;g__;s__
k__Bacteria;p__Cyanobacteria;c__Chloroplast;o__;f__;g__;s__
k__Bacteria;p__Cyanobacteria;c__Chloroplast;o__Chlorophyta;f__Chlamydomonadaceae;g__Acutodesmus;s__obliquus
k__Bacteria;p__Cyanobacteria;c__Chloroplast;o__Chlorophyta;f__Trebouxiophyceae;g__;s__
k__Bacteria;p__Cyanobacteria;c__Nostocophycideae;o__Nostocales;f__Nostocaceae;g__Cylindrospermopsis;s__
k__Bacteria;p__Cyanobacteria;c__Oscillatoriophycideae;Other;Other;Other;Other
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__;g__;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__02d06;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridium;s__intestinale
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Blautia;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Mogibacteriaceae];g__;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Mogibacteriaceae];g__Anaerovorax;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Devosia;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__Methylobacterium;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__Methylobacterium;s__hispanicum
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylocystaceae;g__Pleomorphomonas;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Anaerospora;Other
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales;f__Rickettsiaceae;g__Wolbachia;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Sphingomonas;s__
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfobacterales;f__Desulfobacteraceae;g__Desulfococcus;s__
k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales;f__Campylobacteraceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Aeromonadales;f__Aeromonadaceae;g__;s__

Table 7. Continued

k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Plesiomonas;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Plesiomonas;s__shigelloides
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Legionellales;f__Coxiellaceae;g__Rickettsiella;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Methylococcales;f__Methylococcaceae;g__Methylomonas;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales;f__Vibrionaceae;g__Grimontia;s__hollisae
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales;f__Vibrionaceae;g__Photobacterium;Other
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales;f__Vibrionaceae;g__Photobacterium;s__rosenbergii
k__Bacteria;p__TM7;c__TM7-3;o__EW055;f__;g__;s__

Table 8. Prokaryotes (16S rRNA OTUs) identified in Treatment 2 but not Treatment 5.

k__Bacteria;p__Actinobacteria;c__Acidimicrobiia;o__Acidimicrobiales;f__EB1017;g__;s__
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;Other;Other;Other
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Frankiaceae;g__;s__
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micromonosporaceae;g__Actinoplanes;s__
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Nocardioidaceae;g__;s__
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;Other;Other
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;Other;Other
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Porphyromonas;s__
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__Blvii28;s__
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__SB-1;g__;s__
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__VC21_Bac22;g__;s__
k__Bacteria;p__Chlamydiae;c__Chlamydiia;o__Chlamydiales;f__;g__;s__
k__Bacteria;p__Chlamydiae;c__Chlamydiia;o__Chlamydiales;f__Rhabdochlamydiaceae;g__Candidatus Rhabdochlamydia;s__
k__Bacteria;p__Chloroflexi;c__Anaerolineae;o__;f__;g__;s__
k__Bacteria;p__Chloroflexi;c__Anaerolineae;o__SBR1031;f__A4b;g__;s__
k__Bacteria;p__Chloroflexi;c__Anaerolineae;o__WCHB1-50;f__;g__;s__
k__Bacteria;p__Cyanobacteria;c__Nostocophycideae;o__Nostocales;f__Nostocaceae;g__;s__
k__Bacteria;p__Cyanobacteria;c__Nostocophycideae;o__Nostocales;f__Nostocaceae;g__Cylindrospermopsis;s__
k__Bacteria;p__Cyanobacteria;c__Oscillatoriophycideae;Other;Other;Other;Other
k__Bacteria;p__Cyanobacteria;c__Oscillatoriophycideae;o__Chroococcales;f__Cyanobacteriaceae;g__Cyanobacterium;s__
k__Bacteria;p__Cyanobacteria;c__Oscillatoriophycideae;o__Chroococcales;f__Gomphosphaeriaceae;Other;Other
k__Bacteria;p__Cyanobacteria;c__Synechococcophycideae;o__Pseudanabaenales;f__Pseudanabaenaceae;g__;s__
k__Bacteria;p__Cyanobacteria;c__Synechococcophycideae;o__Pseudanabaenales;f__Pseudanabaenaceae;g__Leptolyngbya;s__
k__Bacteria;p__Cyanobacteria;c__Synechococcophycideae;o__Synechococcales;f__Synechococcaceae;g__Paulinella;s__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__;g__;s__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Bacillus;Other
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Bacillus;s__flexus
k__Bacteria;p__Firmicutes;c__Bacilli;o__Gemellales;f__Gemellaceae;g__;s__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__02d06;s__

Table 8. Continued

k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridium;s__intestinale
k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Fusobacteriaceae;g__u114;s__
k__Bacteria;p__Nitrospirae;c__Nitrospira;o__Nitrospirales;f__[Thermodesulfovibrionaceae];g__HB118;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__BD7-3;f__;g__;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__Methylobacterium;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__Methylobacterium;s__hispanicum
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylocystaceae;g__Pleomorphomonas;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Rhodobacter;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales;f__Rickettsiaceae;g__Wolbachia;s__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales;f__mitochondria;Other;Other
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Sphingomonas;s__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__;f__;g__;s__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__;f__;g__;s__
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Syntrophobacterales;f__Syntrophobacteraceae;g__Syntrophobacter;s__
k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales;f__Campylobacteraceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Legionellales;f__Coxiellaceae;g__Rickettsiella;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Methylococcales;f__Methylococcaceae;g__Methylomonas;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales;f__Pseudoalteromonadaceae;g__;s__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales;f__Vibrionaceae;g__Grimontia;s__hollisae

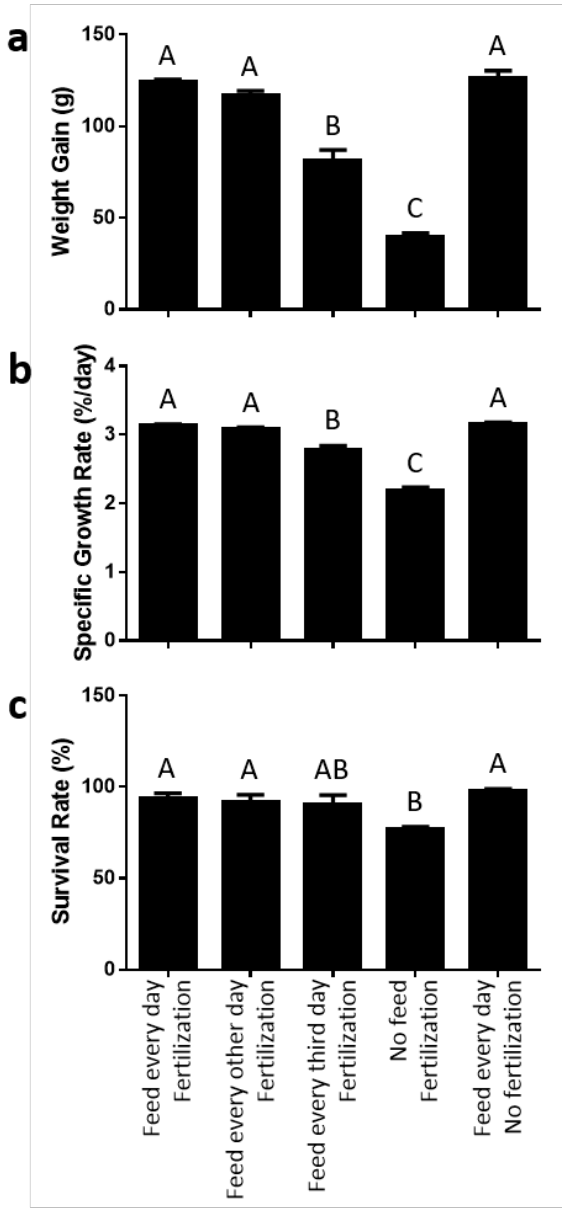

Figure 1. Differences in growth parameters of Nile tilapia in varying pulsed-feeding regimes (mean ± SEM). (a) Weight gain; (b) Specific growth rate (SGR); (c) Survival rate. Treatments with different letters are significantly different ($p < 0.05$).

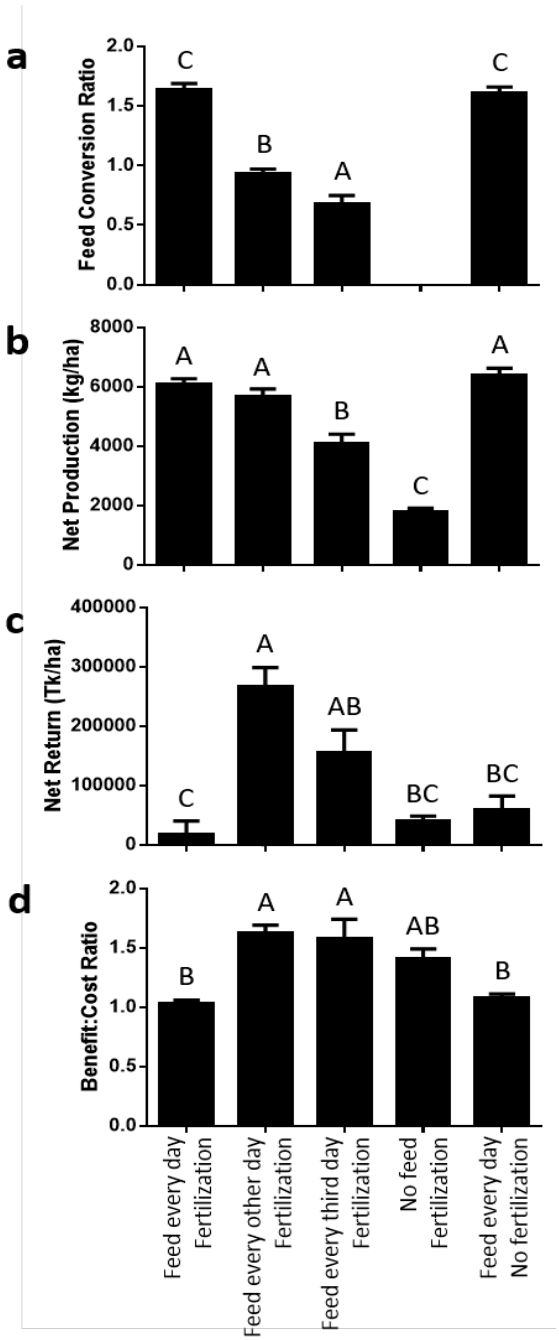

Figure 2. Differences in production parameters of Nile tilapia in varying pulsed-feeding regimes (mean ± SEM). (a) Feed conversion ratio (FCR); (b) Net production; (c) Net return; (d) Benefit to Cost ratio (BCR). Treatments with different letters are significantly different ($p < 0.05$).

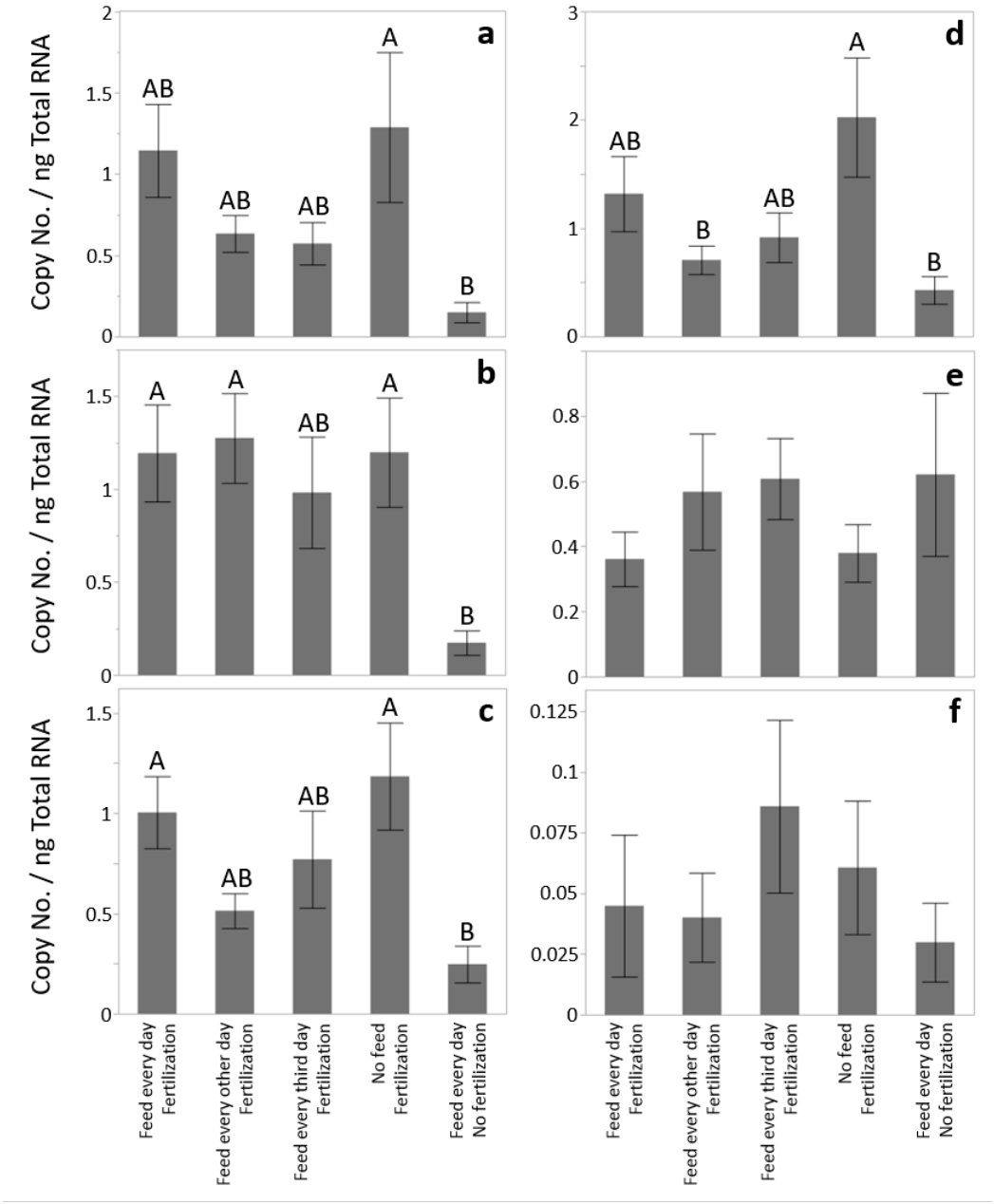

Figure 3. Change in weight (a) and length (b) of Nile tilapia in varying pulsed-feeding regimes throughout the study (mean ± SEM).

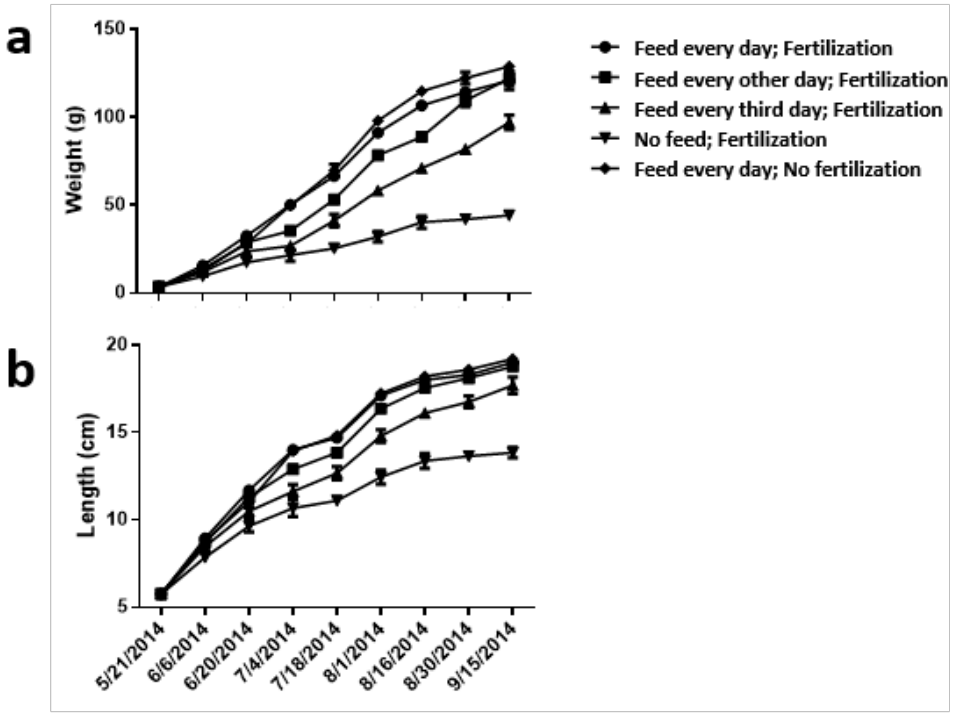

Figure 4. Gene expression of nutrient transporters in the intestine of Nile tilapia subjected to varying pulsed-feeding regimes (mean ± SEM). (a) *SLC2A5*; (b) *SLC2A6*; (c) *SLC27A4*; (d) *SLC38A2*; (e) *SLC38A4*; (f) *SLC43A1*. Treatments with different letters are significantly different ($p < 0.05$).

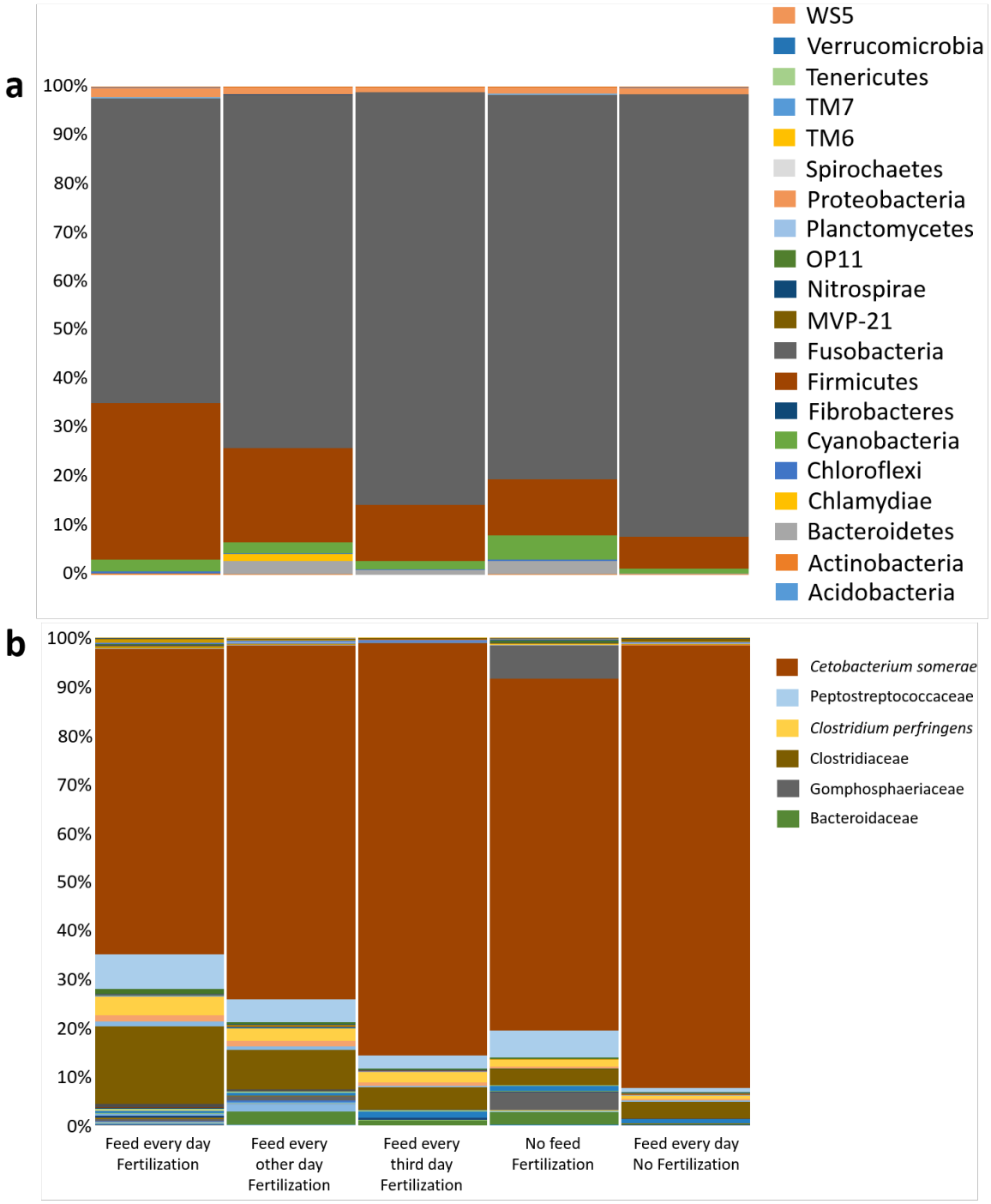

Figure 5. Relative abundance of bacteria in the fecal material of Nile tilapia subjected to varying pulsed-feeding regimes at the level of Phylum (a) and Family/Species (b).

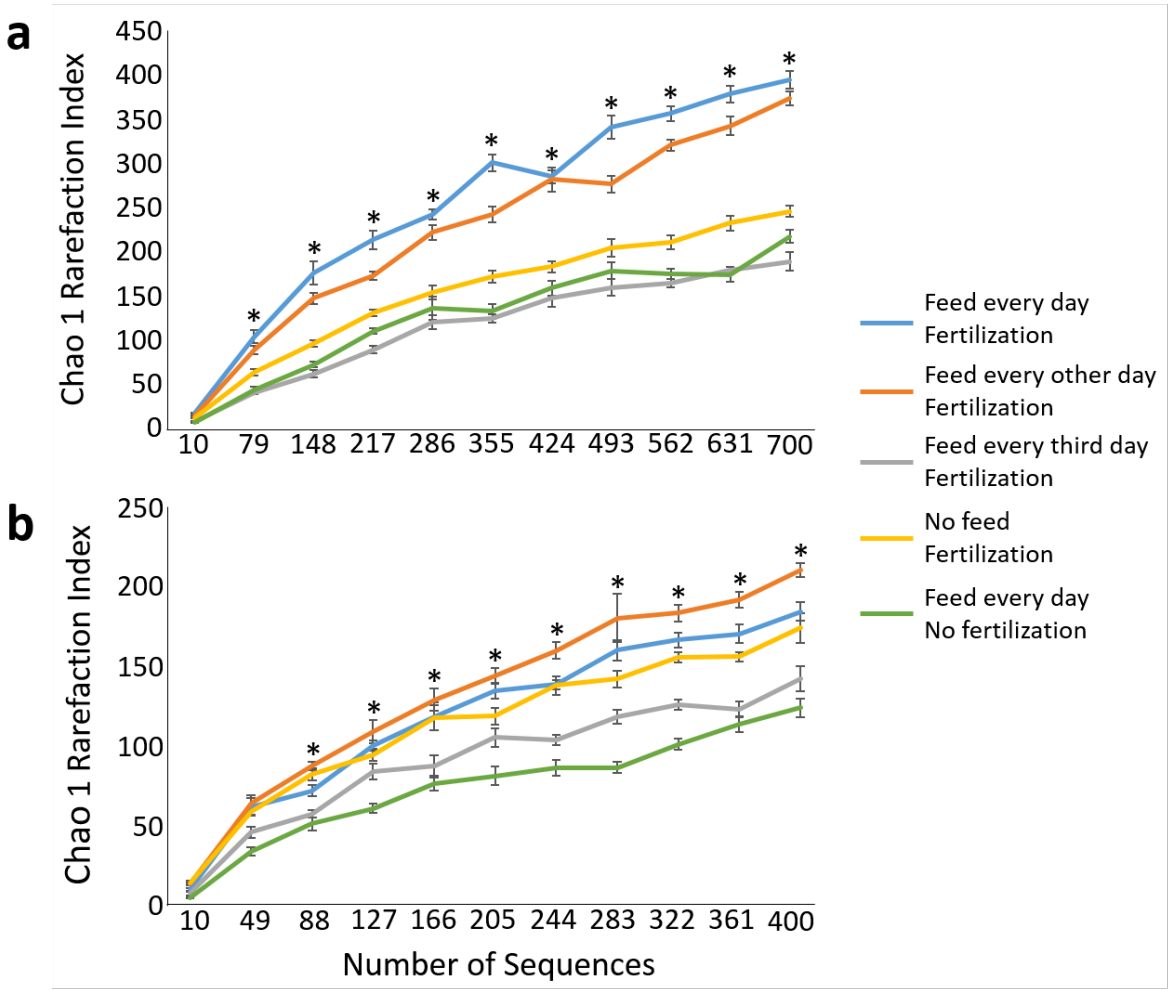

Figure 6. Diversity indices (Chao 1) of microbes found in the gut of Nile tilapia subjected to varying pulsed-feeding regimes (mean ± SEM). (a) 16S rRNA (prokaryote); (b) 18S rRNA (eurkaryotes). Asterisks indicate significant differences between treatments ($p < 0.05$).

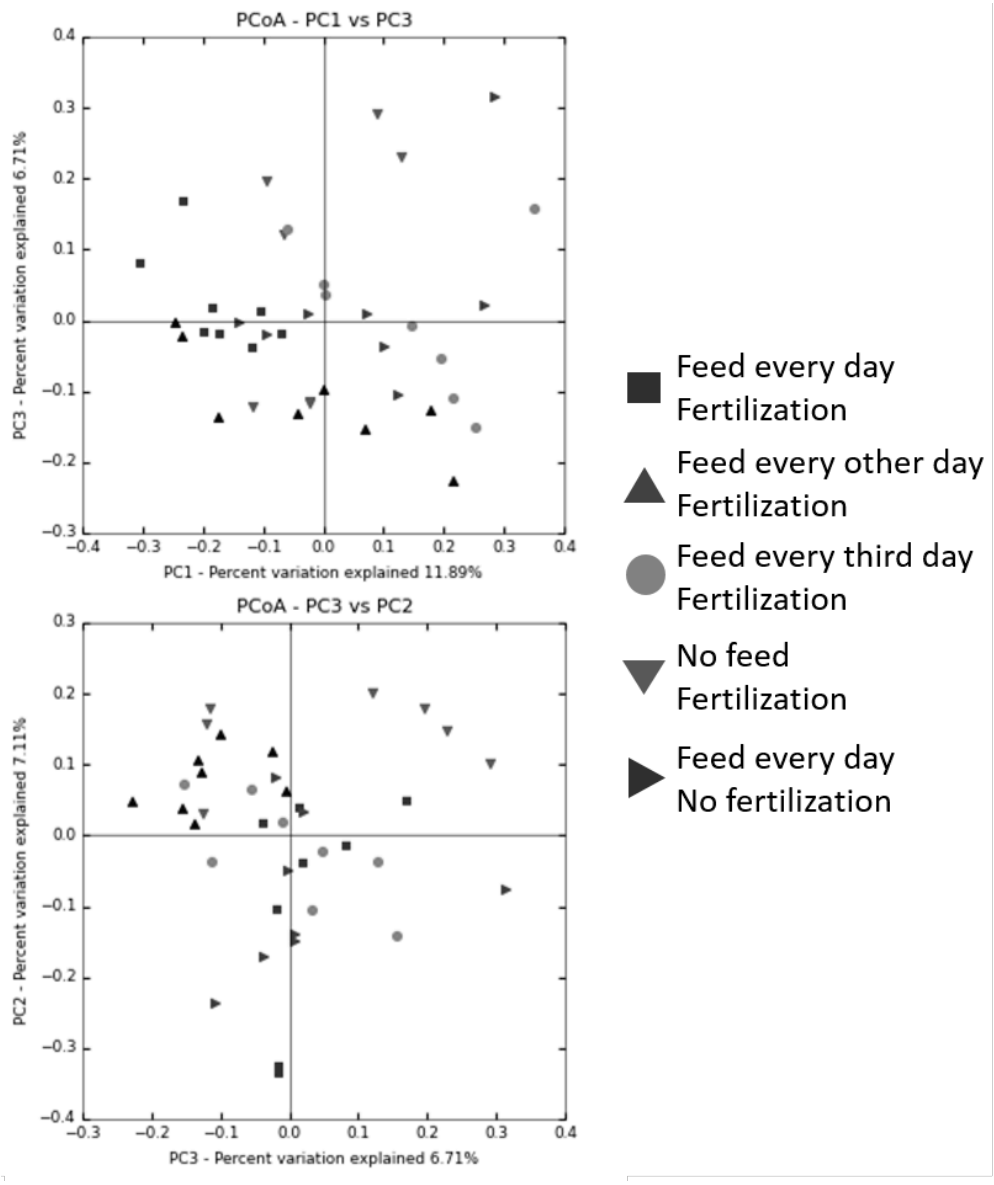

Figure 7. Principal coordinates analysis (PCoA) plots of bacterial communities found in the fecal material from Nile tilapia subjected to different pulsed-feeding regimens. Each replicate sample is represented in the plot.

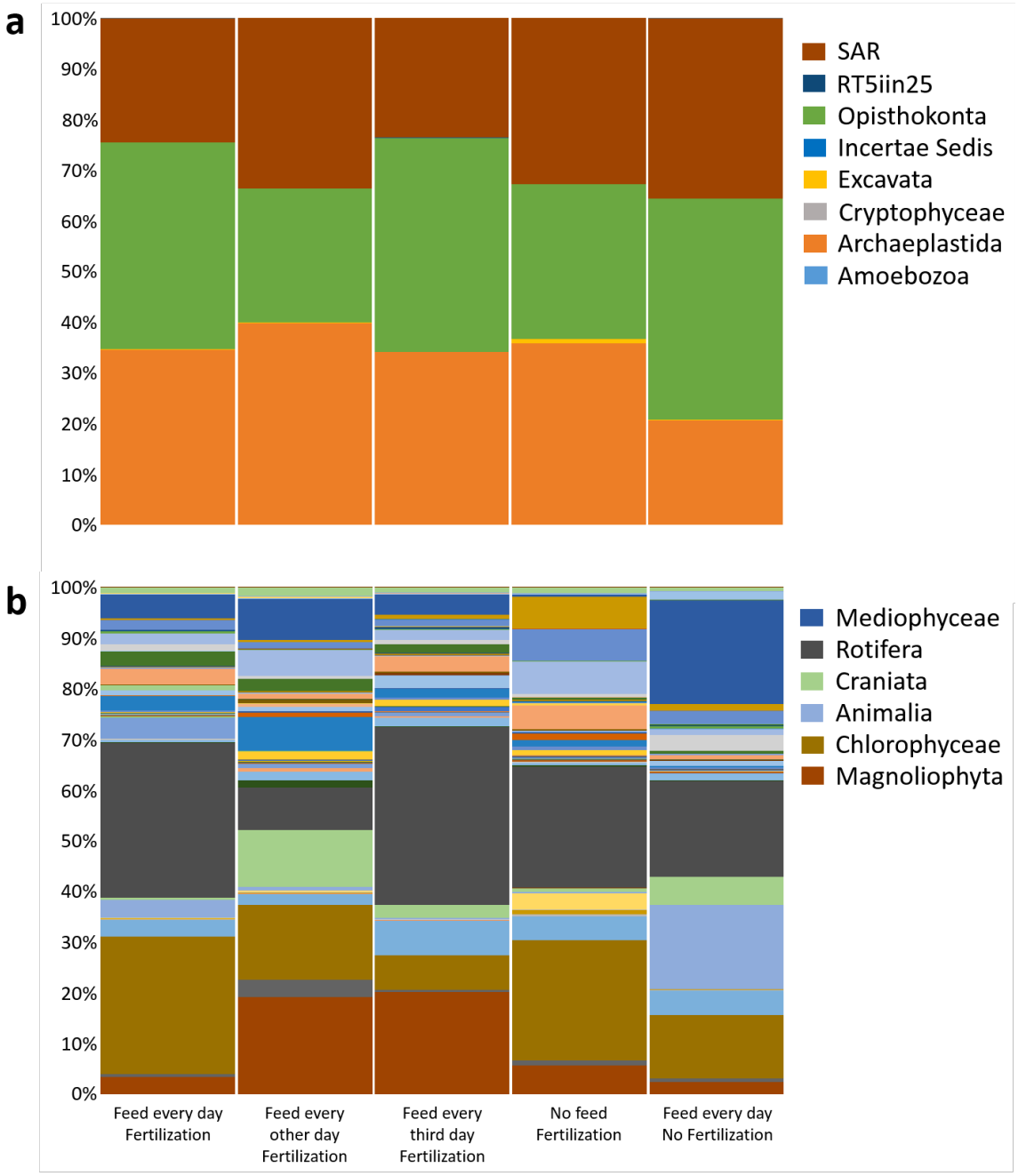

Figure 8. Relative abundance of phyla (a) and higher groups (b) of eukaryotes in the fecal material of Nile tilapia subjected to varying pulsed-feeding regimes.

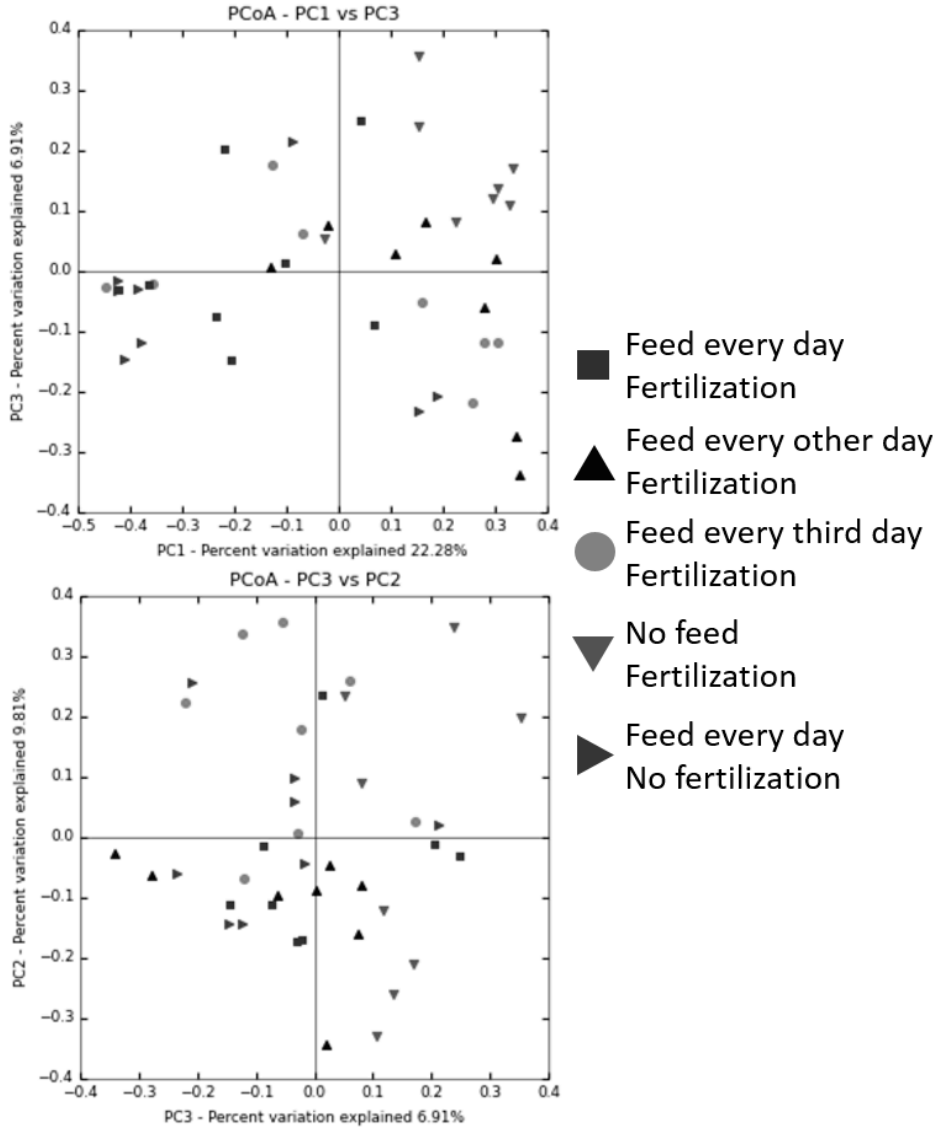

Figure 9. Principal coordinates analysis (PCoA) plots of eukaryotic communities found in the fecal material from Nile tilapia subjected to different pulsed-feeding regimens. Each replicate sample is represented in the plot.